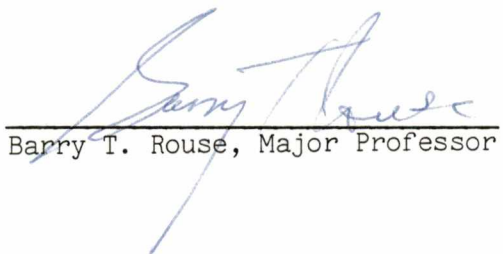
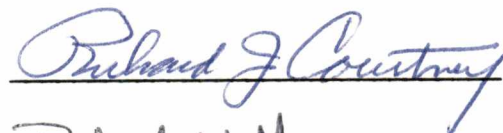


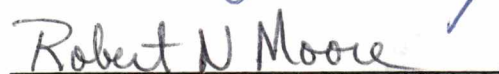
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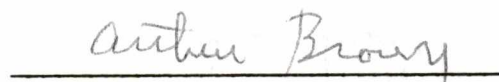
I am submitting herewith a dissertation written by David W. Horohov entitled "The Regulation of Herpes Simplex Virus Specific Cytotoxic T Lymphocyte Induction by a Suppressor Cell Circuit." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.


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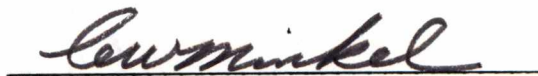

Richard J. Courtney


Robert N. Moore


Arthur Brown


Alexander Osmund

Accepted for the Council:


Vice Provost
and Dean of the Graduate School

THE REGULATION OF HERPES SIMPLEX VIRUS SPECIFIC CYTOTOXIC T
LYMPHOCYTE INDUCTION BY A SUPPRESSOR CELL CIRCUIT

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

David W. Horohov

June 1985

To my wife, Janice, and my son, Nikolai, for their support.

In memory of my father.

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ABSTRACT

Evidence for a suppressor cell circuit regulating herpes simplex virus (HSV) specific cytotoxic T lymphocyte (CTL) induction is presented. Thus the stimulation of HSV immune murine splenocytes with HSV antigens results in the induction of suppressor cells which inhibit both HSV-specific CTL induction and lymphoproliferation. Through the use of coculture experiments it was possible to identify a radioresistant, Thy 1⁺, Lyt 2⁺, T cell responsible for the suppression. The induction of this cell required the participation of several distinct splenocyte populations. Thus Lyt 1⁺ and Lyt 2⁺ T cells as well as I-E⁺, I-J⁺ antigen presenting cells (APC) were required for the induction of the Lyt 2⁺ suppressor cell. Only the Lyt 2⁺ cell had to come from HSV-immune mice, both the Lyt 1 cells and the APC required for suppressor cell induction could be provided by HSV naive mice. In addition to the cellular requirement for suppression, several soluble factors were involved in the suppression. These factors were present in the supernatant fluids from HSV-stimulated, HSV-immune splenocyte cultures. Separation of the suppressive supernatant by gel filtration chromatography identified both non-specific and antigen-specific suppressor activities. The specific suppressor activity exhibited both antigen-specificity and genetic restriction in its action. Western blotting analysis of the specific suppressor activity indicated that it was both I-J⁺ and anti-idiotypic. Production of the suppressor factors required Lyt 2⁺ splenocytes. The ultimate suppression mediated by the specific factor was non-specific in its action and required the

participation of a HSV-primed Lyt 1⁺ cell. A model is thus presented which describes the regulation of HSV-specific immune responses by a suppressor cell circuit which is specifically induced, but which mediates its activity non-specifically.

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LIST OF ABBREVIATIONS

APC:	Antigen presenting cell
CMI:	Cell mediated immunity
CTL:	Cytotoxic T lymphocyte
CY:	Cyclophosphamide
DTH:	Delayed type hypersensitivity
FPC:	Factor presenting cell
HI:	Heat inactivated virus
HSV:	Herpes simplex virus
HSV-SF:	HSV-specific suppressor factor
IL-2:	Interleukin 2
MO:	Macrophage
NP:	4-hydroxy-3-nitrophenyl acetyl
nsSF:	Non-specific suppressor factor
PCL:	Picryl chloride
PSA:	Picrylsulfonic acid
SC:	Suppressor cell
SF:	Suppressor factor
SF-200:	S-200 fractionated SF
Ts ₁ :	Suppressor inducer cell
Ts ₂ :	Suppressor effector cell
Ts ₃ :	Suppressor acceptor cell
TsF ₁ :	Suppressor inducer factor
TsF ₂ :	Suppressor effector factor
TsF ₃ :	Suppressor acceptor factor

CHAPTER I

INTRODUCTION

Literature Review

The immune response to an antigen is finely regulated by interactions between various mediators and cells (Cantor and Boyse, 1977; Cantor and Gershon, 1979; Unanue, 1981). Some of these interactions are involved in the induction of the response while others suppress it. The interplay between induction and suppression determines the extent and nature of the immune response, and consequently affects the status of immunity. Under normal circumstances, the predominance of induction or suppression depends upon considerations such as: the physical state of the antigen, the route of administration, and the host's genetic background. Thus antigens may contain suppresserogenic and immunogenic determinants which preferentially activate different cell types (Schwartz et al., 1976; Adorini et al., 1979; Naor, 1983; Kolsch, 1984). Intravenous injection of immunogenic antigens often results in immunological non-responsiveness (tolerance) (Battisto and Bloom, 1966; Weigle et al., 1975; Eardley et al., 1979). Finally, the responsiveness to certain antigens is genetically controlled such that non-responder hosts preferentially develop suppression upon exposure to the antigen (Kapp, 1978; Ikezawa et al., 1983).

In 1970, the concept emerged that a lack of a detectable immune response could be mediated by the direct action of suppressor cells which in most systems studied turned out to be subpopulations of T cells

(Gershon and Kondo, 1970). Suppressor cells have been described which modulate the induction and activity of B cells, helper T cells, delayed type hypersensitivity and contact sensitivity, lymphoproliferation, cytotoxic T lymphocyte induction, and natural killer cells (Truitt et al., 1977; Moorhead, 1978; Herzenberg et al., 1980; Germain and Benacerraf, 1981; Schaaf-Lafontaine and Degiovani, 1981; Aune and Pierce, 1982a,b). The regulation can be selective and antigen specific or can be nonspecific accounting in some cases for immunological anergy (Mehra et al., 1982; Scott and Farrell, 1981). Immune responses to all types of antigens appear to be regulated by suppressor mechanisms but most of our knowledge of the topic results from studies of noninfectious antigens and mitogens.

Working with simple antigens and haptens, and murine cells, several groups have demonstrated that a cascade of interacting cells constitute a suppressor cell circuit (reviewed by Germain and Benacerraf, 1981; Green et al., 1983; Dorf and Benacerraf, 1984; Asherson et al., 1985). From such studies the following working model of a suppressor cell circuit has been compiled (Figure 1). The major feature of this model, and suppressor cell circuits in general, is the sequential interaction of distinct T cell subsets and macrophages in the induction, propagation, and execution of suppression. These interactions can be both antigen-specific and genetically restricted. Antigen specificity is determined by the presence of antigen-binding (idiotypic) and receptor-binding (anti-idiotypic) receptors which are present on the cells and their soluble factors. Genetic restriction is determined either by Igh-linked genes, by the I-J antigen, or both (Dorf and

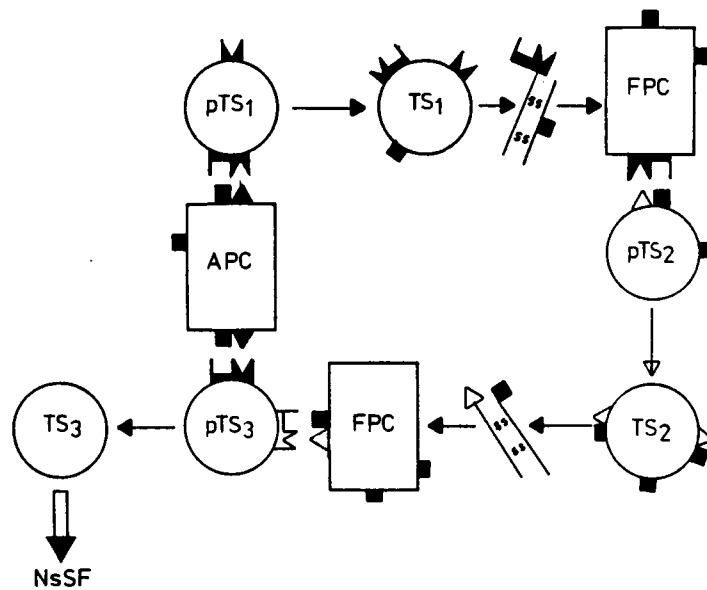


Figure 1. A suppressor cell circuit. To initiate the circuit, an APC presents antigen to the afferent acting suppressor-inducer cell (pTs1). Once activated this cell then produces its soluble factor which is both I-J+ and idiotypic. This factor is presented to an efferent acting suppressor effector cell (pTs2) by an I-J+ FPC. The Ts2 cell and its factor are I-J+ and anti-idiotypic. This anti-idiotypic factor is presented via a FPC to a third cell (Ts3), a nonspecific suppressor acceptor cell which, when also presented with antigen, releases a non-specific inhibitor of DNA synthesis.

Benacerraf, 1984). The I-J antigen has served as the impetus for much interest in suppressor cell regulation and has also generated a great deal of controversy along the way. Therefore before addressing the model, a brief discussion of I-J follows.

The I-J antigen is an enigmatic molecule originally thought to be encoded in the I-region of the murine major histocompatibility complex (MHC) at a region between the I-A and I-E loci (Murphy et al., 1976). This interpretation was the result of work done with two pairs of B10 congenic recombinant strains of mice. These strains were thought to differ at a single subregion (I-J) of the Ir gene locus. Thus reciprocal immunization of the I-J disparate B10 strains results in the production of alloantiserum and monoclonal antibodies which react with the putative product of this region, the I-J antigen (Kanno et al., 1981; Waltenbaugh, 1981). However, genomic analysis of the Ir gene complex indicated that the I-A and the I-E subregions are almost directly adjacent, thus allowing for only 2 kb of DNA to account for the I-J gene (Steinmetz et al., 1982). Further, mRNA transcripts from I-J+ hybridomas failed to hybridize with cloned DNA probes representing the Ir-gene region (Kronenberg et al., 1983). These observations have resulted in what is commonly referred to as the "I-J paradox" (Munro, 1983). Several groups have proposed mechanisms to explain away the paradox (reviewed by Hansen et al., 1984), but to date it still remains. The most recent resolution of the paradox has been submitted by Cantor's group (Klyczek et al., 1984). These authors propose that the I-J gene lies outside of the MHC complex, in fact on a separate chromosome, and a controlling element which regulates I-J expression is linked to the I-

region, possible the I-E subregion. Regardless of where the I-J gene is located, its expression can serve as a usable marker for suppressor cells and their products (Murphy, 1978). The significance of I-J antigens in suppressor cell activity is also uncertain. It appears that I-J expression on macrophages determines the interaction of these cells with suppressor T cells in much the same way that I-A antigens restrict macrophage: helper T cell interactions (Minami et al., 1982). It should be noted that the I-J antigen is also found on suppressor T cells which do not appear to require macrophage assistance in the recognition of antigen (Taniguchi et al., 1980). It has been proposed that in such instances the I-J antigen serves as a portion of the T cell receptor (Ikezawa et al., 1983). It has also been suggested that the I-J molecule is the suppressive signal itself (Ptak et al., 1982), though this assertion requires further study.

As was indicated above, the role of macrophages in suppressor cell induction and activity is controversial. Some authors have indicated that macrophages are not involved in the induction of suppressor T cells since some suppressor T cells appear to interact with antigen directly, without the intercession of an antigen presenting cell (APC) (Feldman and Kontiainen, 1976; Taniguchi et al., 1980). This may account for the propensity of certain routes of immunization to induce suppressor cells. Injection of antigen intravenously presumably bypasses the macrophages encountered during a subcutaneous injection and instead allows the antigen to interact directly with suppressor T cells present in the spleen and in circulation (Weigle et al., 1975). However, as our

understanding of suppressor cell circuits increases it becomes apparent that macrophages do play a major role in suppressor cell induction either as APC or factor presenting cells (FPC) (Ptak et al., 1978; Nakamura et al., 1982; Usui et al., 1984a,b; Granstein and Greene, 1985) also as the effector cells (Ptak et al., 1981). It is still uncertain whether these activities are mediated by a single macrophage population, or whether there are several macrophage subpopulations which present antigens and soluble factors to suppressor cells (Usui et al., 1984a). It does appear that there are distinct macrophage subpopulations which present antigens to helper T cells and those which present antigen to suppressor T cells. Thus, those APC involved in the induction of helper cell activity are sensitive to UV-irradiation, insensitive to cyclophosphamide pretreatment in vivo, and express the I-A antigen whereas the APC involved in suppressor cell induction are resistant to UV-irradiation, may be sensitive cyclophosphamide, and bear I-J antigenic determinants (Lowy et al., 1983; Granstein and Greene, 1985; Noma et al., 1985). However the exact phenotype of suppressor APC is uncertain, in particular it is unclear whether the suppressor APC are also I-A⁺ (Granstein and Greene, 1985; Noma et al., 1985). Thus the APC involved in Ts₁ and Ts₃ induction may be either I-A⁻ I-J⁺ (Schatten et al., 1984; Granstein and Greene, 1985) or I-A⁺ I-J⁺ (Usui et al., 1984a; Noma et al., 1985). It is also uncertain what role other macrophage surface antigens and soluble factors may have in suppressor cell induction.

In the following model, the initial interaction between antigen (Δ) and the suppressor cell circuit involves presentation by an I-J⁺ APC to

the suppressor inducer cell (Ts_1). The Ts_1 cell is $Lyt\ 1^+$, $I-J^+$, and expresses idiotypic receptors for the antigen. Once activated, the Ts_1 cell produces a soluble factor (TsF_1) which contains I-J antigen and the antigen-specific idiotypic receptor. Both Ts_1 and TsF_1 can suppress the immune response to the antigen if they are present at the time of antigen priming. Consequently, they are referred to as afferent suppressors since they suppress the afferent limb of the immune response.

The TsF_1 is then presented via an $I-J^+$ FPC to a suppressor effector cell (Ts_2). The Ts_2 cell is $Lyt\ 2^+$, $I-J^+$, and expresses anti-idiotypic receptors which recognize TsF_1 . Once activated, Ts_2 produces its soluble factor, TsF_2 , which is also $I-J^+$ and anti-idiotypic. Unlike Ts_1 and TsF_1 , Ts_2 and TsF_2 suppress the efferent limb of the immune response and are thus referred to as efferent suppressors. The temporal relationship between afferent and efferent suppressor cell activity in vivo is probably the result of a requirement for the expansion of the Ts_2 population once it is activated. This is consistent with the observation that Ts_2 cells are not present in the spleens of unprimed mice. The requirement for expansion may also account for the sensitivity of some of these cells to cyclophosphamide treatment. Whether Ts_2 , or any suppressor cell, expansion requires a growth factor, such as interleukin 2, also remains to be clarified. However, it is known that the anti-idiotypic TsF_2 is not the ultimate suppressor signal.

TsF₂ is presented to an Lyt 2⁺, I-J⁺, idiotypic suppressor acceptor cell (TS₃), by an I-J⁺ APC. Ts₃ is also presented with an antigen by an I-J⁺ APC. These two signals are necessary for Ts₃ activation. Once activated, the Ts₃ cell produces the ultimate suppressor signal. In this model, the signal is the production of a soluble factor (nsSF) which non-specifically suppresses the immune response (Zembala et al., 1982). Alternatively, a more specific inhibition may be mediated by the Ts₃ cell and its product (Sherr et al., 1983; Dorf and Benacerraf, 1984).

The above mentioned model for suppressor cell circuits has resulted from the study of immune responses to haptens, alloantigens, and other "non-infectious" antigens. While it is uncertain whether similar suppressor cell circuits regulate the immune responses to infectious antigens (bacteria, protozoans, fungi, and viruses), several pieces of indirect evidence do support the existence of such regulatory circuits in the responses to these complex antigens. Thus suppressor cells have been demonstrated during infections with various bacteria (Collins and Watson, 1979; Mehra et al., 1982; Petit et al., 1982; Mustafa and Godal, 1983), fungi (Murphy and Moorhead, 1982; Murphy et al., 1983), protozoans (Charoenvit et al., 1981; Scott and Farrell, 1981; Suzuki and Kobayashi, 1983), helminths (Rocklin et al., 1981; Doughty and Phillips, 1982; Mitchell, 1984) and viruses (Tosato et al., 1979; Leung et al., 1980; Liew and Russell, 1980; Greene and Weiner, 1980; Rinaldo et al., 1980; Nash et al., 1981; Sheridan et al., 1982; Iwasaka et al., 1983; Schrier et al., 1983; Chan et al., 1983; Chong and Mims, 1983; Mathur et al., 1984; Nash, 1985). The role these suppressor cells have in the

disease process remains unclear. Nevertheless in certain occasions suppressor cells can be prognostic of either a resolving (Tosato et al., 1979; Crawford et al., 1981; Evans and Nierderman, 1982) or worsening condition (Rocklin et al., 1981; Modlin et al., 1983). For the purposes of this dissertation I shall focus on the role of suppressor cells and suppressor cell circuits in the immune response to the viruses.

Viruses may be thought of as complex infectious antigens which can gain access to the host via a variety of routes. It is well established observation that resistance to virus infections is genetically controlled (Lopez et al., 1981). Since immunological responsiveness is genetically controlled (Ikezawa et al., 1983), it is predictable that the immune response to viruses is regulated by the interaction of inducer and suppressor cells. However, our knowledge of suppressor cell regulation of responses to viral antigens is fragmentary, and what is known comes from incomplete studies on a small number of viruses. Those which have been studied include Reoviruses (Greene and Weiner, 1980), Orthomyxoviruses (Leung et al., 1980; Liew and Russell, 1980; Ada et al., 1981), Flaviviruses (Mathur et al., 1983; Arenaviruses (Chan et al., 1983), and Herpesviruses (Nash et al., 1981; Schrier et al., 1983; Iwasaka et al., 1983). Those viruses which have been studied most extensively include the orthomyxovirus influenza A and herpes simplex virus types 1 and 2.

Suppressor cell regulation of influenza specific cell mediated immunity (CMI) has been demonstrated by several groups. Leung et al (1980) reported that the intravenous injection of UV-inactivated

influenza A virus caused the generation of suppressor T cells which inhibited the induction of influenza-specific CTL, but did not influence the induction of delayed type hypersensitivity (DTH) mediating T cells or the production of either IgM and IgG antibodies, a phenomenon known as "split tolerance" (Nash and Ashford, 1982). The suppression was type specific for the strain of virus used to induce the suppressor cells. The cell responsible for the suppression was non-phagocytic, plastic non-adherent, and susceptible to lysis by anti-Thy 1.2 antibody and complement. The transfer of suppressor cell activity to recipient mice required compatibility at the H-2 loci. A converse situation was reported by Liew and Russell (1980). Intranasal inoculation of live influenza resulted in the induction of suppressor cells which inhibited DTH responses to viruses of the same HA type. Suppression was mediated by Thy 1⁺, Lyt 1⁻2⁺3⁺ H-2K, H-2D restricted T cells. Again, "split tolerance" was evident by the fact that the induction of humoral immunity and CTL responses to the same strain of the virus were unaffected (Ada et al., 1981).

The possible biological significance of suppressor cell regulation of virus-specific DTH responses was recently demonstrated (Liew and Russell, 1983). During an attempt to determine the role of DTH-mediating T cells in influenza infections, in vitro expanded DTH reactive T cells were adoptively transferred into recipients which had received an intranasal challenge of influenza. Instead of conferring protection, the adoptively transferred cells exacerbated the infection resulting in increased mortality. The recipient mice exhibited extensive lung consolidation similar to human influenza pneumonitis. If

the induction of the DTH mediating cells was instead suppressed by incubating the cells in vitro with suppressor cells from intranasally infected mice, the pathologic effect of the adoptive transfer was negated and the recipients recovered. Thus with influenza, suppressor cell mediated depression of the induction of the DTH response prevented immunopathologic reactions. Since the natural exposure to influenza occurs via the respiratory tract, it appears likely that the induction of DTH-inhibiting suppressor cells is a normal component of the immune response. A similar protective role for suppressor cell induction has been suggested to occur in other virus infections which are characterized by adverse reactions leading to immunopathology (Doughty and Phillips, 1982; Nash, 1985). Furthermore, the lack of induction of an appropriate suppressor cell response may be the reason for some examples of vaccine complications. For instance, administration of inactivated vaccines for measles and respiratory syncytial viruses resulted in an exacerbation of disease upon subsequent natural infection (Kowprowski, 1962; Fulginiti et al., 1967; Chanock et al., 1970). Although the reason for these adverse reactions was not determined, it is possible that the inactivated vaccines sensitized for DTH, but failed to stimulate a protective suppressor cell response. Thus exposure to the virus via the natural route of infection resulted in immunopathology.

The intravenous injection of live or UV-inactivated herpes simplex virus (HSV) also results in the generation of suppressor cells which inhibit the induction of HSV-specific DTH responses (Nash et al., 1981;

Schrier et al., 1983). Neither antibody responses or virus-specific CTL induction is affected, hence "split tolerance" (Nash and Ashford, 1982). Further the suppression is type specific, induction of tolerance to HSV type 1 does not affect responsiveness to HSV type 2 (Nash et al., 1981). At least two different suppressor cell populations appear to be responsible for suppression, afferent acting suppressor T cells are detectable in the spleens of infected mice one week after injection. These cells are Thy 1⁺, Lyt 1⁺, I-J⁺ and sensitive to cyclophosphamide. Four weeks after injection a second population of Lyt 2⁺ suppressor cells is detected. These cells are able to suppress the efferent phase of the DTH response. The latter cells appear to account for the long term maintenance of suppression (Nash, 1985). Moorhead's group reported that injecting mice with different preparations of HSV antigens also induced two different suppressor cell populations (Schrier et al., 1983). Thus sonicated virus particles induce the afferent acting suppressor cells while virus-infected cells induced both afferent and efferent suppressor cells. It should be noted that regardless of which preparation is used, both populations of suppressor cells are present in the spleens of the mice by the fourth week post injection. It should also be noted that suppressor T cells are not the only lymphocytes which downregulate immune responses to HSV antigens. Suppressor B cells have also been described which can inhibit the efferent arm of the HSV-specific DTH response (Nash and Gell, 1980). How these cells mediate suppression of the DTH response is unclear, though it may involve idiotype: anti-idiotype interactions (Nash, 1985). The role these suppressor cells have in the pathogenesis of HSV infections is unknown.

It is possible that like the influenza model, DTH suppression may prevent immunopathologic reactions to the virus (Nash and Gell, 1983). Thus tolerization of the DTH response may result in a decreased severity of stromal reactions in ocular HSV infected mice (Rouse, 1984) and a decrease in the incidence and severity of demyelination of local sensory nerve fibers in tolerized mice infected in the pinna (Nash, 1985). Alternatively suppressor cells may also play a role in the pathogenesis of HSV infections by inducing immune dysfunction which predisposes the host to recrudescence lesions.

The hallmark of herpesvirus infections is the ability of the virus to establish a latent infection in a ganglion of the host's nervous system (Stevens and Cook, 1971; Stevens, 1979). Following reactivation of the virus, by a variety of causes (Stevens et al., 1975; Blyth et al., 1976; Hill et al., 1978), the virus travels down the axon to the site of the original infection and causes a recurrent lesion. The exact mechanism whereby reactivation and recrudescence lesions occur in the face of both specific humoral and cellular immunity to the virus is unknown. However it has been proposed that there may be a regulatory defect in virus specific CMI which accompanies recurrent HSV disease (Sheridan et al., 1982; Sheridan and Aurelian, 1983). This hypothesis is supported by studies of HSV-specific CMI during recrudescence disease in man (Thong et al., 1975; Shillito et al., 1977; Sheridan et al., 1982; Rola-Pleszczynski and Lieu, 1984) and an animal model (Iwasaka et al., 1983). Thus HSV recrudescence in man is associated with an alteration in helper cell: cytotoxic / suppressor cell ratios in vivo

and decreases in HSV-induced lymphokine production (Sheridan et al., 1982), lymphoproliferation (Shillito et al., 1977), and cell mediated cytotoxicity (Rola-Pleszczynski and Lieu, 1984) in vitro. However, the best evidence for suppressor cell involvement in the aberration of HSV-specific CMI during recrudescence disease comes from the study of the guinea pig model for HSV type 2 recrudescence (Iwasaka et al., 1983). The splenocytes from guinea pigs undergoing recurrent HSV disease exhibit diminished lymphoproliferative responses to HSV antigens. This diminution of the response was due to suppressor cells found in the spleens of animals undergoing recrudescence disease and convalescence, but not in the spleens of quiescent animals or those without a history of recurrent disease. Further, it was also demonstrated that the suppression could be mediated by cell-free supernatant fluids of cultures of suppressive splenocytes. Those cells which mediated the suppression of the lymphoproliferative response, and the cells responsible for the production of the suppressive supernatant fluid were not identified.

Thus it appears that suppressor cells may influence the nature of the virus-host relationship in two ways; either by preventing immunopathologic reactivity (Liew and Russell, 1983; Nash, 1985) or by enhancing the expression of pathology (Iwasaka et al., 1983; Nash, 1985). An additional role for suppressor cells in virus infections may be seen in the murine model for HSV immunobiology. The demonstration of certain HSV-specific cellular immune responses in the mouse has been problematic. Though HSV-specific lymphoproliferative (LP) responses are readily demonstrated in man, previous attempts to demonstrate HSV-

specific LP responses in the mouse have met with failure (Kirchner et al., 1978; Mochizuki et al., 1981; Kirchner, 1982). While some of these problems have been due to the apparent mitogenicity of some viral stocks for murine B lymphocytes (Mochizuki et al., 1981), even when mitogenicity has not been a problem the results have been inconclusive (Kirchner, 1982). Likewise the demonstration of HSV-specific cytotoxic T lymphocytes has also proven difficult. In order to demonstrate HSV-specific CTL activity it is necessary to either pretreat the mice with cyclophosphamide before infection (Pfizenmaier et al., 1977a) or culture the lymphocytes from infected animals in vitro (Pfizenmaier et al., 1977b). Likewise the demonstration of secondary or memory CTL requires in vitro stimulation with HSV antigens (Lawman et al., 1980). These various observations indicate that the induction of HSV-specific CTL is regulated by suppressor cells. This supposition is based on the reported sensitivity of suppressor cells to cyclophosphamide and the fact that in vitro cultivation often results in the breaking of suppressor cell mediated tolerance (Rollinghoff et al., 1977). Some recent data from limiting dilution analysis of HSV-specific CTL precursors (CTLp) also indicated that HSV-specific CTL induction was regulated by suppressor cells (Rouse and Wagner, 1984). At very low responder cell numbers it appeared that there was a higher frequency of CTLp than was actually calculated, such a biphasic curve is characteristic of suppressor cell activity (Goronzy et al., 1981).

Taken together the preponderance of indirect evidence indicates that the induction of HSV-specific CTL activity is regulated by

suppressor cells. Owing to the fact that CTL play a major role in the host's immune response to various viruses (Zinkernagel and Doherty, 1979), including HSV (Rouse and Horohov, 1984), direct information regarding the regulation of CTL activity by suppressor cells is important for our understanding of host: virus interactions. Further, since it is possible that recrudescence could result from the alteration of CMI by virus induced suppression, any information regarding the regulation of HSV-specific CMI by suppressor cells might have eventual therapeutic applications.

Rationale for the Present Study

Numerous studies have shown that the induction of immune responses to non-infectious antigens are regulated by suppressor cell circuits (Cantor and Gershon, 1979; Germain and Benacerraf, 1981; Greene et al., 1983; Dorf and Benacerraf, 1984; Asherson et al., 1985). It has also been shown that immune responses to several viruses are also regulated by suppressor cells (Nash et al., 1980; Leung et al., 1980; Liew and Russell, 1980; Schrier et al., 1982). The role such suppressor cells may play in virus infections is not certain. Though in the case of immunity to influenza suppressor cells may be involved in the prevention of immunopathology (Liew and Russell, 1983), there is also evidence that suppressor cells may be involved in the pathogenesis of virus infections (Iwasaka et al., 1983). Suppressor cells are also thought to account for the difficulty in demonstrating CTL responses to HSV antigens (Pfizenmaier et al., 1977a,b; Lawman et al., 1980; Rouse and Wagner, 1984), however the evidence supporting this notion is strictly

inferential. The work presented in this dissertation was designed to demonstrate directly that the in vitro induction of HSV-specific CTL is regulated by suppressor cells which are specifically induced following in vivo priming with viral antigens.

Suppressor cell regulation of secondary HSV-specific CTL induction in vitro was tested by generating suppressor cells in one culture and adding them to a second culture in order to suppress HSV-specific cell mediated immune responses. Similar experimental approaches have been used to investigate the regulation of immune responses to haptens and alloantigens (Konttiainen and Feldman, 1976; Susskind et al., 1983). Once it was possible to demonstrate suppressor cell activity, the cellular requirements for suppressor cell induction were investigated. These experiments were important since they directly demonstrated the regulation of HSV-specific CMI by a suppressor cell and that the induction of the suppression involved a suppressor cell circuit analagous to those circuits which are involved in the regulation of immune responses to non-infectious antigens.

It is hoped that the work performed in this study will provide new insight into the regulation of virus specific immune responses. This information may facilitate the development of safer, more efficacious vaccines against viruses. Further, it may indicate the mechanism whereby recurrent herpesvirus infections occur inspite of active immunity.

Specific Aims

1. The determination of suppressor cell regulation of secondary in vitro cytotoxic T lymphocyte responses to herpes simplex type 1.
2. The determination of the surface antigen profile of those cells which mediate suppression.
3. The determination of the surface antigen profile of those cells which are involved in the induction of suppressor cells.
4. Analysis of the soluble factors involved in the generation and execution of suppression.
5. To propose a mechanism for suppressor cell regulation of herpes simplex virus-specific cytotoxic T lymphocyte induction.

CHAPTER II

MATERIALS AND METHODS

Virus Preparations

HSV type 1 strain KOS was propagated in HEp-2 cells as described (Lawman et al., 1980). The viral stock had an infectivity titre of 4×10^8 plaque forming units per ml (pfu/ml). UV-inactivated virus (UV-HSV) was prepared by exposing 0.5 ml of the viral stock to a germicidal lamp (Sylvania Electric Products, Danver, MA) at a distance of 3 cm for two minutes. This resulted in a reduction of the viral titre to fewer than 10^2 pfu/ml. Heat inactivation was performed by incubating 0.5 ml of the viral stock at 60°C for 30 minutes. The infectivity titre of the heat inactivated virus (HI-HSV) stock was less than 10 pfu/ml. Sonicated HEp-2 cells were used as sham controls in all experiments.

Influenza virus A/Puerto Rico/8/34 was obtained from Dr. J. Bennick (Wistar Institute, Philadelphia, PA) and propagated in embryonated chicken eggs. Influenza virus stock had a hemagglutination titre 1200 units per ml (HAU/ml). Strain L929 cells (H-2^k) were obtained from the American Type Culture Collection (Rockville, MD), 3T3 strain A31 cells (H-2^d) were obtained from Dr. Ray Tennant, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN. EL4 thymoma cells (H-2^b) were obtained from Dr. M. Hilfiker (Cleveland Clinic Foundation, Cleveland, OH). All cells were cultured in McCoys 5A supplemented with 5% donor calf serum (Grand Island Biological Company, Grand Island, NY). The

cells and viral stocks were routinely tested for mycoplasmata contamination by the method of Kaplan et al (1984).

Antisera and Preparation of Plastic for Panning

Murine derived anti-Thy 1.2, -Lyt 1.1, -Lyt 2.1, -I-J^k antisera and complement were purchased from Cedarlane Laboratories (Accurate Chemical and Scientific Corp., Westbury, NY). Rabbit anti-asialo GM₁ antiserum was purchased from Wako Chemical, Dallas, TX. Goat anti-mouse immunoglobulin was purchased from Cappel Laboratories, West Chester, PA. Thirty-five cm flasks (Corning Glass Works, Corning, NY) were coated with anti-immunoglobulin antisera by incubating the plates overnight with 1.0 ml of the antiserum (1.2 mg protein) at 4°C. Before use, the flasks were rinsed three times with phosphate buffered saline (PBS; pH 7.2) and incubated for 45 minutes with 5 mls of PBS containing 40% fetal calf serum (FCS; GIBCO Laboratories, Grand Island, NY).

Mouse Immunization and Splenocyte Cultures

Four to six week old C3H/HeJ mice were obtained from the breeding colony of the University of Tennessee Memorial Research Center Hospital, Knoxville, TN. BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from Cumberland View farms, Clinton, TN. The mice received a single injection of 1×10^6 pfu of HSV in 0.1ml volume by various routes. HSV-immune mice received an intraperitoneal injection 4 weeks prior to use. Tolerized mice received an intravenous injection followed 7 days later with a subcutaneous injection. DTH primed mice received a subcutaneous

injection 14 days before ear challenge. Influenza primed mice received a single intraperitoneal injection of 40 HAU of 4 weeks prior to use. In some experiments the mice received an intravenous injection of 0.1 ml of a 1/5 dilution of anti-asialo GM₁ antiserum (Wako Chemical, Dallas, TX) 18 hours prior to sacrifice. This treatment effectively eliminated natural killer cell activity in the splenocyte cultures (Biron et al., 1982).

The preparation of single cell suspensions of splenocytes has been described elsewhere (Lawman et al., 1980). The splenocytes were cultured in RPMI-1640 (GIBCO) containing 5% heat inactivated FCS, 2 mM glutamine, penicillin (100 u/ml), streptomycin (100 ug/ml), gentamicin (50 ug/ml), and 5×10^{-5} mM 2-mercaptoethanol. Bulk cultures consisted of 10^7 cells in 5 ml of media per well of a six-well cluster plate (Costar, Cambridge, MA). Microcultures consisted of 5×10^5 cells in 0.2 ml of media per well of a 96-well flat bottomed microtitre plate (Costar). Virus stimulated cultures were incubated with HSV at a multiplicity of infection (moi) of 1.0 pfu/cell calculated before inactivation.

Measurement of Lymphocyte Proliferation

The incorporation of tritiated thymidine (³H-TdR) into cellular DNA was used as a measure of lymphocyte proliferation. Typically, 0.5 uCi of ³H-TdR (New England Nuclear, Boston, MA) was added to the appropriate wells of the 96-well plate during the final 6 hours of incubation. The cells were then harvested onto glass fiber filters (Skatron Inc., Steding, VA) with a semi-automated cell harvester (Flow Laboratories, McLean, VA). The filter papers were immersed in 0.5 ml of ScintiVerse E

(Fisher Scientific Co., Fair Lawn, NJ) and counted in a Beckman (Irvine, CA) LS 7000 liquid scintillation spectrophotometer. Results are expressed as the mean value obtained from four replicate wells.

Cell Fractionation

Splenocytes were separated into surface immunoglobulin positive (sIg⁺) and negative (sIg⁻) populations by panning onto anti-immunoglobulin (Ig) coated plates (Wysocki and Sato, 1978). Briefly, 5×10^6 viable stimulated splenocytes obtained after centrifugation on Lympholyte-M (Cedarlane Laboratories) were incubated in 1.0 ml of medium containing 4.0 uCi of ³H-TdR for four hours. After washing several times with medium, the cells were added in 1.0 ml volumes to anti-immunoglobulin coated 35 cm culture flasks and incubated for 1 hour at room temperature. Non-adherent cells were gently removed with a pipette and transferred to a 96-well plate for harvesting. The adherent cells were eluted with forceful pipetting action with Ca⁺/Mg⁺ free Hank's basic salt solution (HBSS) containing versene. Following elution, the adherent cells were washed one time, resuspended in 1.0 ml of medium and then transferred to a 96-well plate for harvesting. The results reported are the total cpm associated with the nonadherent and adherent cells. Following elution, less than 1% of the total cpm incorporated by the cells remained associated with the flasks.

Thy 1⁺ and Thy 1⁻ cells (as well as Lyt 1⁺ and Lyt 1⁻ and Lyt 2⁺ and Lyt 2⁻ cells) were separated on the anti-immunoglobulin plates with the following procedure. Radiolabelled, sIg⁻ cells, prepared as

described above, were incubated with specific antiserum for 45 minutes on ice. The cells were washed twice with ice-cold medium and then incubated in the anti-immunoglobulin flasks for one hour at 4°C. The nonadherent cells were removed with gentle pipetting and transferred to a 96-well plate for harvesting. The adherent cells were eluted by vigorous washing with warm HBSS containing versene and then transferred to a 96-well plate for harvesting. Again, less than 1% of the incorporated counts remained affixed to the flasks.

Negative depletion of the microculture wells was performed by transferring 100 ul of the cells to "V"-bottomed Linbro plates (Flow Laboratories). The cells were pelleted by centrifugation (200 x g, 5 minutes) and the supernatant fluid discarded. The cells were resuspended in 100 ul of the diluted antisera and incubated for 45 minutes on ice. The cells were again pelleted, the supernatant discarded, and the cells resuspended in 100 ul of rabbit complement diluted 1/13 in cytotoxicity medium (Cedarlane Laboratories). The plates were incubated 30 minutes at 37°C. Afterwards the cells were washed with cytotoxicity medium and resuspended in complete RPMI-1640 containing 0.5 uCi of ³H-TdR. The cultures were incubated six hours and then harvested onto glass fiber filters.

Negative depletion of the bulk cultures was performed as described (Larsen et al., 1984). Briefly, 1×10^7 splenocytes were resuspended in specific antiserum and incubated for 45 minutes at 4°C. The cells were washed with cytotoxicity media and resuspended in complement. After incubating at 37°C for 30 minutes, the cells were washed several times with cytotoxicity medium and then resuspended in complete RPMI.

Viability of the treated cultures was determined by trypan blue exclusion. The number of cells/ml was adjusted appropriately after viability determinations were performed.

Nylon wool purified T cell populations were collected according to the method of Julius et al (1974). The cells collected from the nylon wool column were determined to be >99% Thy 1.2 positive. Furthermore, the nylon wool purified cells did not respond to either the T cell mitogen concanavalin A (Con A) or specific antigen stimulation unless supplemented with resident peritoneal cells from HSV-naive mice.

Delayed Type Hypersensitivity Response In Vivo

Primary DTH to HSV was determined as described by Nash et al (1980). Mice primed with a subcutaneous injection of 1×10^6 pfu of HSV were challenged in the right pinna with the virus and in the left ear with the HEp-2 sham. Differences in swelling between the two ears 24 hours later were recorded. Unprimed mice demonstrated insignificant swelling when challenged with either virus or the sham.

Adoptive transfer of in vitro primed immune tolerant splenocytes was done as described by Larsen et al. (1984). Either 1×10^7 splenocytes were injected intravenously or 1×10^6 cells were injected into the pinna. In the former procedure, virus was injected four hours later into the right pinna and the sham into the left, in the latter procedure the virus was injected with the cells. The left pinna received splenocytes from HSV-naive mice while the right pinna received splenocytes from HSV-immune mice. For both procedures, differences in

the swelling between the two ears 24 hours post challenge were reported. Naive splenocytes cultured with HSV failed to generate DTH activity in the recipient.

CTL Activity of Microcultures

The cytotoxic activity of the bulk cultures was assessed as previously described (Lawman et al., 1980). The cytotoxic activity of the microcultures was also assessed after a five day incubation period. The microcultures were split such that half of the cells were transferred in 100 ul of medium to corresponding wells of a round bottomed 96-well plate (Linbro). The cells remaining in the original flat bottomed plate were pulsed for six hours with ^3H -TdR and harvested for liquid scintillation spectrophotometry. The cells in the round bottomed plate were washed one time with fresh medium. 1×10^4 ^{51}Cr -labeled, HSV-infected syngeneic or allogeneic cells were added to each of the four replicate wells. The targets were prepared as described by Lawman et al. (1980). The plates were centrifuged ($200 \times g$; 2 minutes) and incubated for three hours at 37° . The ^{51}Cr -released from the lysed cells into the supernatant fluid was then measured. Specific cytotoxic activity was determined as: $((\text{Experimental} - \text{Spontaneous}) / (\text{Total} - \text{Spontaneous})) \times 100\%$. Each determination was performed in quadruplicate. Spontaneous release did not exceed 15% of the total release. The effector to target ratio (E:T) in the microculture wells approximated 12:1 (data not shown).

Coculture Experiments

Suppressor inducer cultures consisted of HSV-immune splenocytes or nylon wool purified Thy 1⁺ cells incubated in the presence of HSV antigens as described above. Nylon wool purified cells were stimulated with HSV-infected resident peritoneal cells from syngeneic mice. In some experiments the cells were treated with specific antiserum and complement prior to viral stimulation. Normal splenocytes were added as filler cells to some of these depleted cultures. Enough filler cells were added to the depleted cultures to replace the cells lysed by the antibody treatment. After three days, the suppressor inducer cultures were irradiated (2000R, X-irradiation) and then 1×10^5 cells were added to 4×10^5 HSV-stimulated, HSV-immune splenocyte. These test cultures were incubated five days and CTL and LP activity was determined as described above.

Interleukin 2 (IL-2) Determination

Supernatant fluids from some of the cultures were analyzed for the presence of the growth hormone IL-2 using the IL-2 dependent cell line CTLL (kindly provided by Dr. K. Smith, Dartford College, NH). Purified IL-2 used for standardization of the assay was prepared according to published procedures (Smith, 1980).

Preparation of Culture Supernatant Fluids

Supernatant fluids from HSV-immune cultures were collected at various intervals after initiation of the bulk culture. The cells were

pelleted by centrifugation (500 x g, 15 minutes) and discarded. The supernatant fluid was dialyzed overnight against RPMI, filter sterilized (0.2 μ m, Acrodisc, Gelman, Ann Arbor, MI) and stored frozen at -70°C. Serum free supernatants for biochemical fractionation were prepared by incubating the splenocytes for 3 days in complete medium, pelleting the cells, and then resuspending them in serum-free RPMI-1640. Following an additional incubation of 48 hrs., the serum-free conditioned medium was concentrated by ultrafiltration on an Amicon (Lexington, MA) PM-10 membrane, and dialyzed overnight against phosphate buffered saline containing 0.05% polyethylene glycol (PBS-PEG).

Assay of Culture Supernatant Fluids

Supernatant fluids were assayed for suppressive activity by adding them to CTL induction microcultures within 24 hours of culture initiation. In some experiments the supernate was added at various intervals after culture initiation. Suppressive supernatant fluids were routinely assayed at a final concentration of 25% (v/v). In some experiments the supernates were assayed at concentrations ranging from 1 to 50% (v/v). The test cultures were assayed on the fifth day of culture for cytotoxic cell activity and lymphoproliferation.

Column Chromatography

Gel filtration chromatography was performed with Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ). Column dimensions were 100 x 1.5 cm, and the gel was equilibrated with PBS-PEG at 4°C. The column was equilibrated with molecular weight standards of 150K, 65K, and 12K

molecular weight. One ml of the concentrated serum-free suppressive supernatant fluid was applied to the column and eluted with PBS-PEG. Two ml fractions were collected, dialyzed against RPMI overnight, filter sterilized, and assayed at a final concentration of 10% using the microculture method.

Immunoaffinity columns were prepared according to published procedures (Axen et al., 1967). Immunoglobulin enriched fractions were prepared from antiserum samples by affinity chromatography using protein A conjugated to Sepharose 4B (Pharmacia). Antibodies were attached to sepharose support beads using cyanogen bromide-activated Sepharose 4B (Pharmacia). Samples to be applied to the immunoaffinity columns were first dialyzed against PBS containing 0.5M NaCl. The samples were mixed with the column gel overnight on a rocking platform at 4°C. The mixture was then applied to a 10 ml disposable column (Pharmacia) and washed with the PBS containing 0.5M NaCl until no detectable protein eluted from the column. The bound material was removed from the immunoaffinity column by the application of a low pH glycine buffer. The eluted fractions were collected directly into 0.2M Tris-base (pH 8.2). The collected fractions were dialyzed against PBS and RPMI prior to assaying.

SDS-PAGE and Western Blotting

The methods used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been described by Compton and Courtney (1984). All running gels were 7.5% acrylamide with 4% stacking gels.

Approximately equal amounts of protein were added to each well in a sample volume of 50 μ l. Protein content of the samples approximated 40 μ g. In some instances, fractions from immunoaffinity columns were concentrated to equal volumes before application to the gels. The proteins resolved by SDS-PAGE were transferred to nitrocellulose paper (Schleicher and Schiell, Inc., Keene, NH), and immunoautoradiography was performed according to published procedures (Towbin et al., 1979). Anti-I-Jk^k antiserum was purchased from Cedarlane Laboratories (Accurate Chemical Co.), anti-HSV antiserum and normal rabbit serum was kindly provided by Janet Lathey, Department of Microbiology, University of Tennessee, Knoxville.

Statistical Analysis

The results presented in this dissertation are representative of experiments that were performed at least four times. In vitro assays were always performed in quadruplicate and in vivo determination of DTH responses involved at least five mice per treatment group. Data were analyzed using Student's t test and an analysis of variance.

CHAPTER III

THE REGULATION OF HERPES SIMPLEX VIRUS-SPECIFIC CTL

INDUCTION BY SUPPRESSOR CELLS

Introduction

Cytotoxic T lymphocytes (CTL) are a subclass of thymus-derived lymphocytes which in the mouse bear Thy 1 and Lyt 23 surface antigens (Cantor and Boyse, 1975). Their pattern of activity of antigen-specific, genetically-restricted lysis of target cells (Zinkernagel and Doherty, 1979) distinguishes CTL from other cells capable of cytolysis including natural killer cells (Herberman and Ortaldo, 1981), macrophages (Morahan et al., 1977), and K cells (Welsh, 1981). Through their ability to specifically lyse virus-infected syngeneic cells, CTL play a role in immune protection and immunopathology of virus infections (Zinkernagel and Doherty, 1979). Against some viruses CTL clearly play a major role in immunity (Zinkernagel and Doherty, 1979; Ada et al., 1981), but with others, such as herpes simplex virus (HSV) the contribution of CTL is less clear.

One reason for the uncertainty surrounding the role of CTL in immunity to HSV has been the difficulty in demonstrating HSV-specific CTL in vivo (Ramshaw, 1975). Only after in vitro cultivation with viral restimulation could CTL activity against HSV-infected syngeneic targets be demonstrated (Pfizenmaier et al., 1977a; Lawman et al., 1980). Cyclophosphamide pretreatment prior to infection also enhances the expression of HSV-specific CTL activity (Lawman et al., 1980).

Conceivably, HSV-specific CTL induction is regulated by a suppressor cell (SC) which is inactivated either by in vitro cultivation (Pfizenmaier et al., 1977a) or low dose cyclophosphamide treatment (Lawman et al., 1980). This hypothesis was supported by limiting dilution analysis of HSV-specific CTL precursors (CTLp) where a biphasic response curve indicative of the activity of SC was observed (Rouse and Wagner, 1984). SC are known to regulate the induction of CTL responses to other viruses (Leung et al., 1980; Ada et al., 1981; Garaci et al., 1981) but details of the cellular interactions necessary for SC induction have not been explored for viral antigens as they have been for simple haptens and alloantigens.

In this chapter, I provide evidence that the intraperitoneal injection of HSV antigens into mice results in the induction of SC which regulates the in vitro induction of secondary HSV-specific CTL. I have characterized the cell involved in this regulation and demonstrate that SC induction involves several distinct populations of leukocytes including I-J⁺ macrophages.

Results

Pretreatment with Either Cyclophosphamide In Vivo or with Anti-I-J^k Antiserum In Vitro Enhances HSV-specific CTL Activity

Suppressor cell regulation of immune responsiveness can sometimes be identified on the basis of enhancement of the response following pretreatment either in vivo with cyclophosphamide or in vitro with anti-I-J antiserum plus complement (Pfizenmaier et al., 1977; Lagrange et

cyclophosphamide (50 mg/kg) 48 hours prior to sacrifice resulted in a significant enhancement of CTL activity (Table 1). It was also possible to enhance CTL activities in the cultures by treating the splenocytes with anti-I-J^k antiserum and complement prior to incubation indicating regulation by SC which are I-J⁺ and cyclophosphamide sensitive.

Demonstration of SC Activity in the HSV-stimulated, HSV-immune Splenocyte Cultures

To directly test for the presence of SC in the stimulated splenocyte cultures, the following co-culture experiments were performed. HSV-immune splenocytes were incubated three days with HSV antigens and then x-irradiated. The irradiated, stimulated cells were then added to HSV-CTL induction cultures at their initiation. Five days later, these test cultures were assayed for HSV-specific CTL activity. The results presented in Table 2 indicate that the three day stimulated HSV-immune splenocyte cultures contained a radioresistant SC which inhibited HSV-specific CTL induction in the test cultures. As few as 1×10^5 irradiated cells added to 4×10^5 splenocytes in the test culture suppressed CTL activity by over 50%. Non-irradiated cells caused an increase in CTL activity in the test cultures presumably due to the contribution of additional helper cells. Unstimulated HSV-immune cells failed to suppress CTL induction in the test cultures. These results indicate that in vitro stimulation of HSV-immune splenocytes with HSV antigens resulted in the induction of SC activity which inhibited CTL induction.

TABLE 1. ENHANCEMENT OF HSV-SPECIFIC CTL ACTIVITY IN VITRO BY
PRETREATMENT WITH EITHER CYCLOPHOSPHAMIDE IN VIVO OR ANTI-I-J
ANTISERUM AND COMPLEMENT IN VITRO^a

Treatment ^b	CTL Activity	
	LHSV	EHSV
None	32.1	11.3
Cyclophosphamide	53.6	16.8
Anti-I-J ^k + C'	65.2	22.0

^aSplenocytes from HSV-immune mice were incubated five days in the presence of HSV antigens and then assayed for CTL activity in a three hour ⁵¹Cr-release assay. Four replicates of syngeneic (LHSV) and allogeneic (EHSV) targets were tested. The above results are representative of experiments performed at least four times. The values presented are the percent specific ⁵¹Cr-released as determined in the methods section.

^bThe non-treatment control mice (None) were injected with PBS 48 hours prior to sacrifice. These splenocytes were also treated with rabbit complement prior to stimulation. The cyclophosphamide pretreated mice received an intraperitoneal injection of cyclophosphamide 48 hours prior to sacrifice. The anti-I-J depletion was performed prior to incubating the splenocytes with the virus.

TABLE 2. THREE DAY OLD, IRRADIATED, HSV-STIMULATED, HSV-IMMUNE SPLENOCYTES SUPPRESS HSV-SPECIFIC CTL INDUCTION IN TEST CULTURES^a

Cells Added	Number of Cells Added ($\times 10^5$)		
	1	2	3
None	22.9	32.8	22.4
Immune	21.3	28.2	24.7
Immune + HSV	35.0	38.0	24.6
Immune (2000R)	30.9	36.2	22.8
Immune + HSV (500R)	18.8	19.8	ND ^b
Immune + HSV (1000R)	21.6	5.5	ND
Immune + HSV (2000R)	11.2	0.6	8.8

^aHSV-immune splenocytes were incubated with or without HSV for three days in complete medium at 37°C. On the third day the cells were suspended in fresh medium and divided into aliquots. Some cells were x-irradiated with different dosages of X-rays. The cells were then added at various concentrations to CTL induction cultures in 96-well plates such that the total number of cells per well was 5×10^5 . The test cultures were incubated five days and the HSV-specific CTL activity was determined. The results reported are the average percent specific lysis of LHSV targets, lysis of EHSV targets by replicate wells did not exceed 13.8%.

^bND = not done.

Characterization of the SC

The SC in the three day-stimulated cultures was neither plastic nor nylon wool adherent (Table 3). In addition the SC failed to adhere to anti-immunoglobulin coated plates. However, treatment of the three day stimulated splenocytes with anti -Thy 1, -Lyt 2, or -I-J antisera but not anti-Lyt 1 antisera followed by panning onto anti-immunoglobulin coated plates removed the SC activity. These results indicated that the suppression was mediated by a T cell which expresses I-J and Lyt 2 surface antigens.

Cellular Requirements for SC Induction

To identify the cellular requirements for SC induction in vitro, HSV-immune splenocytes were fractionated into Thy 1⁺ enriched populations by passage over nylon wool. The nylon wool purified cells (> 99% Thy 1⁺) were treated with specific antisera and complement prior to incubation with HSV-infected APC. Nylon wool purified Thy 1⁺ cells from normal mice were added to the depleted cultures to replace the lysed cells. HSV-infected resident peritoneal cells, depleted of Thy 1⁺ cells by antibody and complement treatment, were used as APC. After three days incubation with the APC, the cells were irradiated and added to test cultures at their initiation. The test cultures consisted of unseparated HSV-immune splenocytes incubated with HSV for five days. Non-depleted suppressor induction cultures developed SC activity which inhibited by 48% the CTL induction in the test cultures (data not shown). The nylon wool purified cells generated suppressor activity only when stimulated with HSV-treated APC (Table 4). Active, UV-

TABLE 3. IDENTIFICATION OF A T SUPPRESSOR CELL IN HSV-STIMULATED, HSV-IMMUNE SPLENOCYTE CULTURES^a

Population added to CTL induction cultures	CTL Activity	
	LHSV	EHSV
None	38.5	11.2
Plastic nonadherent	15.4	3.2
Nylon wool nonadherent	12.6	4.9
Anti-Ig nonadherent	11.8	3.8
Anti-Thy 1.2 nonadherent	42.4	15.8
Anti-Lyt 1.1 nonadherent	19.7	9.9
Anti-Lyt 2.1 nonadherent	35.2	10.4
Anti-I-J ^k nonadherent	34.9	11.9

^aHSV-stimulated, HSV-immune splenocytes were incubated three days to induce suppressor cell activity. The cultures were then depleted of various subpopulations based on adherency or antigen expression. The remaining cells were then irradiated (2000R, x-rays) and subsequently added to CTL induction cultures. The test cultures consisted of 4×10^5 HSV-immune splenocytes incubated with HSV and 1×10^5 cells from the suppressor induction cultures. The test cultures were incubated five days and CTL activity against syngeneic (LHSV) and allogeneic (EHSV) targets was determined. Each value represents the mean percent specific ⁵¹Cr-released for four replicate wells.

TABLE 4. THE CELLULAR REQUIREMENTS FOR THE INDUCTION OF T SUPPRESSOR CELLS IN HSV-STIMULATED, HSV-IMMUNE T CELL CULTURES^a

Immune T	Cells Added		CTL Activity ^b	
	Normal T	APC	LHSV	EHSV
-	-	-	24.8	3.5
+	-	-	28.2	4.6
+	-	+	10.2	7.7
Lyt 1-	-	+	25.7	8.6
Lyt 1 ⁻	+	+	11.1	4.4
Lyt 2 ⁻	-	+	28.5	5.2
Lyt 2 ⁻	+	+	24.3	8.3
I-J ⁻	-	+	21.9	8.1
I-J ⁻	+	+	19.3	2.8

^aSC induction cultures were prepared using nylon wool purified HSV-immune T cells as described in the text and in the Materials and Methods. Some of the SC induction cultures were depleted of Lyt 1⁺ or Lyt 2⁺ or I-J⁺ cells prior to incubation with the virus. Normal nylon wool purified Thy 1⁺ cells were added to some of the depleted cultures in order to replace the cells which were lysed by the antibody and complement treatment. Thy 1-depleted HSV-stimulated peritoneal cells (APC) from normal mice were used to stimulate the cultures. APC negative cultures received free HSV. As before, the cultures were irradiated on the third day and then added to test cultures. 1×10^5 SC were added to 4×10^5 cells of the test culture.

^bThe results represent the mean specific ^{51}Cr released for four replicate wells of syngeneic (LHSV) and allogeneic (EHSV) targets.

inactivated, and HI-HSV preparations induced equivalent levels of SC activity (data not shown). For the remainder of the experiments HI-HSV was used to induce SC activity. In the absence of added APC, free HSV failed to generate suppression. Pretreatment of the suppressor induction cultures with either anti-Lyt 1.1, -Lyt 2.1, or -I-J^k antisera and complement abrogated SC activity. It was possible to restore SC activity in the Lyt 1.1-depleted suppressor induction cultures by replacing the lysed cells with Thy 1⁺ cells from normal mice. In contrast, normal Thy 1⁺ cells could not restore SC activity to the Lyt 2- or I-J-depleted cultures. Thus to generate suppression both the Lyt 2⁺ and the I-J⁺ cells needed to be expanded by antigen exposure in vivo as well as in vitro. This requirement for both in vivo activation and in vitro HSV-stimulation for SC induction resembles the requirement for HSV-specific CTL induction (Lawman et al., 1980).

Characterization of the APC Involved in SC Induction

Macrophages play a major role in the induction of SC (Ptak et al., 1978; Nakamura et al., 1982). Thus I-J⁺ macrophages have been shown to present antigen and soluble factors to suppressor T cells (Dorf and Benacerraf, 1984). We next characterized the APC in the HSV-specific SC induction cultures. APC were treated with specific antisera and complement before infection with HSV and added to the nylon wool nonadherent cells. Upon depletion of the APC population of either I-E⁺ or I-J⁺ cells prior to HSV exposure, SC activity was not generated in the nylon wool nonadherent suppressor induction cultures (Table 5).

TABLE 5. APC REQUIREMENT FOR THE INDUCTION OF T SUPPRESSOR CELLS IN HSV-STIMULATED, HSV-IMMUNE T CELL CULTURES^a

APC Added	CTL Activity ^b	
	LHSV	EHSV
C'	10.2	1.2
Anti-I-A	8.7	3.1
Anti-I-E	23.8	8.0
Anti-I-J	23.4	5.5
None	22.6	9.2

^aNylon wool purified T cell cultures were incubated with HSV-infected Thy 1⁺ APC from normal mice. The APC population was treated with various antisera and complement prior to virus infection. The suppressor inducer cultures stimulated with these various APC populations were tested on the third day for suppressor cell activity as described in the preceding tables.

^bHSV-specific CTL activity in the test cultures was determined on the fifth day. The results are the average ⁵¹Cr-released by four replicate cultures for syngeneic (LHSV) and allogeneic (EHSV) targets.

Conversely IA⁺ depletion of the APC population actually enhanced the suppressor cell induction.

The Suppression Mediated by the HSV-induced SC is Non-specific

The above results indicated that the induction of SC requires several leukocyte populations. Some of these cells had to come from HSV-primed mice, suggesting a requirement for in vivo activation. Suppressor cell induction also required stimulation with HSV-antigens in the context of either I-J or I-E. Together these results indicate that HSV-induced suppressor activity is specifically induced following re-exposure to the virus. In order to determine if the suppression mediated by these SC was also specific, three-day induced SC were added to influenza-specific CTL induction cultures at their initiation. Addition of 1×10^5 irradiated HSV-induced SC inhibited influenza-specific CTL induction by 58% (Table 6), the same cells added to HSV-specific CTL induction cultures caused 43% suppression (data not shown). It is also evident from Table 6 that the cells involved in the SC activity against influenza appear to be I-J⁺, T cells.

Discussion

Although several recent observations indicate that immune responses to viruses are regulated by SC (Greene and Weiner, 1980; Leung et al., 1980; Nash and Gell, 1980; Schrier et al., 1983), direct evidence for this notion needs to be established. In this chapter it is shown that the in vitro induction of anti-HSV CTL by primed splenocytes is regulated in part by an Lyt 2⁺ I-J⁺ T cell. Furthermore, several

TABLE 6. HSV-INDUCED SUPPRESSOR CELLS MEDIATE NON-SPECIFIC SUPPRESSION OF INFLUENZA-SPECIFIC CTL INDUCTION^a

Cells Added	Percent Suppression ^b
None	0
Anti-Ig nonadherent	58.2
Anti-Thy 1.2 nonadherent	4.5
Anti-I-Jk nonadherent	15.5

^aHSV-stimulated, HSV-immune splenocytes were incubated three days to induce suppressor cell activity. The cultures were then incubated with various antisera and then panned on anti-Ig coated plates. The nonadherent cells were gently removed and then assayed for suppressor cell activity; 1×10^5 suppressor cells were added to 4×10^5 influenza-stimulated immune splenocytes. The influenza-specific test cultures were incubated five days and CTL activity against syngeneic and allogeneic targets was determined. The specific CTL activity of the untreated control cultures was 14.8%. Each value represents the mean percent suppression for four replicate wells.

^bPercent suppression was calculated as $((\text{Control} - \text{Experimental}) / \text{Control}) \times 100\%$.

distinct leukocyte populations are involved in the generation of the SC. These results provide the first direct evidence for a suppressor cell regulating the induction of HSV-specific CTL.

The demonstration of an anti-HSV CTL response has been problematical and several observations have indicated that suppressor mechanisms exist which could account, in part, for the difficulty in measuring CTL. Thus, in the initial report of an anti-HSV CTL response, it was necessary to culture primed cells for a period in vitro before CTL activity became detectable (Pfizenmaier et al., 1977a). Furthermore, the response was elevated by administering cyclophosphamide prior to antigen exposure (Pfizenmaier et al., 1977b). In this chapter it was demonstrated that cyclophosphamide administration to primed animals also enhances antigen-specific secondary CTL responses. These results indicate that the induction of secondary HSV-specific CTL is also regulated by SC. To obtain direct evidence for the operation of SC in the CTL response, I exploited observations made in several nonviral systems that most SC express I-J alloantigens (Murphy, 1978). When I-J+ cells were removed from CTL induction cultures prior to antigen stimulation, the MHC restricted CTL response was increased significantly. Thus during an ongoing secondary in vitro CTL response, I-J+, cyclophosphamide sensitive SC are presumably also generated. It was logical to suppose that the activity of such effector SC might be best demonstrated if the cells were transferred to a test CTL induction culture at its initiation. Using this approach, reproducible suppressor activity (up to 90%) was obtained. The SC mediating the suppression was identified as Thy 1+ Lys 2+ I-J+. Cells with a similar phenotype

mediate suppression in several nonviral systems and have been referred to as efferent suppressor cells (Germain and Benacerraf, 1981; Green et al., 1983; Dorf and Benacerraf, 1984; Asherson et al., 1985). In such cases the actual suppressor mechanism may be due to the production of a non-specific soluble factor by other SC (Zembala et al., 1982) and this appears to be the case in the anti-HSV CTL system. Evidence for an anti-idiotypic, I-J+ HSV-specific suppressor factor produced by HSV-immune Lyt 2+ I-J+ T cells which induces the production of non-specific suppressor factors HSV-immune splenocytes is presented later.

The generation of suppressor activity involved several cell types presumably interacting as a circuit such as has been suggested to occur for a number of nonviral systems (Germain and Benacerraf, 1981). At least two distinct T cell subpopulations were required for SC induction. Thus SC activity was not generated if Lyt 1, Lyt 2, or I-J+ cells were depleted prior to antigen stimulation. The Lyt 1 and Lyt 2 cells were considered distinct since whereas the requirement for the Lyt 1+ cell in the induction culture could be replaced by nonimmune T cells, this was not the case for the Lyt 2+ cell. Because the requirement for the I-J+ cell could also not be replaced by normal T cells, I suspect that the Lyt 2+ cell additionally expressed I-J.

In addition to the requirement for the T cell subpopulations, HSV-infected antigen presenting cells (APC) were also necessary for SC induction. Thus nylon wool purified Thy 1+ cells failed to generate SC activity unless stimulated with HSV-infected Thy 1- APC. The APC involved were identified as I-J+ I-A- I-E+ by depletion with specific

antisera and complement. The role of APC in the induction of SC in nonviral systems has only recently been determined (Usui et al., 1984; Granstein and Greene, 1985; Noma et al., 1985). However, most of these studies have not demonstrated unequivocally that the APC population necessary for SC induction was distinct from the APC required for the induction of immunity. In fact, several groups had reported that I-A⁺ APC were required for both SC and T helper cell induction (Sherr et al., 1980; Usui et al., 1984; Noma et al., 1985; Granstein and Greene, 1985). In the HSV system, cells required for helper cell induction (I-J⁻ I-A⁺) (Schmid et al., 1981) appear distinct from those which are involved in SC induction.

The evidence for the operation of a cell circuit to generate SC remains indirect until suitable hybridomas or T cell clones are developed, one cannot indicate how the three cell types interact directly in the circuit. It will be important to establish if the regulatory suppressor mechanisms involved in CTL induction resemble those regulating anti-HSV delayed type hypersensitivity responses (Nash and Gell, 1983; Schrier et al., 1983). It is of interest to note that the suppression in these cases also involves Lyt 1⁺ and Lyt 2⁺ cells. However, I suspect that the mechanisms differ since their Lyt 1⁺ SC was only demonstrable in cell populations from HSV-tolerized animals. In contrast, in the system described here, HSV-naive Lyt 1⁺ cells functioned in the generation of suppression.

It is important to understand how the SC system is activated and expresses its activity in vitro since clues may emerge how to manipulate the system that will prove of value in vivo. For example, SC could

serve to inhibit protective aspects of immunity prior to the development of recrudescence. There is some evidence that helper to suppressor T lymphocyte ratios do change around the time of recrudescence in man (Sheridan and Aurelian, 1982) and recently it has been shown in the guinea pig model of HSV type 2 recrudescence that SC are present in the spleen at the time of recrudescence (Iwasaka et al., 1983). If SC and their products are indeed involved in the modulated immunity to HSV in man, this could provide a useful target for treatment aimed at breaking the cycle. Whether or not the operation of a similar suppressor circuit accounts for the variation of host responsiveness that accompanies the development of herpesvirus recrudescence requires further investigation.

Summary

The incubation of HSV-immune murine splenocytes with HSV antigens resulted in the induction of suppressor cells which inhibited HSV-specific and influenza-specific CTL induction. The cell mediating the suppression was a Thy 1⁺, Lyt 2⁺, I-J⁺ T cell. The induction of the suppressor T cell required the participation of at least three different leukocyte populations. Thus, depleting the culture of either Lyt 1⁺ or Lyt 2⁺ splenocytes resulted in a failure to induce suppressor cell activity. Likewise the removal of macrophage-like APC, in particular I-A⁻, I-J⁺, I-E⁺ APC, resulted in an abolition of suppressor cell activity in the cultures. Though the Lyt 2⁺ cells had to be derived from HSV-immune mice, both the APC and the Lyt 1⁺ cells could be provided by HSV-

naive mice. The involvement of these different cell types in the induction of a non-specific suppressor cell indicates the existence of a suppressor cell circuit. The implication of these results for the understanding and future manipulation of herpesvirus disease is briefly discussed.

CHAPTER IV

HERPES SIMPLEX VIRUS-SPECIFIC LYMPHOPROLIFERATION BY MURINE SPLENOCYTES: CHARACTERIZATION OF THE PROLIFERATING POPULATIONS AND THEIR REGULATION BY SUPPRESSOR CELLS

Introduction

Studies of animal models for human viral diseases have clearly demonstrated the critical role that cell mediated immunity (CMI) plays in recovery from various infections (Rouse, 1984). Thus the murine model for herpes simplex type 1 (HSV) has provided valuable information regarding the role of cytotoxic T lymphocytes (CTL) and delayed type hypersensitivity reactions (DTH) in recovery from these infections (Pfizenmaier et al., 1977; Lawman et al., 1980; Nash et al., 1980; Kirchner, 1982; Schrier et al., 1982; Sethi et al., 1983; Larsen et al., 1984). In humans, as in the mouse, aspects of cell mediated immunity (CMI) play a major role in immunity to herpesvirus infections (Kirchner, 1982; Shore and Nahmias, 1982). The principal means of assessing anti-HSV specific CMI is the lymphoproliferation assay (LP). However, previous attempts to use the LP assay to measure HSV-specific CMI in the mouse have proven inconclusive (Kirchner, 1982). One reason for this inconclusiveness is the apparent mitogenicity of some strains of the virus for murine B lymphocytes (Kirchner et al., 1978; Mochizuki et al., 1981), however, even when mitogenicity is precluded, LP results often are not reproducible (Kirchner, 1982). The reasons for this are not

clear. One possible explanation is that there are suppressor cells which regulate the LP response to HSV antigens. Evidence for suppressor cell regulation of HSV-specific LP was in fact reported in a guinea pig model for HSV type 2 infection (Iwasaka et al., 1983).

In man, recrudescence disease is a characteristic feature of herpesvirus infections and many have assumed that recrudescence occurs because of some subtle alteration in the effectiveness of antiviral immunity (Sheridan et al., 1982). Currently, one idea courting favor is that changes in immunoregulation resulting from suppressor cell activity would precede lesion development (Shillito et al., 1977). Support for this idea includes the observation that changes in the ratio of suppressor to helper T cells may occur around the time of recrudescence (Sheridan et al., 1982).

In this chapter, the value of LP as a measure of T cell mediated immunity to HSV in the mouse was reassessed and the conditions for reproducible, antigen-specific T cell mediated LP response to HSV antigens are defined. The phenotype of the proliferating population is determined and an attempt is made to correlate LP with DTH and CTL activity. Evidence is also presented that the LP response to HSV is regulated by suppressor T cells.

Results

Comparison of Various Preparations of HSV Antigens to Stimulate Lymphocyte Proliferation

Splenocytes from normal and HSV-immune mice were incubated with active, UV-inactivated (UV-HSV), and heat-inactivated (HI-HSV)

preparations of HSV (moi = 1.0 before inactivation) and the incorporation of ^3H -TdR by the splenocytes determined at daily intervals. Each preparation of viral antigen tested induced significant levels of ^3H -TdR incorporation by HSV-immune, but not by the splenocytes from normal mice. The characteristic time course of this antigen-specific proliferation is shown in Figure 2.

Phenotypic Characterization of the Proliferating Population

When UV-HSV stimulated splenocytes were examined on the fifth day for the proportion of ^3H -TdR incorporated by surface immunoglobulin negative (sIg⁻) and positive (sIg⁺) cells, it was observed that sIg⁻ cells accounted for the majority of the incorporation (Table 7). Similar results were obtained for active and HI-HSV stimulated cultures (data not shown). Both positive and negative selection procedures were employed to further characterize the phenotype of the proliferating population. ^3H -TdR pulsed sIg⁻ cells were incubated with various antisera and then panned on anti-Ig coated plates and the amount of ^3H -TdR associated with the adherent and non-adherent populations was determined (Table 7). As expected almost all of the incorporated ^3H -TdR was associated with those cells which adhered to the plates after treatment with anti-Thy 1.2 antiserum. When the cells were treated with anti-Lyt 1.1 approximately 60% of the ^3H -TdR activity adhered to the anti-Ig plates, conversely anti-Lyt 2.1 pretreatment resulted in approximately 40% of the activity remaining bound to the plates. Since the ^3H -TdR associated with the adherent cells after anti-Lyt 1.1 and anti-Lyt 2.1 treatment approximated the total ^3H -TdR incorporated by the

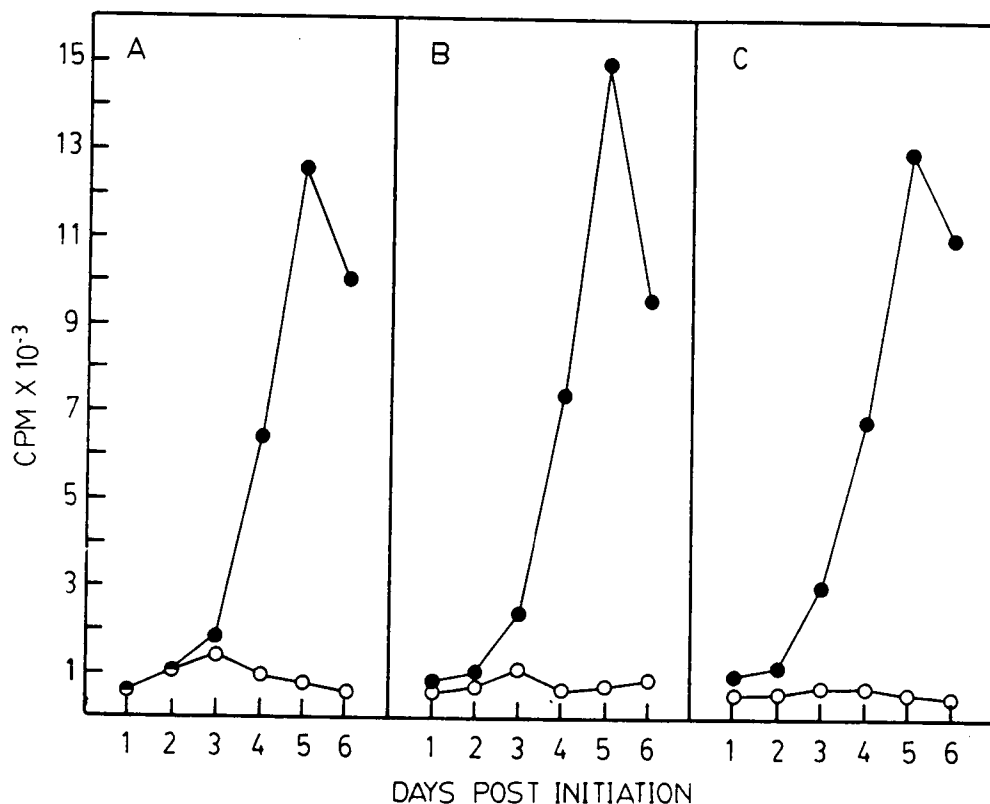


Figure 2. Time course of ^3H -TdR incorporation. Five $\times 10^5$ normal (o) and HSV immune (●) splenocytes were incubated with active (A), UV-inactivated (B), and heat-inactivated (C) preparations of HSV in 0.2 ml of medium in 96-well microtitre plates. At daily intervals the cells were pulsed for four hours with 0.5 uCi of ^3H -TdR and harvested onto glass fiber filters for liquid scintillation counting. Each point represents the mean of four determinations.

TABLE 7. IDENTIFICATION OF THE PROLIFERATING CELLS USING POSITIVE SELECTION PROCEDURES^a

Antisera ^b	Adherent	Non-Adherent
None	3,140	42,940
Anti-Thy 1.2	38,707	875 ^c
Anti-Lyt 1.1	27,540	12,260 ^c
Anti-Lyt 2.1	15,696	24,778 ^d

^a 1×10^7 HSV-immune splenocytes were incubated with UV-inactivated HSV for five days. The cells were then washed and incubated with 4.0 uCi. of ^3H -TdR for four hours at 37° C. The radiolabeled cells were washed and panned onto rabbit anti-mouse Ig coated plates for 1 hour at room temperature. The non-adherent cells (sIg⁻) were gently removed with a pipette. The adherent cells were removed by vigorous pipetting with HBSS containing versene. The amount of [^{3}]H-TdR associated with each population was determined after transferring the cells to 96 well plates for harvesting. Each value represents the average from 4 replicate panning efforts.

^b Radiolabeled sIg⁻ cells were incubated with specific antiserum for 45 minutes on ice prior to panning. The incubation of these cells in the anti-Ig coated flasks was performed at 4° C in order to prevent capping of the antibody. The amount of radioactivity associated with the adherent and non-adherent cells was determined as described above.

^c Significantly different from the untreated controls at $p < .01$.

^d Significantly different from the untreated controls at $p < .05$.

sIg⁻ splenocytes, it appeared that these two cell types accounted for the majority of the lymphoproliferation induced by UV-irradiated virus preparations. This conclusion was supported by negative depletion experiments in which the splenocytes were treated with specific antisera and complement just prior to ³H-TdR pulsing (Table 8). Depleting the cultures of Thy 1⁺ cells resulted in an almost total abrogation of ³H-TdR incorporation by the cultures. Removal of either Ig⁺ or Ia⁺ cells only slightly reduced the level of incorporation. Treatment with the anti-Lyt antisera again indicated that Lyt 1⁺ cells accounted for 60% of the proliferating activity and Lyt 2⁺ cells accounted for the remainder. These results were in agreement with the results obtained from the panning experiments (Table 7).

The culture conditions employed for the above experiments can sometimes result in the generation of natural killer (NK) cell activity as indicated by non-MHC restricted cytolytic activity. Splenocytes were depleted of NK cells by treatment either in vivo or in vitro with anti-asialo GM₁ antiserum to assess the contribution of NK cells to the proliferative response. An intravenous injection of 0.1 ml of antiserum (diluted 1/5) 18 hours prior to collecting the splenocytes effectively abrogated NK activity as assessed by the killing of the NK-sensitive YAC cells (data not shown). Pretreatment with the antiserum neither affected the total level of ³H-TdR incorporation by the splenocytes, nor the distribution of the isotope between the Lyt 1⁺ and Lyt 2⁺ populations (data not shown). Similarly, anti-asialo GM₁ and complement treatment of cells prior to ³H-TdR pulsing also failed to affect the level of incorporation (Table 8).

TABLE 8. IDENTIFICATION OF THE PROLIFERATING CELLS USING NEGATIVE SELECTION PROCEDURES^a

Antisera	³ H-TdR	% Total
None	17,545	100.0
Anti-Thy 1.2	1,713 ^b	9.7
Anti-Lyt 1.1	8,305 ^c	42.1
Anti-Lyt 2.1	10,942 ^c	62.4
Anti-Ig	15,850	90.3
Anti-Ia ^k	15,650	89.2
Anti-asialo GM ₁	17,405	99.2

^aHSV-immune splenocytes were incubated with UV-inactivated HSV for five days. The cells were then washed and incubated with specific antiserum and complement prior to pulsing with ³H-TdR. Each value represents the average cpm for four replicate wells per treatment group. The % Total was calculated as the level of ³H-TdR incorporated by the treated culture divided by the ³H-TdR incorporated by the complement control.

^bSignificantly different from the complement control at $p < .01$.

^cSignificantly different from the complement control as $p < .05$.

Association of Lymphoproliferation with T Cell Effector Functions

The immunization protocol and the in vitro conditions used in these experiments results in the production of both HSV-specific CTL and cells that mediate DTH responses (T_{DTH}) (Lawman et al., 1980; Larsen et al., 1984). The former cells have the phenotype $Thy\ 1^+ Lyt\ 1^{-}2^+$ and the latter are $Thy\ 1^+ Lyt\ 1^{-}2^-$. Since the relationship between LP and CMI in vivo is unclear, it was of interest to determine if proliferation could be correlated with the induction of either CTL or T_{DTH} .

The contribution of $Lyt\ 2^+$ cells to the proliferative response could imply a correlation between lymphoproliferation and CTL activity. However, when the incorporation of ^3H -TdR and the CTL activity of individual wells was analyzed using regression analysis, significant levels of correlation were not observed ($R^2 = 0.01$, $p > 0.5$). To further test the relationship between CTL induction and LP, the splenocytes were stimulated with a viral preparation (HI-HSV) which failed to induce CTL activity. As presented in Table 9, although the HI-HSV failed to stimulate CTL activity, it nevertheless induced significant levels of ^3H -TdR incorporation (see also Figure 2). The level of incorporation by the $Lyt\ 2^+$ cells was only slightly diminished when HI-HSV was compared to UV-HSV stimulation.

The relationship between LP and T_{DTH} induction was examined exploiting the fact that the intravenous injection of HSV results in the tolerization of only the HSV-specific DTH response (Nash and Ashford, 1982; Schrier et al., 1982). When the splenocytes from tolerized mice were stimulated in vitro with HSV they failed to adoptively transfer HSV-specific DTH reactivity (Table 10; Nash and Gell, 1981). Antigen

TABLE 9. COMPARISON OF VARIOUS VIRUS PREPARATIONS TO STIMULATE CTL ACTIVITY AND PROLIFERATION^a

	Total CPM	Antisera to ^b		% specific 51Cr-release	
		Lyt 1	Lyt 2	LHSV ^c	EHSV ^c
Live	16,030	4,384	9,772	42.3	14.1
UV-inactivated	13,636	5,194	7,222	48.9	10.5
Heat inactivated	15,662	5,448	9,564	7.7	5.2

^aHSV-immune splenocytes were incubated with various preparations of HSV antigens. On the fifth day the cells were tested for ³H-TdR incorporation and CTL activity. The results are the average of four replicate cultures per treatment group.

^bSplenocyte cultures were treated with specific antiserum and complement prior to pulsing with the ³H-TdR.

^cOne x 10⁴ 51Cr-labelled, HSV-infected syngeneic (LHSV) and allogeneic (EHSV) targets were added to the five day old splenocytes for a three hour cytolytic assay. Percent 51Cr-release was determined as described in the Methods section.

TABLE 10. EFFECT OF TOLERIZATION ON DTH RESPONSES AND PROLIFERATIVE ACTIVITY^a

	DTH ^b	Adoptive Transfer ^c	Proliferation	% Incorporated ³ H-TdR ^e		
				sIg ⁺	Lyt 1 ⁺	Lyt 2 ⁺
Immune	0.45	0.30	18,160	8.5	59.5	36.6
Tolerant	0.05	0.10	16,126	14.3	41.1	45.4

^aMice were injected intravenously (tolerized) or subcutaneously (immune) with 1×10^6 PFU of HSV. One week later both groups received 1×10^6 PFU subcutaneously. Seven days later both groups were either challenged with 2×10^5 PFU in the pinna or sacrificed for in vitro culture.

^bDTH results are expressed as the difference in swelling (mm) between virus challenged right ears and sham challenged left ears at 24 hours post challenge. Five mice were used for each treatment. Differences greater than 0.15 mm are considered significant.

^cThe splenocytes from tolerant and immune mice were cultured for five days with UV-HSV. The stimulated cells were then used in adoptive transfer experiments as described in the Methods.

^dThe five day stimulated immune and tolerant splenocytes were pulsed with ³H-TDR and then fractionated into various subpopulations using the positive selection procedure described in the footnote to Table 7.

stimulated tolerized splenocytes also incorporated significantly ($P < 0.05$) less ^3H -TdR than immune controls and the pattern of responsiveness differed. Thus levels of ^3H -TdR incorporation by Lyt 1^+ and Lyt 2^+ were approximately equal and sIg $^+$ cells accounted for significantly more ^3H -TdR than did the sIg $^+$ cells from immune controls. Since the relative amount of radiolabel associated with Lyt 2^+ cells was the same for both groups of mice, these results indicate tolerization resulted in an overall decrease in the proliferation of Lyt 1^+ cells. However, Lyt 1 proliferation was merely reduced, not abrogated by the tolerization. Presumably the remaining proliferating Lyt 1^+ cells represented helper T cells. To test this notion, tolerized and immune splenocytes were antigen stimulated and supernatant fluids were assayed for the helper cell product interleukin 2 (IL-2); both tested positive. To determine whether Lyt 1^+ or Lyt 2^+ cells were producing the IL-2, the bulk splenocyte cultures were depleted by negative selection of one cell type or the other on the fourth day and the supernatant harvested on the fifth day. As expected Lyt 1^+ but not Lyt 2^+ depletion prevented IL-2 production in both tolerized and immune cultures (Table 11). Thus tolerization failed to affect IL-2 producing helper cell function.

Regulation of the LP Response to HSV

The part played by Lyt 1^+ and Lyt 2^+ cells in the LP response was further analyzed by depleting a given cell type from the cultures prior to antigen stimulation then testing levels of ^3H -TdR incorporation after five days of culture. Treatment with anti-Lyt 1.1 and complement to remove the Lyt 1^+ cells completely abrogated lymphoproliferation (Table

TABLE 11. PRODUCTION OF IL-2 BY LYT 1+ CELLS^a

Culture	Antisera	Units of IL-2 ^b
Immune	-	16
	anti-Lyt 1	<1
	anti-Lyt 2	32
Tolerant	-	32
	anti-Lyt 1	<1
	anti-Lyt 2	16

^aSplenocyte cultures were incubated for 4 days in the presence of HSV antigens and then washed one time and resuspended in cytotoxicity medium containing antiserum. After incubating the cells for 45 minutes on ice, the cells were pelleted and resuspended in cytotoxicity medium containing rabbit complement. The cells were incubated an additional 30 minutes at 37° C and then washed several times with cytotoxicity medium. After the final wash the cells were counted and resuspended at 4 x 10⁶ cells per ml. in RPMI. 2.5 mls. of the cells were incubated in 6-well cluster plates (Costar) for 24 hours at 37° C. Afterwards the supernatant was collected and later assayed for IL-2 activity.

^bA unit of IL-2 is designated as that dilution of the culture supernatant which resulted in 50% of the maximal ³H-TdR incorporation by CTLL cells when incubated with our reference IL-2 stock.

12). But the response could be partially restored by the addition of exogenous lymphokines. Presumably the partially restored LP represented Lyt 2⁺ proliferation and in fact, the level of ³H-TdR incorporated by the Lyt 1⁻ cultures approximated that achieved by Lyt 2⁺ cells in the undepleted cultures (compare Table 7 with Table 12).

When the splenocytes were depleted of Lyt 2⁺ cells the levels of ³H-TdR incorporated by the remaining cells were not diminished but instead were significantly greater than that of the untreated controls (Table 12). Thus in the absence of Lyt 2⁺ cells, the remaining cells exhibited enhanced proliferation on the fifth day of culture. One interpretation of these results is that an Lyt 2⁺ suppressor cell regulates the proliferative response of the other cells to antigen. Further support for this hypothesis was obtained by removal of I-J⁺ cells prior to culture. The I-J antigen is a well known marker of certain subsets of suppressor T cells (Murphy, 1978). As shown in Figure 3, removal of either Lyt 2⁺ or I-J⁺ cells both provided a significant enhancement of ³H-TdR incorporation. This was particularly marked on the sixth and seventh days of culture. These results support the role of a Lyt 2⁺ I-J⁺ suppressor cell regulating the LP response.

Identification of the T Suppressor Cell Regulating the LP Response to HSV

To better identify the suppressor cell regulating the LP response to HSV, the following co-culture experiments were performed. HSV-immune splenocytes were incubated with HI-HSV for three days and then separated into various subpopulations based on adherence and antigen expression. The selected cells were then irradiated (2000R, X-irradiation) and added

TABLE 12. LYT 1 DEPLETION INHIBITS PROLIFERATION WHILE LYT 2 DEPLETION CAUSES AN ENHANCEMENT^a

Treatment	³ H-TdR Incorporation
-	18,125
Anti-Lyt 1.1	895 ^b
Anti-Lyt 1.1 + IL-2	18,650
Anti-Lyt 2.1	19,685 ^c
C	18,650

^aHSV-immune splenocytes were depleted of Lyt 1⁺ or Lyt 2⁺ cells prior to incubation with HSV. 20 units of IL-2 were added to some of the Lyt 1⁻ cultures at their onset. All cultures were incubated 5 days and pulsed with ³H-TdR six hours prior to harvesting. Each value represents the average of four replicate wells.

^bThe results are significantly different from the complement control at $p < 0.001$.

^cThe results are significantly different from the complement control at $p < 0.01$.

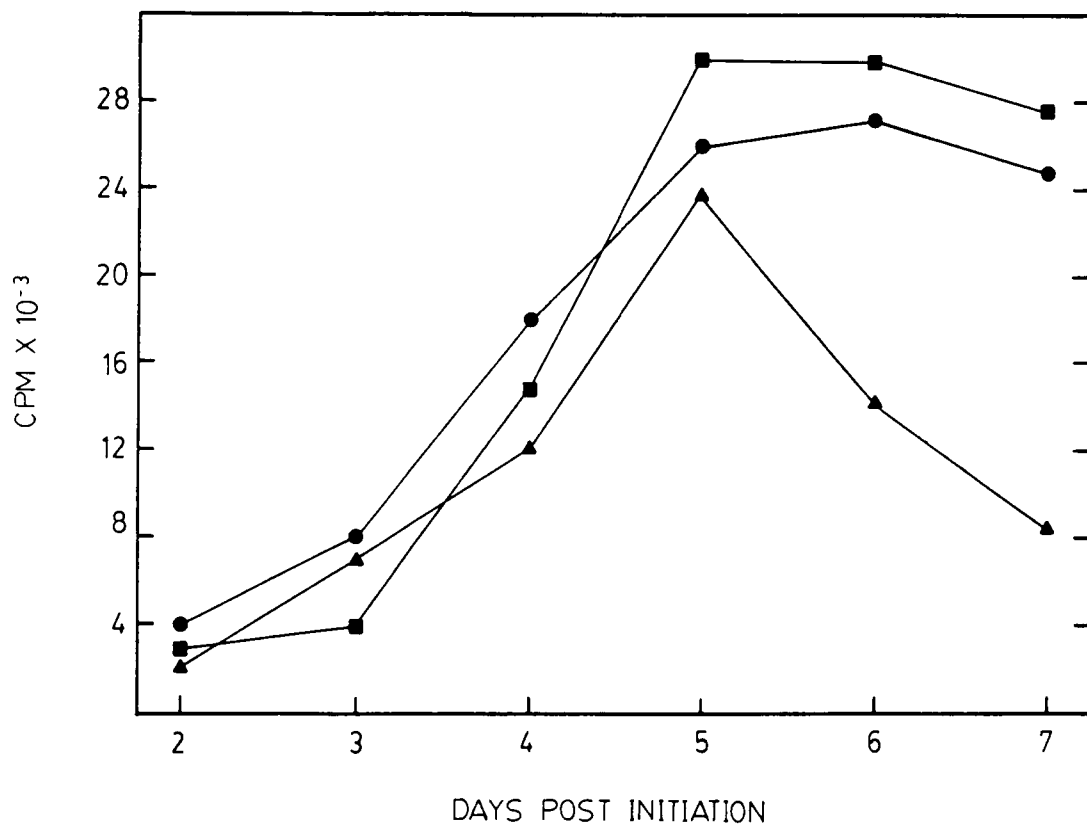


Figure 3. Time course of ^3H -TdR incorporation by depleted splenocyte cultures. Splenocytes were obtained from immune mice and were depleted of Lyt 2⁺ (■) or I-J⁺ (●) cells prior to culturing. Controls were treated with complement alone (▲). ^3H -TdR incorporation was determined at daily intervals. Each point represents the mean for four replicates.

to test cultures at their initiation. On the fifth day of culture the test cultures were pulsed with ^3H -TdR and harvested as described above. As shown in Table 13, three day old, x-irradiated cells from HSV-stimulated, HSV-immune splenocyte cultures markedly (up to 90%) suppressed the incorporation of ^3H -TdR by the test cultures. The suppressor cells were neither plastic- nor nylon wool-adherent and expressed the Thy 1.2, Lyt 2.1 and the I-J antigens.

Whereas suppressor cells from antigen stimulated immune cultures markedly suppressed LP, Lyt 2⁺ cells from non-immune or unstimulated HSV-immune mice failed to inhibit the LP response in the test cultures (data not shown). This requirement for both in vivo activation and in vitro HSV-stimulation for suppressor cell induction resembles the requirement for HSV-specific CTL induction (Lawman et al., 1980).

The Cellular Requirements for Suppressor Cell Induction

The next series of experiments were designed to identify the cellular requirements for suppressor cell induction in vitro. HSV-immune splenocytes were treated with specific antisera and complement prior to incubation with HSV. After three days incubation with the virus, the cells from these suppressor induction cultures were irradiated and added to test cultures at their initiation. The test cultures consisted of unseparated HSV-immune splenocytes incubated with HSV for five days and pulsed with ^3H -TdR six hours prior to harvesting. Non-depleted suppressor^{*} induction cultures developed suppressor cell activity which inhibited the proliferation in the test cultures by 75%. Pretreatment of the suppressor induction cultures with either anti-Lyt

TABLE 13. A T SUPPRESSOR CELL IN HSV-STIMULATED, HSV-IMMUNE SPLENOCYTE CULTURES REGULATING LYMPHOPROLIFERATION^a

Cells Added	³ H-TdR Incorporation
None	22,150
Plastic nonadherent	2,235 ^b
Nylon wool nonadherent	1,350 ^b
Anti-Thy 1.2	24,785
Anti-Lyt 1.1	4,550 ^b
Anti-Lyt 2.1	26,550
Anti-I-J ^k	23,450

^aHSV-stimulated, HSV-immune splenocytes were incubated three days to induce suppressor cell activity. The cultures were then depleted of various subpopulations based on adherency or antigen expression. The remaining cells were then irradiated (2000R, x-rays) and subsequently added to test cultures. The test cultures consisted of 4×10^5 HSV-immune splenocytes incubated with HSV and 1×10^5 cells from the suppressor induction cultures. The test cultures were incubated five days and pulsed with 0.5 uCi of ³H-TdR six hours before harvesting. Each value represents the average cpm for four replicate wells.

^bThe results are significantly different from the non-depleted control at $p < 0.001$.

1.1, -Lyt 2.1, or -I-J^k antisera and complement abrogated suppressor cell activity (Table 14). It was possible to restore suppressor cell activity in the Lyt 1.1⁻ suppressor cell induction cultures by replacing the lysed cells with splenocytes from normal mice. In contrast, normal splenocytes could not restore suppressor cell activity to the I-J⁻ or Lyt 2⁻ cultures. Thus Lyt 2⁺ and I-J⁺ cells needed to be expanded by antigen exposure in vivo (as well as in vitro) to generate suppression.

To further analyze the role of various cell types in suppressor cell induction, nylon wool nonadherent cells (consisting of >99% Thy 1⁺ cells) were used for the suppressor cell induction cultures. Such cells generated suppressor activity only when stimulated with HSV-treated peritoneal cells to act as antigen presenting cells (APC) (Table 15). In the absence of added APC, antigen stimulation failed to generate suppression. Upon depletion of either Lyt 1⁺ or Lyt 2⁺ or I-J⁺ cells from these cultures prior to antigen stimulation suppressor cell activity was not generated. Once again it was possible to restore suppressor cell activity to the Lyt 1⁻ cultures by the addition of normal T cells. Normal T cells would not, however, restore suppressor activity to either the I-J⁻ or Lyt 2⁻ cultures.

Several groups have reported that the APC involved in the induction of suppressor cells can be separated from those APC involved in induction of immune effector cells on the basis of the expression of the I-J antigen by the former population (Noma et al., 1985; Granstein et al, 1985). To test whether the APC involved in suppressor cell induction in the HSV system also expressed I-J the APC population used to stimulate the nylon wool purified suppressor cell induction cultures

TABLE 14. THE CELLULAR REQUIREMENTS FOR THE INDUCTION OF T SUPPRESSOR CELLS IN HSV-STIMULATED, HSV-IMMUNE SPLENOCYTE CULTURES^a

Cells Added	³ H-TdR Uptake	% Suppression
None	17,128	-
Immune	4,181 ^d	75.6
Lyt 1 ⁻	15,892	7.2
Lyt 1 ⁻ + Normal	9,237 ^d	46.1
Lyt 2 ⁻	18,522	-8.1
Lyt 2 ⁻ + Normal	18,340	-7.1
I-J ⁻	20,228	-18.1
I-J ⁻ + Normal	20,075	-17.2

^aSuppressor cell induction cultures were prepared as described in the text and in the footnotes to Table 7. Some of the suppressor cell induction cultures were depleted of Lyt 1⁺ or Lyt 2⁺ or I-J⁺ cells prior to incubation with the virus. Normal splenocytes were added to some of the depleted cultures in order to replace the cells which were lysed by the antibody and complement treatment. As before, the splenocytes were irradiated on the third day and then added to test cultures. 1×10^5 suppressor cells were added to 4×10^5 cells of the test culture.

^bThe results represent the average cpm for four replicate wells of each treatment group.

^c% suppression was calculated as $1 - (\text{experimental cpm} / \text{control cpm}) \times 100$.

^dThe results are significantly less than the untreated test culture at $p < 0.05$.

TABLE 15. THE T CELL REQUIREMENT FOR THE INDUCTION OF T SUPPRESSOR CELLS IN HSV-STIMULATED, HSV-IMMUNE T CELL CULTURES^a

Cells Added	³ H-TdR Uptake	% Suppression
None	27,328	-
Immune + C	11,172 ^d	59.1
Lyt 1 ⁻	2,210	18.7
Lyt 1 ⁻ + Normal	14,738 ^d	46.1
Lyt 2 ⁻	28,238	-3.3
Lyt 2 ⁻ + Normal	28,780	-5.3
I-J ⁻	23,520	13.9

^aSuppressor cell induction cultures were prepared with nylon wool purified Thy 1⁺ cells as described in the Methods section. HSV-infected Thy 1⁻ peritoneal cells were used as stimulator cells for the T cell cultures. Some of the cultures were depleted of Lyt 1⁺ or Lyt 2⁺ or I-J⁺ cells prior to incubation with the stimulator cells. Nylon wool purified cells from HSV naive mice were added to some of the depleted cultures in order to replace the cells which were lysed by the antibody and complement treatment. As before, the putative suppressor cells were irradiated on the third day of culture and then added to test cultures. 1×10^5 suppressor cells were added to 4×10^5 cells of the test culture.

^bThe results represent the average cpm for four replicate wells of each treatment group.

^c% suppression was calculated as $1 - (\text{experimental cpm} / \text{control cpm}) \times 100$.

^dThe results are significantly less than the untreated test culture at $p < 0.05$.

was treated with anti-I-J^k plus complement prior to HSV exposure and then added to the suppressor induction cultures. Following such treatment, suppression was not generated in the nylon wool nonadherent suppressor induction cultures (Table 16). Conversely IA⁺ depletion of the APC population actually enhanced suppressor cell induction.

Discussion

This chapter reports the characterization of the HSV-specific lymphoproliferative response of murine splenocytes in terms of responding cell type, its value as an in vitro correlate with parameters of CMI and the extent of its regulation by suppressor cells. Whilst widely used as an in vitro measure of T cell immunity against HSV in man (Berle and Thorsby, 1980), LP has seldom been used for a similar purpose in the mouse especially against HSV-1. Some of the previous reports, in fact, indicated that the LP response measured mainly the mitogenic effect of HSV-1 on murine B lymphocytes (Kirchner et al., 1978; Mochizuki et al., 1981). This group subsequently indicated that the B cell mitogenicity was attributable to mycoplasma contamination of viral stocks rather than the virus itself acting as a mitogen (Kirchner, 1982). Using viral stocks lacking mycoplasmata as antigen, and evaluating the contribution of different cell types to the proliferation by positive and negative selection techniques we found virtually no B cell proliferation in our system. Furthermore, only splenocytes from mice immunized with HSV proliferated. The cells involved were exclusively Thy 1⁺, with Lyt 1⁺ and Lyt 2⁺ cells accounting for

TABLE 16. THE ANTIGEN PRESENTING CELL REQUIREMENT FOR THE INDUCTION OF T SUPPRESSOR CELLS IN HSV-STIMULATED, HSV-IMMUNE T CELL CULTURES^a

PC Added	³ H-TdR Incorporated ^b	% Suppression ^c
C'	10,164	40.7
Anti-I-A	8,262	51.8
Anti-I-J	19,616	-14.5
None	19,766	-14.0

^aNylon wool purified T cell cultures were incubated with HSV-infected Thy 1- PC from normal mice. The PC population was treated with various antisera and complement prior to virus infection. The suppressor inducer cultures stimulated with these various PC populations were tested on the third day for suppressor cell activity as described in the preceeding tables.

^b³H-TdR incorporation by the test cultures was determined on the fifth day. The results are the average cpm incorporated of four replicate cultures.

^c% suppression was determined as described in the footnote to the preceding table.

approximately 60% and 40% respectively. Few (<2%) proliferating cells were Lyt 1⁺2⁺.

In analyzing the interrelationship of the two T cell subsets in the proliferative response two facts emerged. First, if the Lyt 1⁺ cells were removed prior to antigen stimulation, proliferation was almost totally eliminated. Presumably, as observed in other systems, the Lyt 1⁺ cells included IL-2 producing helper cells since the proliferative response could be restored in part by the addition of an exogenous source of IL-2. Proliferating cells in these latter cultures were exclusively Lyt 2⁺ cells. We assume that HSV-1 stimulated Lyt 2⁺ cells fail to produce IL-2, unlike some other systems (Miller and Stutman, 1982; Guerne et al., 1984).

The second notable feature was that upon removal of Lyt 2⁺ cells prior to antigen stimulation, levels of ³H-TdR incorporation were enhanced, presumably due to the removal of a regulatory suppressor cell. This aspect is discussed subsequently.

Since in man, HSV induced LP is assumed to be an in vitro measure of CMI, we tested if the LP response correlated with other aspects of CMI in the mouse system. To measure the correlation with in vivo DTH, advantage was taken of the observation that mice can be selectively tolerized for a HSV specific DTH response (which is mediated by Lyt 1⁺ - Nash and Gell, 1983) by exposing them to virus intravenously. Other aspects of immunity function normally following tolerization (Nash and Ashford, 1982), an observation we have confirmed. When comparing the proliferative response of tolerized with immune splenocytes, the level of ³H-TdR incorporation was somewhat diminished and Lyt 1⁺ cells

accounted for a lower percentage of the response. However, levels of ^3H -TdR incorporation by Lyt 1⁺ cells from tolerized mice were still substantial indicating that the bulk of the proliferating cells represent a cell population not involved in the DTH response. In fact cells from tolerized mice produced similar levels of antigen induced IL-2 as did immune cells. Thus LP is not exclusively an in vitro correlate of DTH activity as some have previously supposed (Morahan et al., 1977).

The relationship between LP and the CTL response was addressed by comparing the level of CTL activity and LP in individual microcultures. There was no significant correlation. Furthermore, certain viral antigen preparations induced significant levels of LP but failed to induce CTL responses. Thus HI-HSV induced similar levels of ^3H -TdR incorporation as active virus with a similar distribution of the radiolabel between the Lyt 1⁺ and Lyt 2⁺ cells, yet the antigen failed to induce CTL. The fact that the Lyt 2⁺ cells were proliferating and that CTL belong to the same subset makes the observation difficult to explain. Possibly once the CTL precursor (CTLp) has matured into the killer competent CTL, the cytotoxic activity of these cells is independent of their ability to further proliferate. In fact, it is known that CTL induction and maturation can proceed in the absence of proliferation (MacDonald et al., 1975), and in our system at the time of peak CTL activity, proliferation is decreasing markedly. It might be better to look for correlation between the LP response and the number of noncytotoxic CTL-P at an early stage of culture.

A more plausible explanation for the lack of correlation between CTL activity and LP could be because of the differential sensitivity of the CTL to the activity of suppressor cells. Our results clearly showed that the HSV-specific LP response was subject to regulation by suppression, and that this regulation appeared to occur late in the culture period at a time when CTL activity was already maximum. This regulation was most notable after the fifth day of culture, indicating that suppressor cell regulation of the responses appeared to modulate the duration of the response more than its extent. Similar examples of this type of regulation by Lyt 2⁺ cells has been described for other systems (Kanellopoulos-Langevin et al., 1984). It was initially suggested that such regulation could be the result of CTL activity in the cultures (Fitch et al., 1976; Al-Adra et al., 1978). Thus suppressor cells are often Lyt 2⁺ (Cantor and Gershon, 1979; Germain and Benacerraf, 1981; Schaaf-Lafontaine and Degiovanni, 1981; Schwartz et al., 1982; Green et al., 1983; Dorf and Benacerraf, 1984) and this was found to be the case in this system. HSV-specific CTL are also Lyt 2⁺ (Lawman et al., 1980). However, suppressor cell induction differed from CTL induction. Most notably, the HI-HSV antigen preparation, which failed to induce CTL, did generate suppressor cells. Also, CTL induction requires I-A⁺ APC (Schmid et al., 1981) whilst suppressor induction required I-A⁻, I-J⁺ APC.

In an attempt to further identify the cell types involved in suppression, experiments were done in which suppression was generated in one culture and those cells, after irradiation, were subsequently used to modulate the LP response of a second antigen stimulated test culture.

After 3 days antigen stimulation, potent suppressor cell activity was generated which could inhibit the induction of LP in test cultures by up to 90%. The cell type responsible for the suppression was an $\text{Lyt } 2^+ \text{ I-J}^+$ cell, but, several cell types appeared necessary for the generation of the suppressor cell. These included cells expressing the $\text{Lyt } 2^+$ antigen, the $\text{Lyt } 1^+$ (or 1^+ plus 2^+) antigen and the I-J antigen. That all markers were expressed on a single suppressor cell precursor seemed unlikely. Rather it appeared on the basis of indirect evidence that SC generation required the interaction of at least three cell types as reported for nonviral systems (Germain and Benacerraf, 1981; Green et al, 1983; Dorf and Benacerraf, 1984; Asherson et al., 1985). Of the three putative cell types required for SC generation both the $\text{Lyt } 2^+$ and the I-J^+ cells, required to be from an HSV immune animals. The $\text{Lyt } 1^+$ cell could be provided by normal splenocytes. Thus when the $\text{Lyt } 1^+$ cells were removed from the suppressor induction culture, normal T cells could restore suppressor cell activity to the depleted cultures. The APC required for suppressor cell induction was most likely an I-J^+ macrophage. Thus the cell was adherent, Thy 1 negative and was present in peritoneal washes from nonimmune mice.

Like the induction of HSV-specific cell mediated immunity, suppressor cell induction also required a subpopulation of cells which had been activated in vivo and restimulated in vitro with HSV antigens. Unlike the induction of immune effector cells though, suppressor cell induction also utilized $\text{Lyt } 1^+$ cells which did not require previous exposure to HSV. That a specific suppressor cell circuit would utilize

unprimed cells in its induction is surprising, perhaps HSV infection of a subpopulation of Lyt 1⁺ T cells provides the specific signal for suppressor cell induction. Thus the signal recognized might be HSV antigen associated with some T cell antigen other than the T cell receptor molecule. This possibility requires further investigation. The fact that naive Lyt 1⁺ cells could provide the induction signal tends to rule out the possibility that IL-2 induced the suppression as has been described in other systems (Thoman and Weigle, 1984; Ting et al, 1984).

Summary

This chapter assesses the value of the lymphoproliferation (LP) assay as an in vitro measure of T cell mediated immunity against herpes simplex virus type 1 (HSV) in mice and demonstrated the extent of its regulation by suppressor cells. Splenocytes from immune mice underwent LP responses upon exposure to virus preparations. Responses peaked on the fifth day and over 90% of the proliferating cells were Thy 1 positive. Of these Thy 1⁺ cells responding, approximately 60% were Lyt 1⁺ and 40% Lyt 2 positive. The extent of LP did not appear to correlate with either delayed type hypersensitivity (DTH) or the cytotoxic T lymphocyte (CTL) response to HSV. One reason for the lack of correlation with these other measures of HSV-specific cell mediated immunity may have been the presence of suppressor cells which regulated the LP response. These cells were shown to be Thy 1⁺, Lyt 2⁺, I-J⁺ T cells. Responses of splenocytes to HSV increased following removal of the suppressor cells with increases most apparent late in the culture

(6-7 days). The generation of suppressor cell activity appeared to depend upon the interaction between three cell types: a Lyt 1⁺ and an Lyt 2⁺ T cells and an I-J⁺ antigen presenting cell (APC). While the Lyt 2⁺ cell was present in the spleens of HSV-immune mice, both the Lyt 1⁺ cell and the APC could be found in HSV-naive mice.

CHAPTER V

PRODUCTION OF SOLUBLE SUPPRESSOR FACTORS BY HERPES SIMPLEX VIRUS (HSV) STIMULATED HSV-IMMUNE SPLENOCYTES

Introduction

Cell mediated immunity (CMI) plays a critical role in recovery from HSV infections (Rouse, 1984) and several models have been described for identifying specific CMI responses to HSV. Anti-herpetic activity by macrophages (Stevens and Cook, 1971), natural killer cells (Lopez et al., 1982), DTH-mediating T cells (Nash et al., 1980), and CTL (Lawman et al., 1980) have been described. Of these, HSV-specific CTL activity has proven the most difficult to demonstrate. Either pretreatment with cyclophosphamide or in vitro cultivation is necessary to demonstrate CTL activity in lymph nodes (Pfizenmaier et al., 1977a,b) and spleens (Lawman et al., 1980) of infected animals. Therefore, it has been suggested that HSV-specific CTL induction is regulated by a suppressor cell which is inactivated by either in vitro cultivation or low dose cyclophosphamide treatment (Pfizenmaier et al., 1977a,b; Lawman et al., 1980). While limiting dilution analysis of HSV-specific CTLp has provided additional indirect evidence of suppression (Rouse and Wagner, 1984), no direct evidence of HSV-specific CTL suppression has been forthcoming in spite of reports of suppressor cells and/or factors regulating HSV-specific DTH responses (Nash and Gell, 1983).

In this chapter suppressor factors are described which regulate the in vitro induction of HSV-specific CTL. HSV-immune splenocytes

stimulated with HSV produced suppressive supernatant fluids which, when fractionated on a gel filtration column, contained multiple suppressor activities. One of these fractions exhibited both HSV-specific and genetically-restricted activity.

Results

Production of a Soluble Suppressor Activity by Stimulated HSV-immune Splenocytes

Incubation of HSV-immune splenocytes with HSV resulted in the induction of HSV-specific secondary CTL (Table 17; Lawman et al., 1980). The supernatant fluids from these cultures also contained a non-dialyzable, heat labile suppressor activity for HSV-specific CTL induction (SF). However, production of the suppressor activity did not always correlate with CTL induction as CTL negative cultures did produce SF (data not shown). Production of this suppressor activity increased throughout the culture period (Figure 4); however, statistically significant ($p < .01$) levels of suppressor activity was noted only with SF obtained after three days incubation. The supernatant fluids from normal, HSV-stimulated normal, HSV-stimulated influenza immune or unstimulated immune splenocytes were neither suppressive nor enhancing.

Identification of the Cell Population Responsible for SF Production

HSV-immune splenocytes were incubated for three days with HSV antigens and then separated into different cell populations. The separated cells were then cultured an additional three days and then their supernatant fluids were collected and assayed for SF activity

TABLE 17. INHIBITION OF HSV-SPECIFIC CTL (H-2^k) INDUCTION BY SUPERNATANT FLUIDS FROM HSV-STIMULATED, HSV-IMMUNE SPLENOCYTE CULTURES.^a

Supernate Tested ^c	Specific ⁵¹ Cr release ^b	
	LHSV (H-2 ^k)	EHSV (H-2 ^b)
None	44.2	14.2
Normal	42.4	13.9
Normal & HSV	46.5	12.5
Immune	38.5	10.1
Immune & HSV	5.8	2.4
Immune & HSV; 60°C, 15 min.	41.5	14.9

^aHSV-specific CTL induction cultures consisted of 4×10^5 HSV-immune splenocytes incubated with HSV (moi = 1.0) in 200 ul volumes in 96 well flat bottomed plates for five days. On the fifth day 100 ul of cells from each well was transferred to a 96 well round bottomed plate and 1×10^4 HSV-infected, ⁵¹Cr-labelled syngeneic (LHSV) or allogeneic (EHSV) targets were added in a 100 ul volume. The plates were then centrifuged (200 x g, 2 minutes) and incubated for a three hour ⁵¹Cr-release assay. Each determination was performed in quadruplicate.

^b% specific ⁵¹Cr-release = [(Experimental - spontaneous)/(Total - spontaneous)] x 100. Standard error of the mean did not exceed 10%.

^cHSV-immune and normal splenocytes were incubated with or without HSV-stimulation for five days. The culture supernate was then harvested, dialyzed, and filter sterilized before adding to the HSV-specific CTL induction cultures (20% v/v). In some cases an aliquot of the supernate from HSV-stimulated, HSV-immune splenocyte cultures was heated at 60°C for 15 minutes prior to dialysis.

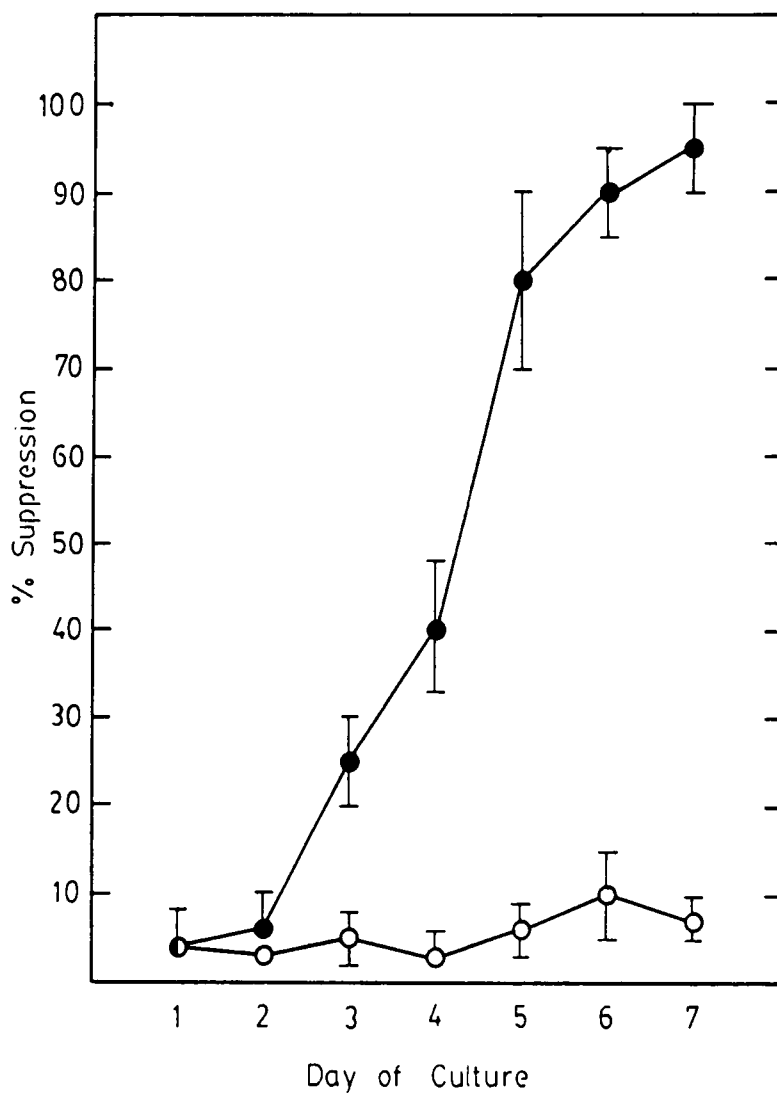


Figure 4. Time course of the production of suppressor activity. Supernatant fluid from HSV-immune splenocytes incubated with (●) or without (○) HSV stimulation were collected at daily intervals and later assayed for suppressor activity against HSV specific CTL induction cultures. Each point represents the mean for four replicates.

(Table 18). The nylon wool purified cell population produced SF as did the plastic nonadherent cells. Treatment with anti-Thy 1.2 antiserum and complement at day zero followed by virus stimulation also abrogated SF production. Together these results indicate the SF production requires T cell participation.

Time Course of Suppression by the Stimulated Immune Splenocyte Supernatant Fluids

In the preceding experiments, the SF was added at the initiation of the test cultures. In experiments in which SF was added to test culture at different times, maximum suppression of CTL induction was observed only when the SF was added during the first 24 hours of incubation (Figure 5). This indicated that the target of the suppressor factor was either an early event in HSV-specific CTL induction or that the suppressive activity represented an afferent acting acting suppressor factor.

Genetic Restriction of the Suppressive Supernatant Activity

A characteristic in other systems of afferent acting suppressor factors is that the activity is genetically restricted (Germain and Benacerraf, 1981; Greene et al., 1984; Dorf and Benacerraf, 1984). To test this in the present system, the SF generated by C3H/HeJ (H-2^k) stimulated-immune splenocytes was assayed against C57BL/6 (H-2^b) and Balb/C (H-2^d) cultures. The results in Table 19 indicate that while the SF sometimes exhibited genetic restriction in its activity (Experiment 1), variations in the genetic restriction were observed among batches of SF, implying the possibility of multiple suppressive factors. To

TABLE 18. T CELL REQUIREMENT FOR PRODUCTION OF HSV INDUCED SUPPRESSOR FACTORS FROM HSV-IMMUNE SPLENOCYTES^a

Treatment ^b	% Suppressor Activity of Supernate ^c
None	100
Nylon Wool Non-adherent	115
Plastic Adherent	0
Plastic Non-Adherent	95
Anti-Thy 1.2 + C'	5
Anti-Ig + C'	105

^aHSV-immune splenocytes were incubated three days with HSV antigens in bulk cultures and then separated into various subpopulations based on adherency to plastic and nylon wool. The cells were incubated for three additional days in fresh medium. The supernates from these cultures were harvested and assayed for HSV-SF activity as described in the Footnote to Table 1. In the case of antibody and complement (C') treatments, the splenocytes were treated on day 0 prior to antigen stimulation. The supernates were harvested on the sixth day.

^bThe treatment procedures are described in detail in the Methods section. Plastic adherent cells were those cells which remained on the culture dishes after rinsing with warm media on day 3. Plastic non-adherent cells were those cells collected by rinsing the plates with warm media.

^c% suppressor activity of supernates was calculated as $(\text{Experimental} / \text{control}) \times 100\%$. The no treatment control (None) reduced CTL activity in the treated cultures from 48.4% specific lysis to 10.2%, a reduction of 79%. This value was used as the control value in the above formula. Four replicates were performed for each determination.

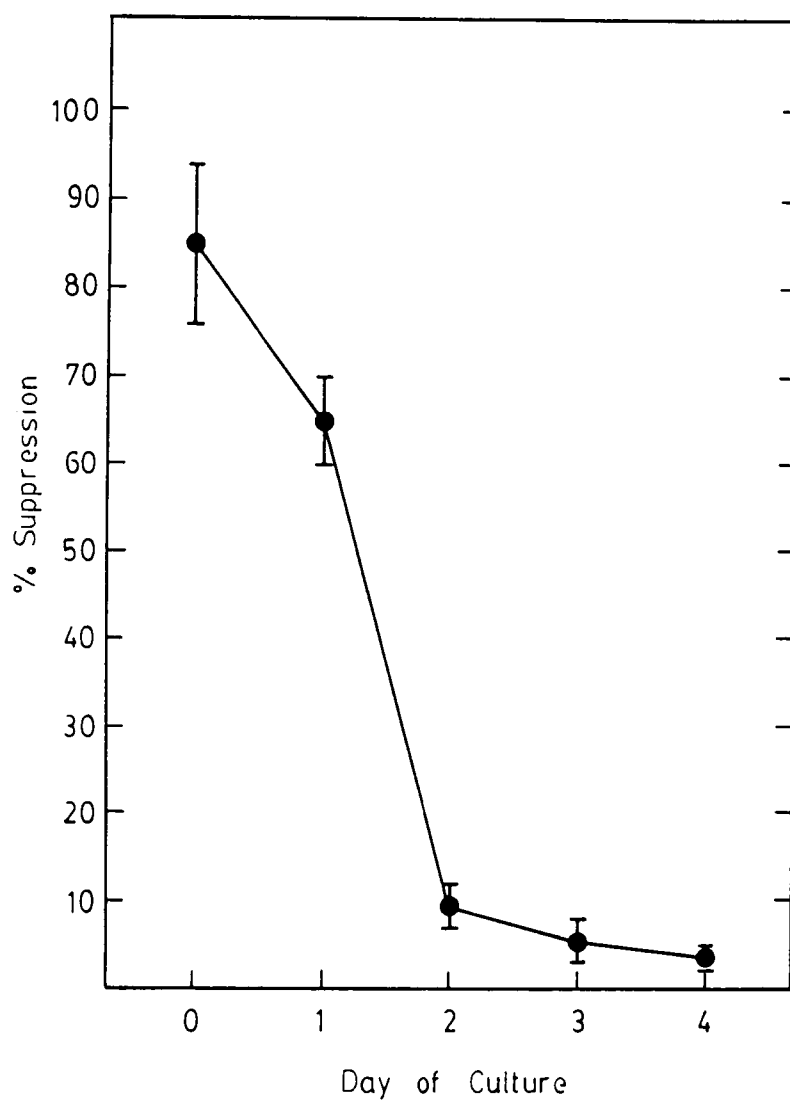


Figure 5. Time course of suppressor activity. Suppressive supernatant from HSV stimulated, HSV-immune splenocyte cultures was added to HSV-specific CTL induction cultures at daily intervals after culture initiation. Specific CTL activity and percent suppression of CTL activity was determined on the fifth day of culture. Each point represents the mean for four replicates.

TABLE 19. GENETIC RESTRICTION OF SUPPRESSIVE SUPERNATE^a

	Strain Tested	H-2	Supernate added	LHSV(H-2 ^k)	EHSV(H-2 ^b)	AHSV(H-2 ^d)
Expt 1	C3H/HeJ	k	-	35.7	15.0	ND
	C3H/HeJ	k	+(A) ^b	5.2	1.7	ND
	C57BL/6	b	-	1.3	33.0	ND
	C57BL/6	b	+(A)	5.4	29.4	ND
Expt 2	C3H/HeJ	k	-	38.4	13.5	ND
	C3H/HeJ	k	+(B)	3.2	0.5	ND
	C57BL/6	b	-	3.0	39.7	ND
	C57BL/6	b	+(B)	2.5	17.7	ND
Expt 3	C3H/HeJ	k	-	48.7	ND	11.4
	C3H/HeJ	k	+(C)	11.9	ND	3.3
	C3H/HeJ	k	+(D)	2.5	ND	0.1
	BALB/c	d	-	4.3	ND	33.7
	BALB/c	d	+(C)	1.7	ND	5.9
	BALB/c	d	+(D)	4.0	ND	34.0

^aSuppressive supernatant fluids were added at 20% (v/v) concentration to HSV-stimulated immune splenocyte cultures at initiation. CTL activity against syngeneic and allogeneic targets was assayed on day 5 as described in Table 1. Data represents average of four replicates per experiment. Standard errors were routinely less than 10% of mean values.

^bLetter in parenthesis indicates different batch of C3H/HeJ (H-2^k) generated suppressive supernate used for given experiment. ND= not done.

separate the genetically-restricted activity from non-restricted activity, serum-free SF was fractionated on a S-200 gel filtration column. When the column fractions were assayed against syngeneic and allogeneic HSV-specific CTL induction and syngeneic influenza-specific CTL induction (Figure 6), several distinct peaks of non-specific suppressor activity were observed. In addition, antigen-specific, genetically-restricted suppressor activities were observed in the fractions corresponding to an apparent molecular weight of 90-150,000. When those fractions containing the antigen-specific, genetically-restricted suppressor activity were concentrated, rechromatographed on S-200 and assayed against C3H/HeJ HSV-specific CTL induction cultures, a single peak of activity at 90-150,000 MW was observed. No suppressive activity was observed against either C57BL/6 HSV-specific CTL cultures or C3H/HeJ influenza-specific CTL cultures (Figure 7), confirming the suggested presence of an antigen-specific, genetically restricted suppressor factor in the SF.

Discussion

Suppressor cells (Al-Adra et al., 1978; Miller, 1980; Schwartz et al, 1982; Susskind et al., 1983) and soluble factors (Kramer and Koszinowski, 1982; Truitt et al., 1978) are known to regulate the induction of alloantigen-specific CTL, but the role such regulatory elements play in virus-specific CTL induction is unknown. Previous evidence that HSV-specific CTL induction is regulated by a suppressor mechanism was indirect (Lawman et al., 1980; Pfizenmaier et al., 1977a,b; Rouse and Wagner, 1984). In this chapter, I provide direct

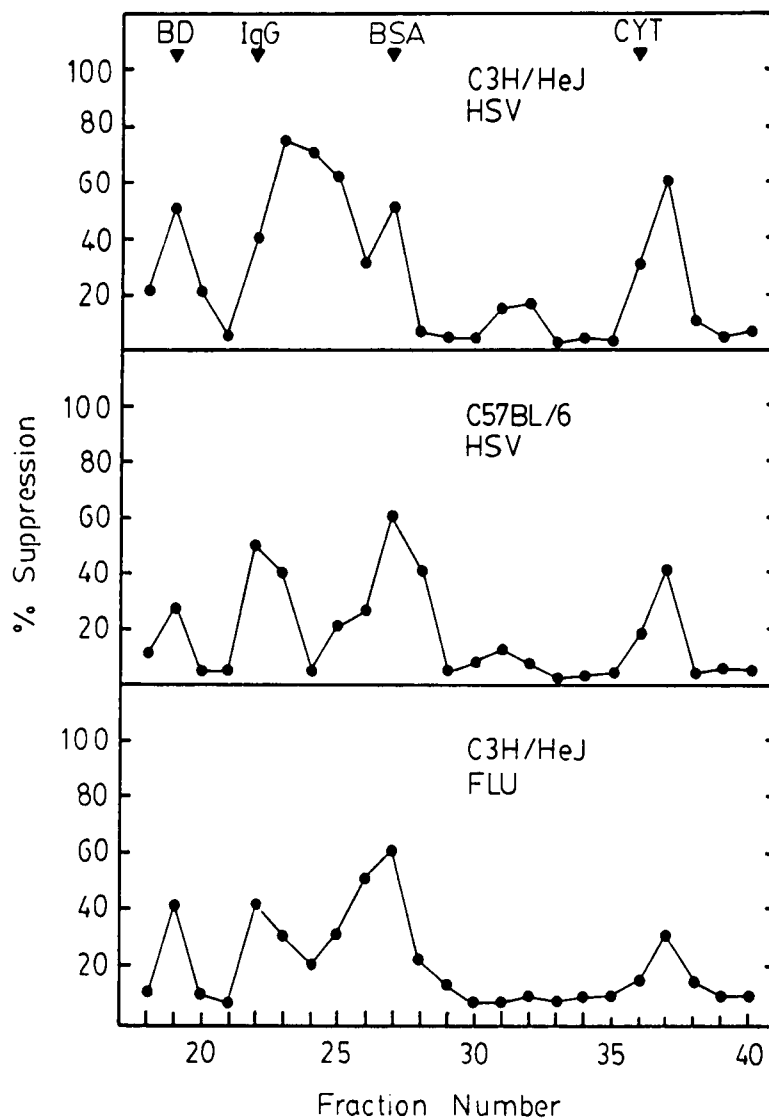


Figure 6. S-200 fractionation of serum-free SF. SF produced by HSV immune C3H/HeJ splenocytes was chromatographed on a Sephacryl S-200 gel filtration column. The eluted fractions were assayed for suppressor activity against C3H/HeJ HSV-specific CTL, C57BL/6 HSV-specific CTL, and C3H/HeJ influenza-specific CTL induction cultures for evidence of genetic restriction and antigenic specificity. Specific ^{51}Cr -releases for the cultures were 43.3%, 34.7%, and 14.2% for C3H/HeJ-HSV, C57BL/6-HSV, and C3H/HeJ-influenza respectively.

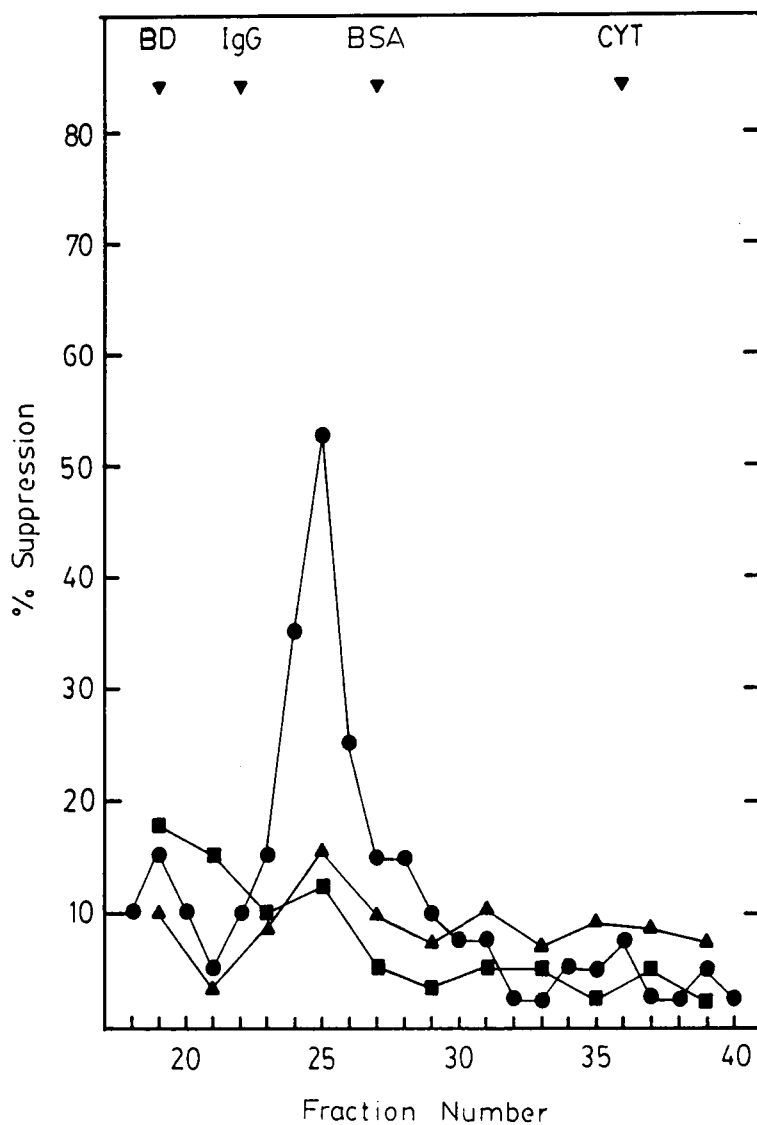


Figure 7. Rechromatography of genetically-restricted, antigen-specific suppressor activity. Fractions 24, 25, and 26 from the S-200 column (Figure 6) were rechromatographed on the S-200 column. The eluted fractions were again assayed against C3H/HeJ HSV-specific CTL (●), C57BL/6 HSV-specific CTL, and C3H/HeJ influenza-specific CTL (■) induction cultures. Specific CTL activities for the cultures were similar to those reported in the legend to Figure 6.

evidence that induction of HSV-specific CTL in vitro is regulated by soluble suppressor factors (SF) produced by HSV-specific T lymphocytes. The SF was generated in cultures of HSV-immune splenocytes following viral stimulation. Similar factors were not produced by HSV-stimulated normal or by HSV-stimulated influenza-specific splenocytes, nor by unstimulated HSV-immune splenocytes indicating that activation by specific antigen is required for both in vivo priming and in vitro activation in order to generate suppressor factors. From cell separation studies, T lymphocytes were shown necessary for SF production, but the role of other interacting cell types and the T cell subset nature of the producing cells requires further investigation. Cultures which produced suppressor factor usually generated CTL indicating that factor production failed to abrogate CTL induction in the factor producing culture. Presumably this was because factor production occurred too late to regulate CTL. Thus SF levels were first detectable on day 3 and did not peak until day 5. In order to effect suppression and abrogate CTL induction, factors had to be added within 24 hours of initiation of culture.

Some batches of SF exhibited genetically-restricted suppressor activity, whereas other batches inhibited CTL induction in a non-specific fashion. These results indicated SF might be heterogeneous in composition. Support for this notion came from results of fractionation of the SF by molecular sieve chromatography. Multiple peaks of suppressor activity were observed. Most of those peaks exhibited non-specific activity, but the suppression mediated by the 90-150,00 MW fractions was antigen-specific and genetically-restricted.

While antigen-specific regulation of CTL induction by suppressor cells has been described (Al-Adra et al., 1978; Schwartz et al., 1982), regulation by soluble factors was not antigen-specific (Truitt et al., 1978; Kramer and Koszinowski, 1982). Thus the observation of an antigen-specific, genetically-restricted suppressor factor regulating HSV-specific CTL induction represents a novel mechanism for CTL regulation. Antigen-specific, genetically-restricted suppressor factors are involved in the regulation of antibody production, contact sensitivity, and DTH reactions and similar factors act as suppressor signals between various subpopulations of T cells which compromise antigen-specific suppressor circuits (Germain and Benacerraf, 1981; Greene et al., 1983; Dorf and Benacerraf, 1984; Asherson et al., 1985). This report of an antigen-specific, genetically-restricted suppressor factor in SF indicates a similar circuit may be involved in the regulation of HSV-specific CTL induction. Furthermore, antigen-specific suppressor circuits are known to culminate in the production of non-specific suppressor factors which mediate suppression (Aune et al., 1982; Zembala et al., 1982). It is possible that the non-specific factors described in this report may be part of the same suppressor circuit.

It will be important to ascertain the mode of action of the specific and nonspecific suppressor factors. However, from analogy with alloantigen systems, we suspect that the target cells could be helper T lymphocytes known to be required for HSV-specific CTL induction (Schmid and Rouse, 1983). Thus the necessity to add the factors within 24 hours

to obtain suppression was also observed in the alloantigen system (Truitt et al., 1978; Kramer and Koszinowski, 1982). In the latter model, helper T cells were identified as targets on the basis of cell separation analysis and because suppression could be overcome by the addition of helper cell products (Kramer and Koszinowski, 1982; Susskind et al., 1983). It was possible to overcome some, but not all of the suppressive effects of SF by the addition of exogenous sources of IL-2 containing supernatant fluids from mitogen stimulated splenocytes (data not shown). These results indicate that more than one mechanism of suppression may be operative in addition to possible effects on helper cell activity.

Although this work demonstrates that CTL induction in vitro can be regulated by suppressor factors, the in vivo significance of such factors remains to be demonstrated. Others have proposed that such factors may be involved in recrudescence disease (Iwasaka et al., 1983). Using the guinea pig model for HSV 2 recrudescence, virus reactivation in vivo was shown to result in the generation of suppressor cells which produce suppressor factors upon in vitro incubation. The authors proposed that similar factors could suppress protective aspects of immunity and so permitting sufficient virus spread to cause lesions. However, whether or not such factors are produced in vivo or whether in vitro generated factors can modulate immunity in vivo has not been shown.

Summary

Indirect evidence indicates herpes simplex virus (HSV)-specific cytotoxic T lymphocyte (CTL) induction is regulated by suppressor cells. To search for such suppressor effects, supernatant fluids from splenocytes cultures from normal and HSV-immune mice cultured either with or without viral stimulation were tested for their ability to inhibit HSV-specific CTL induction. Only the supernatant fluid from the HSV-stimulated, HSV-immune cultures contained suppressor activity. The suppressor activity was produced by nylon wool-purified Thy 1⁺ cells. Suppressor activity was detectable in the supernatant fluids after three days of culture and would only suppress CTL induction if added within twenty-four hours of initiation of the test cultures. The suppressor activity was neither dialyzable nor heat stable. Molecular sieve chromatography of the supernates yielded multiple peaks of suppressor activity. While most of these peaks exhibited non-specific suppressor activity, the suppression mediated by the 90-150,000 molecular weight fractions was both antigen-specific and genetically restricted. These results provide direct evidence for the regulation of HSV-specific CTL induction by a novel suppressor factor.

CHAPTER VI

THE REGULATION OF HERPES SIMPLEX VIRUS-SPECIFIC CMI RESPONSES

BY AN I-J+ ANTIIDIOTYPIC SUPPRESSOR FACTOR

Introduction

Immune responsiveness is regulated by suppressor cell circuits composed of various subpopulations of T lymphocytes and macrophages (Herzenberg et al., 1980; Germain and Benacerraf, 1981; Greene et al., 1983; Dorf and Benacerraf, 1984). Soluble factors transmit the suppressogenic signal between the T cell subsets (Ptak et al., 1978; Zembala et al., 1982; Aoki et al., 1983; Tokuhisa et al., 1984; Jayaraman et al., 1985) and mediate the ultimate suppressive effect mechanism (Rich and Pierce, 1974; Kapp and Araneo, 1982; Zembala et al., 1982; Ikezawa et al., 1983; Malkovsky et al., 1983). These factors may be either antigen-specific or non-specific in their action (Tada and Okumura, 1980; Aune et al., 1982; Taniguchi et al., 1982; Asherson et al., 1985). The non-specific factors contain neither genetic restriction elements nor receptor structures (Aune and Pierce 1982; Kramer and Koszinowski, 1983). In contrast, the antigen-specific factors possess either idiotypic or anti-idiotypic receptors and may also express various genetic restriction elements (Czitrom et al., 1980; Taniguchi et al., 1980; Flood et al., 1982; Ptak et al., 1982; Adorini et al., 1983; Ikezawa et al., 1983; Kitamura et al., 1984; Sumida et al., 1984; Zembala et al., 1984; Asherson et al., 1985). The antigen specific suppressor factors may be composed of separate subunits joined

by disulfide bonds (Taniguchi et al., 1980; Flood et al., 1982). In such cases either the idiotypic or anti-idiotypic receptor is present on one subunit while the genetically-restricted element (such as I-J) is found on the second subunit (Flood et al., 1983; Lei et al., 1983). However, some suppressor factors consist of a single chain that expresses both I-J determinants and antigen-binding activity (Kapp and Araneo, 1982; Jayaraman and Bellone, 1985).

The above information regarding the characterization and biological activity of suppressor cell circuits and suppressor factors results from studies of responses to non-infectious antigens (Tada et al., 1979; Taniguchi et al., 1980; Moorhead, 1982; Zembala et al., 1982; Kapp et al., 1983; Ptak et al., 1983; Jayaraman and Bellone, 1985). However immune responses to a variety of infectious agents appear to be regulated by suppressor mechanisms (Iwasaka et al., 1983; Murphy et al., 1983; Mitchell, 1984) but details of this regulation and its relevance to pathogenesis remain to be determined. Thus, soluble suppressor factors regulate herpes simplex virus (HSV) specific-lymphoproliferation (Iwasaka et al., 1983) and cytotoxic T cell (CTL) induction (Chapter 3), but these factors were poorly characterized and their role in the pathogenesis of HSV disease can only be surmised. In this chapter, the nature of suppressor factors which regulate HSV-specific responses and are investigated and an antigen-specific T suppressor factor which bears I-J antigenic determinants and expresses either HSV antigens or anti-idiotypic determinants is described. This suppressor factor regulates specific the induction of cell-mediated immune responses to HSV in vitro.

Results

Generation and Demonstration of Antigen-specific Suppressor Factor

Supernatant fluids from HSV-stimulated, HSV-immune T lymphocyte cultures significantly inhibited HSV-specific CTL induction and lymphoproliferation when added to test cultures (Table 20). Whereas the unfractionated suppressor supernate also inhibited responses to influenza virus, fractionation by Sephacryl S-200 gel filtration revealed a peak of molecular weight range 90 - 100,000 (SF-200) which inhibited both CTL generation and lymphoproliferation in an antigen-specific and genetically restricted fashion (Figure 7; page 85). Thus the CMI responses to HSV were suppressed but the same responses to influenza were barely affected. SF-200 activity was not generated by antigen stimulated normal splenocytes nor by unstimulated immune cells.

Characterization of the Antigen-specific Suppressor Factor

SF-200 was electrophoretically fractionated by SDS-PAGE and subsequently Western blotted with polyspecific anti-HSV antiserum. This resulted in the detection of several proteins (Figure 8A). However, if the blot was reacted with anti-I-Jk alloantibodies, a single protein band of approximately 77,000 molecular weight was detected which coincided with one of the proteins detected by the anti-HSV antiserum (Figure 8B). To further investigate the immunochemical and immunobiological characteristics of the antigen-specific suppressor activity of SF-200, the material was fractionated on an immunoadsorbent column. Exposure to an anti-I-Jk columns resulted in the removal of the

TABLE 20. ANTIGEN-SPECIFIC SUPPRESSION OF HSV-SPECIFIC CELL-MEDIATED IMMUNITY BY A SUPPRESOR FACTOR PRODUCED BY HSV-STIMULATED, HSV-IMMUNE T LYMPHOCYTES^a

Sample Tested	CTL Activity		³ H-TdR Uptake	
	HSV	FLU	HSV	FLU
None	37.5	18.9	24,235	18,245
IVS	6.5	3.1	4,350	2,225
IVS Δ	35.0	19.9	24,580	18,090
SF-200	11.2	19.3	3,575	17,250

^aSplenocytes from HSV-immune mice were incubated with HSV antigens for three days and then depleted of macrophages and B cells by passage over nylon wool columns. The non-adherent cells were determined to be greater than 95% Thy 1.2⁺ by antibody and complement mediated cytotoxicity. The Thy 1⁺ cells were then cultured an additional three days in serum-free medium. Afterwards the cells were pelleted by centrifugation and the supernatant (IVS) was assayed for suppressor activity. In some experiments the supernatant was heated at 60° C for 15 minutes (Δ). Serum-free supernatants were concentrated 1000 fold and applied to a Sephacryl S-200 cel filtration column. Those fractions which suppressed HSV-specific but not influenza-specific CTL induction were pooled (SF-200). All supernatant fluids and column fractions were assayed at a final concentration of 20% (v/v) after dialysis overnight against serum-free RPMI.

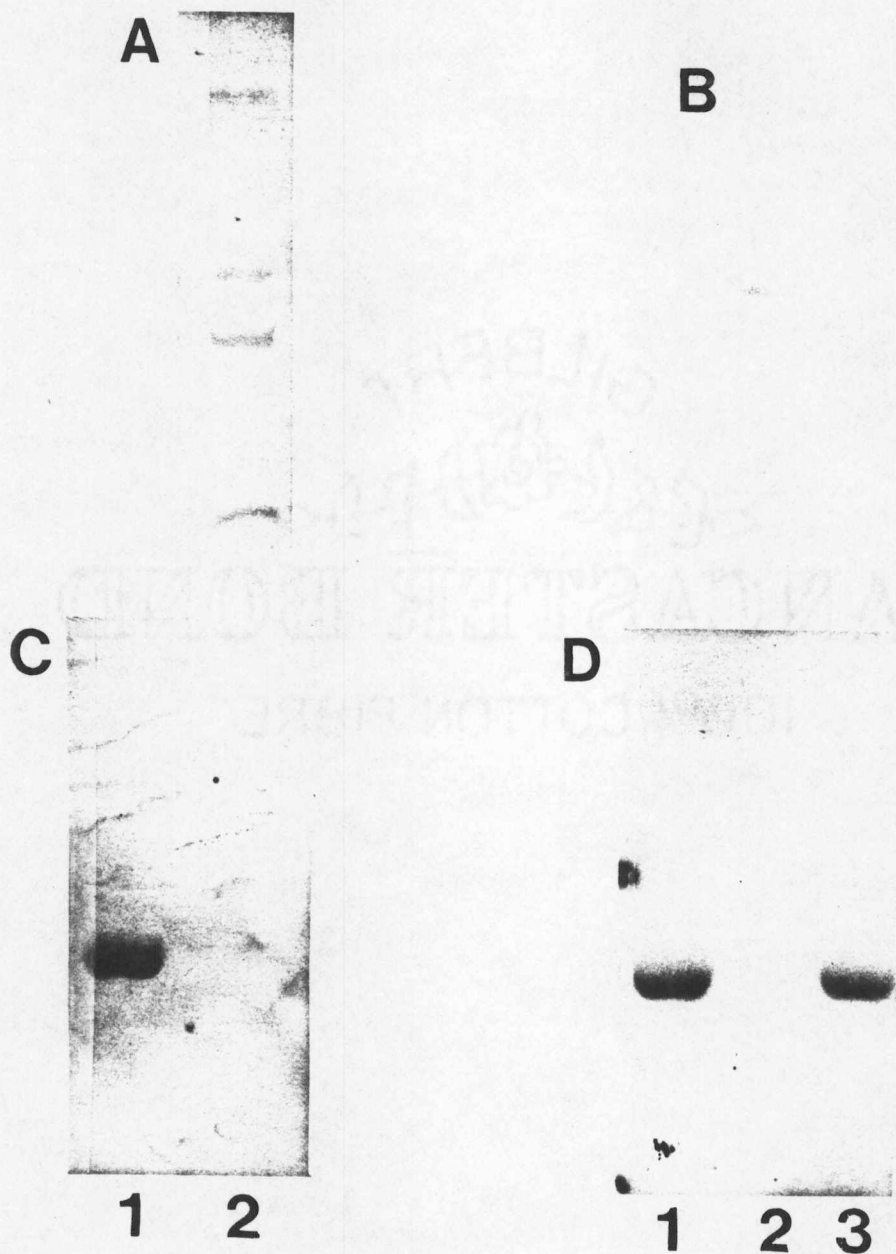


Figure 8. Western blot analysis of HSV-specific suppressor factor. Suppressive supernatant fluid (A,B) or immunoaffinity column fractions (C,D) were electrophoretically separated on a 7.5% SDS-PAGE gel. The separated proteins were electroblotted onto nitrocellulose and then reacted with either anti-HSV (A,C) or anti-I-J (B,D) antiserum. The bound antibody was detected with radiolabelled protein A. Lanes C1, D1 bound and then eluted; Lanes C2, D2 unbound eluant; Lane D3 unfractionated supernatant fluid.

suppressor activity (Table 21). In contrast, exposure to an anti-Ig immunoadsorbent column failed to significantly reduce suppressor activity. The material adsorbed by the anti-I-J^k column was eluted by application of low pH glycine buffer, dialyzed, and tested for suppressor activity against HSV and influenza specific CTL induction cultures. This I-J⁺ eluate contained antigen-specific suppressor activity (Table 21). The eluted material was also analyzed by SDS-PAGE and Western blotting. As shown in Figure 8C and 8D, the eluted material reacted with anti-HSV and anti-I-J antiserum. Reduction of the suppressor material with 2-mercaptoethanol abrogated both the suppressor activity of the specific suppressor factor and its ability to react with either antiserum. Several very low molecular weight peptides were also detected following reduction (data not shown) indicating that the suppressor factor contained disulfide bonds and that it was partially degraded.

The I-J⁺, HSV-specific Suppressor Activity Induced Non-specific
Suppressor Activity in HSV-immune, HSV-stimulated Splenocyte Cultures

Concentrated eluates from the anti-I-J immunoaffinity column (HSV-SF) were incubated with various splenocyte populations for three days. These were either control or antigen-stimulated HSV immune splenocytes, control or antigen stimulated influenza immune splenocytes, or antigen stimulated HSV immune splenocytes depleted of I-J⁺ cells prior to stimulation. Following the three day treatment, supernatant fluids were collected and tested for suppressor activity against test HSV specific and influenza specific CTL induction cultures. As is apparent in Table

TABLE 21. ANTIGEN-SPECIFIC SUPPRESSION OF HSV-SPECIFIC CELL-MEDIATED IMMUNITY BY THE ANTI-I-J ADHERENT SUPPRESSOR ACTIVITY OF SF-200^a

Sample Tested	CTL Activity		³ H-TdR Uptake	
	HSV	FLU	HSV	FLU
None	37.5	18.9	24,235	18,245
I-J ⁻	43.3	18.5	22,755	18,880
I-J ⁺	3.2	19.0	6,445	18,555
Ig ⁻	2.4	19.7	7,257	17,987
Ig ⁺	42.1	21.5	24,895	18,535

^aSF-200 fractions were concentrated and applied to anti-I-J^k and NMS immunoadsorbent columns. The unadsorbed fractions (I-J⁻, Ig⁻) and those fractions collected after elution with low pH buffer (I-J⁺, Ig⁺) were concentrated fifteen-fold and dialyzed against PBS and then RPMI before assaying. All samples were tested at a final concentration of 10% (V/V).

22, incubation of I-J⁺, incubation of the I-J⁺ HSV-SF with antigen stimulated HSV immune splenocytes generated suppressor factors that nonspecifically inhibited both HSV and influenza specific CTL induction. The generation of the nonspecific suppressor factor required I-J⁺, HSV stimulated cells from HSV immune mice and in addition was markedly reduced if the cells were pretreated in vivo with cyclophosphamide.

Discussion

The in vitro stimulation of HSV-immune splenocytes with HSV-antigens results in the production of a suppressive supernatant. This suppressive supernatant contained both non-specific and HSV-specific soluble suppressor factors. It was possible to separate the HSV-specific suppressor factor from the non-specific activities by gel filtration chromatography. In this report we have further characterized this specific suppressor activity. SDS-PAGE electrophoresis and Western blotting of the suppressive supernatant demonstrated the presence of a 60-70,000 molecular weight protein which reacted with HSV-specific and I-J^k specific antiserum. Thus it was also possible to selectively adsorb the HSV-specific suppressor activity by passage over anti-I-J immunoadsorbent columns. The material eluted from these columns both specifically suppressed HSV-specific CTL induction and also reacted with HSV-specific antiserum on Western Blots. It also appeared that the specific suppressor factor did not directly suppress HSV-specific CTL

TABLE 22. PRODUCTION OF NON-SPECIFIC SUPPRESSOR FACTORS BY HSV-SF TREATED SPLENOCYTES^a

Cells Treated	Cells	Specific CTL	Percent
With HSV-SF	Tested	Activity	Suppression
None	HIV ^b	33.6	---
HI	HIV	34.0	0.0
HIV	HIV	21.0	37.5
HIV CY	HIV	29.6	11.9
HIV I-J-	HIV	32.4	3.6
FIV	HIV	31.2	7.1
None	FIV	25.8	---
HI	FIV	24.0	7.0
HIV	FIV	15.8	38.8
HIV CY	FIV	21.2	17.8
FIV	FIV	23.3	9.7

^aI-J⁺ HSV-SF was added to various splenocyte cultures. Three days after culture initiation the supernatant fluids from these cultures was replaced with fresh medium. After an additional incubation of 3 days, the supernatant fluid was collected and assayed for suppressor activity against C3H/HeJ HSV-specific and C57BL/6 influenza-specific CTL induction cultures. The supernatant fluids were added to the cultures at a final concentration of 25% (v/v). Specific CTL activity of the test cultures and percent suppression were calculated as described in the footnote to Table 1.

^bHI = HSV-immune splenocytes; HIV = HSV-stimulated HSV-immune splenocytes; FIV = influenza-immune, influenza-stimulated splenocytes; CY = cyclophosphamide pretreated.

induction, but rather induced non-specific suppression in the HSV-immune splenocyte cultures.

At this time we can only speculate on how this soluble suppressor factor may be involved in the regulation of HSV-specific CTL activity. It is tempting to suggest that this factor is a part of a suppressor cell circuit which regulates immune responsiveness. We base this suggestion on the following observations. Second order suppressor cells (Ts_2) produce antigen specific, anti-idiotypic, $I-J^+$ soluble factors which cause cyclophosphamide-sensitive, $I-J^+$ third order suppressor T cells (Ts_3) to produce soluble factors which mediate non-specific suppression (Malkovsky et al., 1983; Asherson et al., 1985). In this report we have demonstrated that HSV-stimulated HSV-immune T lymphocytes produced an antigen-specific, $I-J^+$ suppressor factor which induces the production of non-specific suppressor activity by cyclophosphamide sensitive, $I-J^+$ splenocytes from HSV-immune mice. Thus the HSV-specific suppressor factor resembles the factors produced by anti-idiotypic $I-J^+$ Ts_2 cells.

Though our results indicate that the induction of HSV-specific CTL in vitro is regulated by a suppressor cell circuit, the actual mechanism of the suppression needs to be established. Again if an analogy is made between this suppressor circuit and those previously described, one would predict that suppression would be a result of the inhibition of DNA synthesis (Malkovsky et al., 1982). However, additional possibilities would also have to be considered, including interference with APC-T helper cell interactions, inhibition of either T helper cell or CTL activation, the neutralization of specific growth factors or

differentiation factors, or the inhibition of CTL activation due to direct interaction of the CTL precursor with the suppressor effector cell.

It is important to understand how the suppressor cell system is activated and expresses its activity in vitro since clues may emerge how to manipulate the system that will prove of value in vivo. For example, suppressor cells could serve to inhibit protective aspects of immunity prior to the development of recrudescent disease. There is some evidence that helper to suppressor T lymphocyte ratios do change around the time of recrudescence in man (Sheridan et al., 1982) and recently it has been shown in the guinea pig model of HSV type 2 recrudescence that suppressor cells are present in the spleen at the time of recrudescent disease (Iwasaka et al., 1983). If suppressor cells and their products are indeed involved in the modulated immunity to HSV in man, this could provide a useful target for treatment aimed at breaking the cycle. Whether or not the operation of a similar suppressor circuit accounts for the variation of host responsiveness that accompanies the development of herpesvirus recrudescent disease requires further investigation.

Summary

The suppressive supernatant of HSV-stimulated, HSV-immune splenocyte cultures contains an antigen specific suppressor activity. SDS-PAGE and western blot analysis of S-200 fractions containing the specific suppressor factor (SF-200) indicated that the SF-200 contained

an I-J+, antiidiotypic protein. It was possible to adsorb the suppressor activity of the SF-200 to an anti-I-J immunoaffinity column. The suppressor activity could be eluted from the immunoaffinity column by lowering the pH of the buffer. The acid-eluted material was determined to be I-J+ and reactive with anti-HSV antiserum by western blot analysis. Both the SF-200 and the I-J+ suppressor activity only suppressed HSV-specific CMI responses, however by incubating the I-J+ suppressor factor with Lyt 1+ splenocytes from HSV immune mice it was possible to generate non-specific suppressor activity. The implication of these results in regards to the model for a suppressor cell circuit regulating HSV-specific CMI responses is discussed.

CHAPTER VII

UNIFYING DISCUSSION AND SPECULATION

At the time these studies began, available evidence of the regulation of HSV-specific CTL induction by suppressor cells was inferential. The experiments outlined in the preceding chapters have directly demonstrated suppressor cell regulation of HSV-specific CTL induction. Included is information regarding the cell populations and soluble factors involved in the induction of suppression. The major assumption of this work has been that these cells and soluble factors constitute a suppressor cell circuit analogous to those regulating immune responses to non-viral antigens. While many of the observations I have made are similar to the published accounts of suppressor cell circuits regulating immune responses to haptens and alloantigens, the combined results of these experiments do not directly match any particular model and indeed, are unique to the HSV model. Though the information regarding the suppressor cells I have described is incomplete, it is possible to propose the following model for a suppressor cell circuit regulating HSV-specific CMI responses (HSV-SC) (Figure 9), the similarities and differences between this model and those described for the regulation of immune responses to non-infectious antigens are discussed below and summarized in Table 23.

Macrophages play a major role as APC and FPC in the induction and propagation of suppressor cell circuits (Ptak et al., 1978; Moser et al., 1983; Dorf and Benacerraf, 1984; Usui et al., 1984; Granstein and Greene,

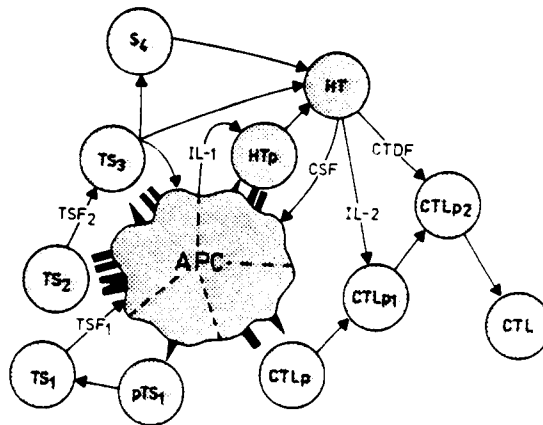


Figure 9. The regulation of HSV-specific CTL induction. The initial interaction of the virus (Δ) with the immune system is via the antigen presenting cell (APC). This cell is illustrated as being a composite of several different cells which may perform accessory cell functions for different effector and suppressor cell subpopulations, alternatively all functions may be performed by an omnipotent APC. The presentation of the viral antigen to the helper cell precursor (HTp) and the suppressor cell (pTs₁) is restricted by the class II antigens (II) of the major histocompatibility complex. Presentation of antigen and the release of interleukin 1 (IL-1) by the APC activates the HTp to its mature lymphokine producing state (HT). Activated HT produce a variety of soluble factors including the T cell growth factor interleukin 2 (IL-2) and the macrophage growth factor, colony stimulating factor (CSF). The former serves to facilitate the expansion of effector T cells, while the latter stimulates IL-1 production by the macrophages. The HT also produce cytotoxic T cell differentiation factors (CTDF) which cause activated CTL to differentiate into killer competent cells. CTLp are activated by the presentation of viral antigen in the context of the class I antigens (I). Once activated the CTLp₁ cells express IL-2 receptors and thus proliferate in the presence of the growth factor. Through the action of CTDF these cells become killer competent CTL. Suppressor cell regulation of CTL activation involves the activation of a Ts₁ cell by presentation of viral antigens by the APC. This cell presumably produces a soluble factor which causes the Ts₂ cell to produce its anti-idiotypic suppressor factor. This anti-idiotypic factor then causes an antigen-primed Ts₃ cell to produce its soluble factor which either directly, or via a fourth cell (e.g. FPC), mediates non-specific suppression. Suppression is directed against the HT and therefore could affect CTL proliferation or differentiation by inhibiting either IL-2 or CTDF production. Alternatively the suppressor factor could act directly with the CTLp and prevent either its proliferation or differentiation.

TABLE 23. A COMPARISON OF THE SUPPRESSOR CELL CIRCUITS REGULATING CELLULAR IMMUNITY TO NP AND HSV^a

Cell and Factor	NP	HSV
APC	I-A ⁺ , I-J ⁺	I-A ⁻ , I-J ⁺
Suppressor-inducer (Ts ₁)	Lyt 1 ⁺ , I-J ⁺	Lyt 1 ⁺ , I-J?
TsF ₁	I-J ⁺ Idiotypic	? ?
Suppressor-effector (Ts ₂)	Lyt 2 ⁺ , I-J ⁺ Primed	Lyt 2 ⁺ , I-J ⁺ Primed
TsF ₂	I-J ⁺ Anti-idiotypic Two chain	I-J ⁺ Anti-idiotypic? Two chain
Suppressor-auxiliary (Ts ₃)	Lyt 2 ⁺ , I-J ⁺ CY sensitive Primed	Lyt 1 ⁺ , I-J ⁺ CY sensitive Primed
TsF ₃	I-J ⁺ Idiotypic	? Non-specific
Suppressor-acceptor (Ts ₃) ^b	Lyt 2 ⁺ , I-J ⁺ CY sensitive Primed	
TsF ₃ ^b	Non-specific	

^aThe suppressor cell circuit described for the regulation of contact sensitivity to 4-hydroxy-3-nitrophenyl acetyl (NP) as described by Dorf et al (1985) is compared to the results reported in this dissertation regarding the regulation of HSV-specific lymphoproliferation and CTL induction.

^bFrom Asherson et al., 1985

1985; Noma et al., 1985). Likewise there is a requirement for APC in the induction of suppressor cells which regulate HSV-specific CTL and LP responses. Thus free HSV alone failed to induce either immune responses or suppressor cell activity. The APC involved in the induction of suppression can be discriminated from those involved in the induction of immune responses to HSV by the presence of the I-J cell surface antigen. Thus, the APC involved in the induction of HSV-specific immunity are I-A⁺, I-J⁻ (Schmid and Rouse, 1983) while those required for suppressor cell induction are I-A⁻, I-J⁺. Other groups have described similar phenotypic characteristics for the APC involved in the induction of suppressor cell circuits regulating immune responses to haptens (Usui et al., 1984; Granstein and Greene, 1985; Noma et al., 1985), though unlike the HSV model these APC may (Moser et al., 1983; Usui et al., 1984; Noma et al., 1985) or may not express I-A antigens (Granstein and Greene, 1985). I-J⁺ macrophages also present soluble factors to suppressor T cells (Dorf and Benacerraf, 1984), it is uncertain whether the APC for the HSV suppressor circuit (HSV-SC) presents antigen, soluble factors, or both. For the purpose of the model, I have assumed that I-J⁺ macrophages serve only as APC, though their function as FPC in this model needs to be addressed.

The T cell responsible for the induction of the suppressor cell circuit in the HSV-SC model is a Lyt 1⁺ splenocyte. Since Lyt 1-depleted cultures fail to produce any evidence of suppressor cells or soluble factors upon exposure to HSV, it is assumed that this cell plays a major role in suppressor induction. This assumption is consistent with the role Lyt 1⁺ cells play in the induction of other suppressor

cell circuits (Germain and Benacerraf, 1981; Miller et al., 1982; Yamauchi et al., 1982; Zembala et al., 1982; Usui et al., 1984; Jayaraman and Bellone, 1985). Such cells, referred to as suppressor inducer (Ts_1) cells, are present in the spleens and lymph nodes of primed mice. In addition to serving as inducer cells for suppression, they may also function as T helper cells (Yamauchi et al., 1982). This is not the case in the HSV-SC model since the $Lyt\ 1^+$ cell suppressor cell activity in $Lyt\ 1$ -depleted cultures could be restored by the addition of normal T cells which lack HSV-specific T helper cell activity. How an unprimed cell may be involved in the induction of a specific suppressor cell circuit is unclear, though similar cells have been described in the suppressor cell regulation of contact sensitivity to picryl chloride (PCL) (Zembala et al., 1984). Mice primed with an intravenous injection of picrylsulfonic acid (PSA) and then skin painted with PCL generate antigen-specific soluble suppressor factors which can inhibit PCL-specific contact sensitivity reactions. However, splenocytes from mice primed with PSA alone fail to produce a suppressor factor unless co-cultured with PCL-haptenized, nylon wool purified T cells from normal mice. The normal $Lyt\ 1^+$ cells, once stimulated in vitro with PCL, produced a cofactor which, along with a factor produced by a PCL-primed $Lyt\ 2^+$, resulted in the suppression of contact sensitivity to PCL. The cofactor provided by the $Lyt\ 1^+$ cell did not bind antigen, but was I-J $^+$. While the possibility of factor production by the $Lyt\ 1^+$ inducer cell in the HSV-SC was not addressed, it is nonetheless possible that this cell is analogous to the suppressor inducer cell in the PCL system. Thus,

stimulation of normal T cells with HSV in the suppressor cell induction cultures activated this cells in much the same way that haptenization activated the cells in the PCL system. It was not determined whether the induction activity of the Lyt 1⁺ cell in the HSV-SC model also involved the production of soluble factors. Such determination will undoubtedly require the isolation of suppressor inducer clones or hybridomas, as has been performed in the hapten system (Dorf et al., 1985).

In the hapten systems, the cell induced by the suppressor inducer cell is an I-J⁺, Lyt 2⁺ suppressor effector cell (TS₂) which expresses anti-idiotypic receptors and produces a characteristic soluble suppressor factor which is also I-J⁺ and anti-idiotypic (Taniguchi et al, 1980; Ikezawa et al., 1983; Dorf and Benacerraf, 1984; Sherr and Dorf, 1984; Sumida et al., 1984; Asherson et al., 1984). The presence of an I-J⁺, anti-idiotypic suppressor factor is indirect evidence of the existence of the suppressor effector cell (Murphy et al., 1983). In the HSV-SC model the existence of the suppressor effector cell is thus inferred from the demonstration of the anti-idiotypic, I-J⁺, HSV-specific suppressor factor in the supernatant fluid of HSV-stimulated, HSV-immune splenocytes. When the supernatant fluid was fractionated by gel filtration chromatography a HSV-specific, genetically-restricted peak of suppressor activity was resolved (SF-200). When the bulk supernatant fluid and SF-200 were subsequently electrophoresed and western blotted, both anti-HSV and anti-I-J^k antibodies reacted with a common protein. Furthermore it was possible to isolate the specific suppressor activity in the SF-200 fractions by affinity chromatography

using an anti-I-J immunoaffinity column. The HSV-specific suppressor activity (HSV-SF) bound to this column and was subsequently eluted by a low pH elution buffer. The eluted suppressor activity was shown to react with anti-HSV and anti-I-J^k antisera. HSV-SF suppressed both HSV-specific CTL induction and lymphoproliferation, however this effect was not directly mediated by this factor. Similarly in the hapten models for suppressor effector cell activity neither this cell nor its factor is the final effector of suppression, rather the suppressor effector cells serve as an intermediary or amplification step in the induction of the final effector cell (Green et al., 1983; Dorf and Benacerraf, 1984; Asherson et al., 1985). Presumably the cell producing the HSV-specific, I-J⁺ factor also represents such an amplification step, however it will be necessary to clone this cell in order to properly address this question.

The inability of suppressor effector cells, and their soluble factors, to directly mediate suppression portends the existence of a third cell which is induced by these cells and factors to mediate suppression. In the DNP model described by Dorf's group (Dorf and Benacerraf, 1984; Dorf et al., 1985) the antigen-specific suppression mediated by the Ts₃ cell, a suppressor auxiliary cell, results from the production of an antigen-specific suppressor factor. In the PCL model described by Asherson's group (Asherson et al., 1985), the Ts₃ cell, a suppressor acceptor cell, mediates non-specific suppression through the production of non-specific suppressor factors (Asherson et al., 1985). The induction of the suppressor acceptor cells is sensitive to

pretreatment with cyclophosphamide (Asherson et al., 1985). HSV-SF also induced the production of non-specific suppressor activity and this process was sensitive to cyclophosphamide pretreatment. However, unlike the PCL-specific suppressor cell circuits, only HSV-stimulated, HSV-immune splenocytes responded in this manner to the specific factor. The HSV-SC thus appears to involve the participation of a suppressor acceptor cell which is distinct from previously described suppressor acceptor cells. The HSV-specific suppressor acceptor cell required prestimulation in vivo with HSV-antigens before it would respond to the suppressor factor. Once activated, this cell produced soluble suppressor factors which suppressed both HSV and influenza specific CMI. The cell responsible for the production of the non-specific suppressor factors was Lyt 1⁺2⁻, I-J⁺ according to negative and positive depletion experiments. The results from these experiments are presented in Table 24. Pretreatment of the splenocytes with Lyt 1, but not Lyt 2 specific antiserum and complement prior to addition of the HSV-specific suppressor factor abrogated production of the non-specific suppressor factors. Thus the phenotype of the suppressor acceptor cells is similar to the suppressor inducer cells, however these cells can be separated on the basis of the former's requirement for priming by HSV in vivo. The non-specific nature of the suppressor factor produced by the suppressor acceptor cells also distinguishes them from the suppressor effector cells which produced the I-J⁺, anti-idiotypic suppressor factor.

Thus the HSV-SC system appears to be composed of at least four distinct splenocyte subpopulations; an I-J⁺ macrophage, a suppressor

TABLE 24. PRODUCTION OF NON-SPECIFIC SUPPRESSOR FACTOR BY LYT 1⁺ SPLENOCYTES TREATED WITH THE I-J⁺, HSV-SPECIFIC SUPPRESSOR FACTOR

Cells Treated With SF	Cells Tested	Specific CTL Activity	Percent Suppression
None	HIV	33.6	-
HIV	HIV	21.0	37.5
HIV Lyt 1 ⁻	HIV	30.0	10.7
HIV Lyt 2 ⁻	HIV	22.2	33.9
HIV I-J ⁻	HIV	32.4	3.6
None	FLU	25.8	-
HIV	FLU	15.8	38.8
HIV Lyt 1 ⁻	FLU	23.3	9.7
HIV Lyt 2 ⁻	FLU	16.6	35.7
HIV I-J ⁻	FLU	23.3	9.7

^aI-J⁺ effluents from affinity chromatographed SF-200 were added to various splenocyte cultures. Some of the splenocyte populations were pretreated with anti-Lyt 1.1 or -Lyt 2.1 or -I-J^k specific antiserum and complement prior to addition of the suppressor factor. The suppressor factor was added to these cultures at a final concentration of 1/40 (v/v). On the third day of culture the supernatant fluid was harvested and tested for suppressor activity against HSV- and influenza-specific CTL induction as described in the footnote to (Table 3 in Chapter 5.)

inducer cell, an anti-idiotypic suppressor effector cell, and a suppressor acceptor cell which produces non-specific suppressor factors which mediate the eventual suppressor mechanism (Figure 9). According to the model, the restimulation of the immune splenocyte cultures with HSV results in the activation of a Lyt 1⁺ population which initiates the suppressor cell circuit. This cell, either directly or via the production of a soluble factor, stimulates a suppressor effector cell to produce its HSV-specific suppressor factor. This factor, along with HSV antigens, stimulated a third cell, the suppressor acceptor cell, to produce non-specific suppressor factors which inhibited CTL induction in the test culture. The sensitivity of the suppressor acceptor cell to cyclophosphamide pretreatment thus accounts for the observation that cyclophosphamide pretreatment of HSV-immune mice enhanced the CTL response.

The above description of a suppressor circuit is only a working model, as additional information is gathered it may prove necessary to modify the model. Such information can be provided through the use of better defined cellular populations. All of the above experiments utilized heterogeneous populations of splenocytes from HSV-immune and normal mice. While it was possible to determine, albeit indirectly, which cell populations appeared to be involved in the induction and execution of suppression, it is not possible to preclude any additional interactions. Only through the use of isolated homogeneous populations of cells is it possible to discount the participation of other cells. Studies of this nature require either the clonal expansion of selected populations or the use of hybridomas (Taniguchi et al., 1980; Dorf and

Benacerraf, 1984; Asherson et al., 1985). Therefore for the further clarification of the suppressor cell circuit which regulates the cellular immune responses to HSV it will be necessary to produce either suppressor cell clones or hybridomas of the inducer, effector, and acceptor populations which I have described. Further, in order to determine whether the APC population involved in suppressor cell induction is distinct from the APC involved in induction of effector cells, it will also be necessary to produce macrophage cell lines which can be used to specifically induce either suppressor or effector cells.

In order to generate suppressor cell clones or hybridomas a selection procedure is needed to identify the suppressor cells. Selection of the effector population may rely upon the presence of both the I-J antigen as well as HSV-specific anti-idiotypic receptors on the cells and their products. Likewise screening for the acceptor cells would depend upon the detection of the non-specific suppressor factors produced by these cells. Identification of inducer cell activity would entail the use of suspected cells or their products to restore suppressor activity to Lyt 1-depleted suppressor induction cultures. Similar approaches have been used to generate suppressor cell hybridomas representing all the cells involved in the suppressor circuit regulation of anti-hapten responses (e.g. Dorf and Benacerraf, 1984). Through the use of such suppressor cell hybridomas it may be possible to determine the role APC play in the induction and execution of the suppressor signal. Macrophage populations, perhaps derived from bone marrow cultures, could be used as antigen presenting cells for hybridomas and

nylon wool purified T cell cultures. The macrophage populations could be selected on the basis of class II antigen expression. Those cells which express I-A antigens might be expected to serve as APC for HSV-specific T helper cell activation, others which are I-A⁻ and I-E⁺ or I-J⁺ might present antigen to suppressor cells. In addition to determining the APC requirement for suppressor cell induction, the antigen requirement for the activation of the various suppressor cell populations should also be determined. Thus whether purified glycoproteins or peptide fragments can serve as the stimulus for suppressor cell activation should be determined, likewise whether the same antigen stimulates inducer and acceptor cells, and whether the anti-idiotypic of the effector cell is the internal image of the antigenic determinant which the suppressor inducer cell recognized.

The proposed circuit imposes several considerations regarding the interactions between the suppressor cells and the immune effector cells induced in the splenocyte cultures. These considerations include the production of suppressor activity by a splenocyte culture which also generates HSV-specific CMI responses. One possibility is that the induction of immunity precedes the induction of suppression. During the in vitro expansion of the suppressor cells, the immune response proceeds to a point where it is unaffected by the developing suppressor mechanism, i.e. 48 hours post-induction. Such a temporal relationship between helper and suppressor cell activity has been described in other systems (Feldmann and Kontiainen, 1976; Parker and Turk, 1982). This also explains why the addition of the suppressor factors 48 hours after culture initiation failed to suppress CTL induction. The time period

involved allowed the immune effector cells (e.g. CTL) to differentiate to a point where they were no longer susceptible to suppressor cell control.

Implicit in the above argument is the notion that suppressor cell regulation of CTL induction occurs early in the differentiation process of either the CTLp or one of its accessory cells. The suppression of CTL activity thus appears to be due to inhibition of CTL maturation, and not due to a blockage of CTL activity by already differentiated cells. Indeed, most published accounts of suppressor cell regulation of CTL activity occur at the point of CTL induction (Truitt et al., 1978; Klimpel and Henney, 1978; Kramer and Koszinowski, 1982; Susskind et al., 1983; Gautam et al., 1984), though others have reported the inhibition of cytolytic activity of fully differentiated CTL (Clark et al., 1976; Gorczynski and MacRae, 1978). One mechanism for the inhibition of CTL maturation would be to inhibit the activity of the T helper cells necessary for CTLp maturation. Since the suppressor cells and soluble factors described in this dissertation suppress both CTL induction and lymphoproliferation, and since lymphoproliferation is a measure of helper cell activity, it seems likely that the target of HSV-SC regulation is the T helper cell. This contention is supported by the following observations. It is possible to overcome, at least partially, the suppression mediated by the HSV-SF by adding IL-2 containing supernatant fluids to the test cultures (Table 25). The addition of the IL-2 to these cultures appears to partially abrogate suppression by accelerating the development of some of the CTL precursors beyond the

TABLE 25. SUPPRESSION OF CTL INDUCTION IS OVERCOME BY THE ADDITION OF INTERLEUKIN 2 CONTAINING SUPERNATANT FLUIDS^a

Treatment	³ H-TdR Incorporated	Specific CTL activity
None	22,326	50.3
HSV-SF	11,743	28.4
IL-2	24,208	50.7
HSV-SF + IL-2	11,310	44.5

^aSF-200 was added to HSV-CTL induction cultures at a final concentration of 20% (v/v). IL-2 containing supernatant fluids from Con A stimulated rat splenocyte cultures were added at a final concentration of 2.5% (v/v). The test cultures were incubated five days and then assayed for ³H-TdR incorporation and HSV-specific CTL activity against HSV-infected L929 cells.

point of suppressor cell regulation, note however that the addition of the supernatant fluid did not restore the lymphoproliferative response. One explanation for this dichotomy is that once activated, CTL cytotoxic activity is independent of proliferation (MacDonald et al., 1975), since IL-2 containing Con A-induced supernatant fluids also contain soluble factors which enable CTLp differentiation (Finke et al., 1981; Raulet and Bevan, 1982; Wagner et al., 1982), the addition of the supernatant fluid also activated the CTL and once activated they were no longer sensitive to suppressor cell regulation. These results indicate that the non-specific suppressor factors, which appear to be the actual mediator of suppression, do so by inhibiting proliferation, similar *modus operandi* have been shown for other suppressor factors with similar molecular weight and origin (Kramer and Koszinowski, 1982; Zembala et al., 1984).

A final point for consideration is the role that specific HSV antigens play in the induction of suppressor cells. Simple antigens, like lysozyme, bear antigenic determinants which specifically induce suppressor and effector cells (Sercarz et al., 1978; Adorini et al., 1979; Kolsch, 1984; Wicker et al., 1984). Thus suppressor cell induction to this protein results from the recognition of specific determinants by suppressor cells. Others have proposed that suppression is the result of feedback to a specifically induced immune response (McDougal et al., 1979; Yamauchi et al., 1982; Green et al., 1983; Hu et al., 1983; Shimamura et al., 1984) or the result of the production of IL-2 (Thoman and Weigle, 1982; Ting et al., 1984). Several observations in this model indicate that HSV-specific suppressor cells are

induced as a result of the presence of specific antigenic determinants, and not the result of IL-2 production or feedback regulation. Firstly, the importance of an I-A⁻, I-J⁺ APC in the induction of suppressor cell activity indicates that some antigen presenting function is necessary for the induction of the Ts₁ cell. And since this particular type of macrophage fails to induce either CTL or LP responses, it appears that suppressor cell induction occurs in the absence of an immune response. Likewise the ability of normal Lyt 1⁺ cells to restore suppressor cell activity to Lyt 1-depleted cultures also indicates suppressor cell induction occurs in the absence of an immune response since the normal cells cannot restore either LP or CTL activity to these same cultures. In light of these observations the pertinent question is, which HSV antigens are responsible for suppressor cell induction? Since HI-HSV also induces suppressor cell activity, it appears that the viral antigen required for suppressor cell activation does not have to be associated with an active virus particle, and is in fact functional when the virus particle is disrupted. Thus this antigenic determinant is distinct from that necessary for CTL induction. Preliminary experiments with L929 cells transfected with the genes encoding the gD glycoprotein, and with gD peptide fragments, indicate that this protein may contain suppressogenic determinants. Addition of these materials to immune splenocyte cultures resulted in the induction of a suppressive supernatant. Whether these cultures contained suppressor cells and soluble factors similar to those induced by HSV remains to be determined.

These results indicate that it may be possible to identify the antigens necessary for suppressor cell induction. Such information would be very useful for both the development of a anti-herpes subunit vaccine and in the treatment of recrudescence. Thus it may be necessary to include such determinants in a vaccine in order to avoid immunopathologic reactions resulting from the absence of appropriate suppressor cell response (Liew and Russell, 1983). Alternatively the removal of such determinants may potentiate the protective effect of such a vaccine, perhaps even preventing latency by invigorating a lethargic immune response. Likewise if suppressor cells play a role in recrudescence disease, it may be possible to limit either the frequency or severity of the lesions by abolishing the induction of suppressor cells, perhaps through the use of blocking antibodies directed against either the responsible viral protein, or a constituent of the suppressor cell circuit. Indeed, if immune regulation does proceed as a series of idio-: anti-idiotypic interactions then antibodies directed against a critical antigen may be sufficient to break the cycle of recrudescence.

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VITA

David W. Horohov was born in Philadelphia, Pennsylvania, on September 2, 1956. He attended Philadelphia public schools and graduated from Central High School in June, 1974. The following September he entered The Pennsylvania State University and in August 1978 received a Bachelor of Science degree in Entomology. He then entered Purdue University and received a Master of Science degree in Entomology in August 1981.

David entered the Graduate School of The University of Tennessee, Knoxville in August of 1981. He received the Doctor of Philosophy degree in the major of Microbiology in June 1985.

Dr. Horohov is married to Janice and they have one son, Nikolai. They currently reside in Rockville, Maryland where David is a Staff Fellow at the Food and Drug Administration in Bethesda, Maryland.