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Vice Chancellor
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CELLULAR AND SOLUBLE FACTOR REQUIREMENTS FOR
INDUCTION OF CYTOTOXIC T LYMPHOCYTES
AGAINST HERPES SIMPLEX VIRUS TYPE 1

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To my family,
for their continued
love and support

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ABSTRACT

The cell types required for the induction of cytotoxic T lymphocyte responses to herpes simplex virus were characterized. The cells were analyzed with respect to their role in induction, activation requirements, and their cell surface antigen profiles. At least three cell types are apparently involved in the generation of anti-HSV CTL: a CTL precursor, a helper T lymphocyte, and an adherent accessory cell. The accessory cell, which could be effectively separated from whole splenocyte suspensions by virtue of its differential adherence to glass, plastic, nylon wool, or Sephadex G-10, needed to express Ia antigens in its membrane. In addition, the adherent accessory cell must be capable of secreting the macrophage-derived soluble factor, interleukin-1. These studies also suggested that the Ia-positive accessory cell has as its role the activation of helper T lymphocytes (HTL), which are also a required cell type for anti-herpes CTL induction.

Helper T lymphocytes exposed to infectious HSV-1 presented on the surface of accessory cells in addition to interleukin-1, respond with the production of nonspecific factors that drive antigen-stimulated CTL precursors on to maturity. Heat or UV light-inactivated preparations of HSV-1 were unable to stimulate helper cell activity in vitro or in vivo. The results suggest that inactivated HSV-1 fails to stimulate due to a lack of recognition by HTL, rather than by the induction of active suppression.

The third cell type required for CTL induction, the CTL precursor, could be stimulated effectively by inactivated forms of HSV-1, at least in vitro. These results thus indicate a differential antigen activation requirement for CTL and HTL precursors.

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I. LITERATURE REVIEW

Introduction

The identification and characterization of the cytotoxic T lymphocyte has been a long and arduous process, which began with the observation of allograft rejection. The intensity with which foreign tissues are rejected following transplantation led naturally to questions regarding the evolutionary pressures involved in the development of the phenomenon. Early on, Thomas proposed that the mediators of allograft reaction might have as their function the recognition and elimination of tumor cells (175). This notion was expanded on more recently by Burnet (22), and termed immunological surveillance.

It could be shown that the phenomenon of graft rejection could be adoptively transferred with alloantigen-primed cells, but not with immune serum. Thus, it was determined that the process was mediated by living cells, and not by antibodies.

Progress in the study of T cell-mediated immunity accelerated considerably with the availability of a variety of congenic and recombinant strains of mice (163), and with the identification of a number of T cell specific surface antigens (28, 132). Until recently the degree of genetic relatedness of two animals could be determined only by the rate with which they rejected grafts. By repeatedly back-crossing animals (having an identified tumor rejection capacity) to the original nonreactive parent

over the course of fifteen or so generations, Snell (163) was able to develop a variety of "congenic" mice which had the background genes of the nonreactive parent and the transplantation antigen genes of the tumor reactive strain. From these congenic strains a number of recombinant strains were also generated. Using these mice, investigators were able to map the location of the transplantation antigen genes (84, 150, 163). The congenic and recombinant strains also permitted the further elucidation of this locus, which is now understood to be a linked series of genes in a tight cluster on chromosome 17 of the mouse. A similar cluster has been identified on chromosome 6 in humans. These gene clusters have been termed major histocompatibility complexes (MHC), and are named H-2 and HLA in the murine and human systems, respectively. The H-2 complex of the mouse can be subdivided into four main regions—K, I, S, and D (150). The K and D regions code for or regulate the production of gene products which serve as the stimulus in allograft rejection. Such antigens are in general referred to as class I MHC antigens. The I-region contains regulatory genes for the control of specific immune responses, and these antigens are called class II MHC antigens. The S region appears to regulate certain components of the complement system, and the gene products of this locus are referred to as class III MHC molecules.

The identification of surface antigen markers specific to T cells permitted for the first time the separation of T and B lymphocytes into distinct populations. Reif and Allen (132) discovered the first such antigen, in 1964. It is expressed on all T lymphocytes and has since

been shown to exist as two alleles, which have been renamed Thy 1.1 and Thy 1.2. In 1975, Cantor and Boyse discovered 3 new antigens (Lyt 1, 2 and 3), and were able to employ antiserum directed against these antigens to define functional subsets of T lymphocytes (28). Helper T lymphocytes, which assist in the generation of both the cell-mediated and humoral responses but do not directly mediate either function, are Thy 1^+ , Lyt $1^+,23^-$. The same phenotype exists in T cells which mediate delayed-type hypersensitivity against nonviable antigens (49). CTL have the phenotype Thy 1^+ , Lyt 1^-23^+ , and are the effector cell subset responsible for cell-mediated immunity and the allograft/alloantigen rejection response.

Even with the identification of the CTL and the transplantation antigens against which it reacts, the question of why cytotoxic T cells exist still remained. Initially, it was possible to explain alloreactivity within the framework of the tenets of Thomas (175) and Burnet (22). That is, CTL were proposed to have the task of patrolling the body searching for tumor cells and eliminating them. But in 1974 the question of the mechanism of T cell recognition took an unexpected turn with the discovery, by Zinkernagel and Doherty, of the phenomenon of MHC restriction (197).

It was well-established that fatal lymphocytic choriomeningitis (LCM) was caused by virus-immune CTL (30, 75, 77, 80, 196). Splenocytes from mice infected with LCMV were tested for their ability to lyse LCMV-infected L cells, a fibroblast cell line derived from C3H (H-2^k) strain mice. It was observed that only immune splenocytes taken from H-2^k strain mice were capable of lysing the infected monolayers (197).

Thus, CTL are dually specific for nominal antigen and for a self antigen encoded for or regulated by the major histocompatibility complex. It therefore became necessary to explain alloreactivity in terms of dual recognition, a problem which will be addressed in a later section of this review.

The finding that CTL have two specificities suggested that they are uniquely suited to the detection of native cells which have been modified (by viral antigen expression or neoantigen), and thus implies they have an important role in the recovery from both viral infections and in the immune surveillance for neoplastic cells. There is now considerable evidence for CTL involvement in both anti-viral and anti-tumor activity (7, 42, 53, 87, 90, 104, 144, 176, 179).

In order to assess the participation of CTL in immunity, convenient methods of assay had to be developed. CTL activity directed against alloantigen, tumor cells and virus-infected cells is now routinely demonstrated in vitro by the chromium-51 release assay, which was developed in the late 1960's (18, 72, 96, 118). Cells that express the appropriate target antigens for CTL are incubated in the presence of the high energy gamma emitter, chromium 51 (in the form of sodium chromate), for a sufficient period of time to permit the uptake of the compound into the cells. The target cells are then washed free of any extraneous chromium, and coincubated with specifically-activated cytotoxic T cells at several effector to target cell ratios. At the end of a short incubation period (generally 4 hours), supernatant fluid is drawn from the test wells and chromium-51 release above background is used as a relative measure of CTL-mediated target cell damage.

The chromium-51 release assay is a reliable and precise method if effector cell activities are shown to be dose-dependent, and also, if appropriate controls are included to ensure that the effector activity is attributable to CTL.

While the chromium-51 release assay provides a convenient way to monitor CTL responses in vitro, it is important to have a means of testing the relevance of these same cells in recovery from infections in vivo. The participation in vivo of CTL in protection against viral infection has been traditionally demonstrated by the adoptive transfer of immune cells (9, 10, 11, 65, 93, 114, 193). Many of these experiments are now viewed with skepticism, owing to the fact that crude mixtures of cells were transferred. Thus, a role for activated macrophages, natural killer cells, and frequently even humoral immunity cannot be ruled out. More recently, attempts have been made to better define the cell populations transferred (65, 93). The question of the role of CTL in recovery will be addressed in detail later in this discussion.

The Requirements for the Induction of Cytotoxic T Cells

The mechanism for the activation of antigen-specific CTL is quite complex. Aside from the requirement for specific antigen, the participation of at least three cell types and/or the soluble protein factors they produce is required. These include an Ia-positive glass adherent cell, probably either a macrophage or dendritic cell (35, 61, 63, 89, 100, 105, 122, 177, 188, 189, 190), helper T lymphocytes (49, 102, 116, 125, 133, 186), and CTL. Specific antigen has at least two key roles to

play in the generation of virus-specific CTL. One role is to stimulate the antigen-specific CTL precursors into a state of preparedness for the reception of nonspecific proliferative and differentiative factors (15, 94, 131, 183). The other apparent role of antigen is for the stimulation of antigen-specific T helper lymphocyte precursors into a proliferative expansion and into the secretion of biologically active proteins, including those involved in the generation of CTL (116, 186, 187).

The induction of CTL by antigen frequently involves only one or several of a number of antigens that may be expressed on the surface of virus-infected cells. For example, during reovirus infection, several of the proteins are expressed in quantity on the surface of infected cells (50). Nevertheless, studies with recombinant viruses demonstrated that all CTL are induced by the S1 gene product—the hemagglutinin molecule (50). In the influenza virus system, most of the activity is directed against the hemagglutinin molecule, and probably no CTL activity is induced by the matrix protein (66, 86). When animals are immunized with infectious influenza A virus, two subgroups of CTL develop: one group that is specific for the infecting strain, and one group which is cross-reactive with all influenza A strains (6, 17, 43, 66, 86, 180). It was initially proposed that the cross-reactive subset of CTL was generated against the matrix protein, that is expressed in small quantities on the surface of infected cells (6). Anti-matrix protein antibody was able to prevent the killing of influenza A-infected targets by immune CTL. However, the antiserum employed was produced by crude

membrane antigen preparations and may have had specificities other than anti-matrix present. A more recent study employing monoclonal anti-matrix antibody has cast serious doubt on the involvement of matrix protein in induction of cross-reactive anti-influenza CTL (66). The monoclonal antiserum was incapable of blocking cross-reactive CTL killing of infected cells. Perhaps the most persuasive evidence arguing against a role for matrix protein comes from experiments conducted by Koszinowski and coworkers (86). Liposomes were prepared which had purified HA and/or N proteins, without any matrix protein present, and typical levels of cross-reactive CTL were still generated. Thus, in spite of its expression on the cell surface, matrix protein is considered to have little or no role in the generation of anti-influenza CTL.

It is now thought that the hemagglutinin and/or neuraminidase molecules bear type common determinants which are not detected readily serologically, and that these determinants are responsible for the induction of cross-reactive anti-influenza CTL (66, 86). At least one study of the HA molecule, in which CNBr fragments were tested for their ability to induce cross-reactive CTL, was unable to demonstrate cross-reactive killing of virus-infected cells (180). However, these experiments do not rule out the involvement of neuraminidase in the induction. Further it is conceivable that CNBr fragmentation destroyed any type common sites that may have been available on the HA molecule.

Virus infectivity is a requirement for CTL induction at least under some conditions. Fusion-deficient inactivated influenza or Sendai virus preparations are not capable of inducing primary in vivo cytotoxic T

lymphocytes (17, 52). In both instances, the same virus preparations were able to induce in vitro secondary CTL activation from live virus-primed mouse splenocytes. In addition, isolated influenza virus hemagglutinin has been shown capable of generating secondary CTL (199). These studies suggest that infectivity, or at least fusion capacity, are necessary for the induction of primary, but not secondary CTL responses. Koszinowski and Gething (87) showed that inactivated Sendai virus with fusion capability was able to stimulate CTL activity for primary responses in vivo but nonfusion-active virus could not. In vitro primary CTL responses could also be induced but, unlike the secondary in vitro responses, were dependent on the presence of adherent cells.

Purified VSV glycoprotein (G protein) which has no fusion capacity, is not capable of stimulating CTL activity when incorporated alone into liposomes (104). Anti-VSV CTL activity could be induced only if the G protein was accompanied in the liposome membrane by products of the major histocompatibility complex, and these CTL were classically H-2 restricted. This suggested that antigen can effectively trigger CTL activity only when presented in the context of the appropriate self histocompatibility antigens.

The herpes virus system is more complicated, owing to the number of proteins produced by the virus. Heat-inactivated, completely UV-inactivated, and detergent-extracted HSV antigens are all incapable of inducing either primary or secondary CTL responses (98). If heat-inactivated virus is accompanied in secondary in vitro cultures with supernatant fluids rich in helper cell products, high levels of CTL

activity result (139). This suggested that the inactivated forms of HSV-1 were failing to enlist T helper activity, a notion which will be reinforced by the work reported here.

The herpes virus system can serve as a representative example for the characteristics of primary and secondary responses both in vivo and in vitro. The most effective means of obtaining high levels of anti-HSV-1 CTL for assay in vitro or for adoptive transfer consists of priming mice in vivo by intraperitoneal injection of infectious HSV-1, removal of the splenocytes 6-12 weeks later and restimulation with infectious HSV-1 in vitro (98). Peak CTL activity can be detected on days 4 to 5 of in vitro culture. Marginal primary in vivo CTL responses can be obtained with HSV-1, whereas primary in vitro responses are not demonstrable (98). Secondary in vivo HSV-1 responses are not high (unpublished observations), but high numbers of secondary in vitro cultured anti-herpes CTL can be obtained via the protocol outlined above.

An absolute requirement for syngeneic, adherent accessory cells has been demonstrated for the generation of CTL in a wide variety of experimental systems, including responses to tumor cells (61, 177, 190), alloantigen (35, 122, 174, 188, 189), and viruses (45, 60, 87, 98, 134, 165).

The addition of anti-Ia antiserum to cultures in the absence of complement almost totally inhibited the CTL response to vaccinia virus (89). Similar experiments using anti-Ia plus complement in the reovirus system resulted in a complete ablation of the CTL response (100). The same result will be demonstrated in this report for the

secondary in vitro response to HSV-1. Sensitivity to anti-Ia antiserum has been shown for the CTL responses to tumor cells (190) and alloantigen as well (122).

In addition to being Ia-positive, the accessory cell for the CTL response has the property of adherence. Spleen cell cultures from which nylon wool (134), glass (188), or Sephadex G10 adherent cells (177) have been removed are not capable of responding to HSV-1 with antigen specific cytotoxic T cells. Such depletion techniques effectively remove at least two populations of cells from splenocyte suspensions—macrophages and dendritic cells (81, 177, 188). Which of these cells, if not both, is required for the activation process of cytotoxic T cells has not yet been resolved, but some of the roles of the accessory cells have been established.

The cells must physically present antigen in the context of the appropriate Ia antigen, probably to resting antigen-specific helper T lymphocyte precursors (100, 188, 189). Letvin et al. (100) showed that the C3H (H-2^k) response to reovirus could be blocked with monoclonal anti-Ia^k, whereas the BALB/c (H-2^d) response was unaffected. Additionally, mice that had been extensively exposed to UV irradiation (a process that depletes the animals of Ia-positive cells (101)) exhibited a depressed ability to be primed for CTL function by reovirus (100).

There is also evidence that the accessory cells may in some fashion modify, or "process" antigen prior to presenting it. Weinberger et al. (189), found that UV irradiation of macrophages impairs their antigen presenting capacity. If macrophages were preincubated with antigen for

12 hours before UV irradiation, they could be used effectively as antigen-presenting cells (provided a substitute for the macrophage-secreted soluble factor, interleukin-1, was also supplied to cultures). In contrast, macrophages incubated with antigen subsequent to UV irradiation were not effective accessory cells. This antigen processing step was required only for the presentation of antigen to the T helper cell precursor. Cytotoxic T cells could be generated with macrophages pulsed with antigen after UV irradiation if T helper cell factors were added extraneously to cultures. As will be shown in this report, the failure of Ia-positive, HSV-1 infectable B-cells to serve as accessory cells (even in the presence of IL-1) serves as evidence for an additional accessory cell function, possibly antigen processing. Interleukin-1 (IL-1), a physiologically active soluble protein factor, is produced by macrophages and is also a requirement for the generation of cytotoxic T lymphocytes (46, 58, 119, 161, 189). Farrar et al. (46) demonstrated that IL-1 can partially replace the requirement for macrophages in the murine primary response to alloantigen. However, the presence of Ia⁺ cells plus IL-1 was required for the optimal stimulation of helper T lymphocytes (162). It may be that the partial restoration of CTL responses, which is sometimes observed after addition of interleukin-1 alone, reflects an incomplete depletion of the adherent cells from the cultures. Work which will be reported here shows that IL-1 alone is incapable of restoring CTL responses in exhaustively depleted splenocyte cultures.

Rosenstreich and Mizel (137) reported in 1979 that phorbol myristic acetate (PMA) could replace macrophages in the T cell proliferative

response to lectins. Weinberger and coworkers (189) adapted this system to the CTL response to alloantigens. PMA alone was not stimulatory in this system, and required the presence of antigen-pulsed UV-irradiated splenic adherent cells.

At least one study has suggested that IL-1 acts by enhancing the ability of T helper lymphocytes to bind specific antigen. Puri and coworkers (129) showed that preincubation of T cell enriched murine splenocytes with IL-1 concomitantly increased the antigen affinity and the lipid viscosity of $\text{Lyt } 1^{+}2^{-}$ T lymphocytes. It was postulated that the IL-1-induced increase in lipid viscosity could influence antigen binding by increasing the accessibility of membrane proteins. Palacios showed that HLA-D antigens (the equivalent to murine Ia antigens) enhance the receptivity of T helper cells to IL-1, followed by increased helper cell proliferation and production of nonspecific helper cell factors (119).

Colony stimulating factor (CSF), a protein factor produced by a number of cell types (121, 130, 165), is known to have a variety of effects on macrophages, including induction of proliferation, stimulation of IL-1 and prostaglandin production, and the enhancement of RNA and protein synthesis (11). In spite of the induction of increased IL-1 production by CSF stimulated macrophages, CSF appears to decrease the ability of macrophages to effectively present antigen (11). The explanation may be that CSF in some fashion inhibits the expression of Ia antigen on the surface of macrophages. Calamai et al. (26) have shown that L cell fibroblast supernatant fluids, which contain high

levels of CSF activity, stimulate the proliferation of macrophages in vitro, but the macrophages do not express Ia antigen. This condition can be reversed by the addition of T cell lymphokines. Lymphokine-induced expression of Ia antigen on Ia-negative macrophages has been demonstrated by others (167, 168), and is now attributed to gamma interferon (167). It appears, therefore, that gamma interferon and colony-stimulating factor may have antagonistic effects on the conditioning of macrophages for their role in antigen presentation.

Thus, the accessory cell required for the induction of CTL must present antigen in the context of Ia antigen, possibly after processing it, probably to the resting antigen-specific helper T cell precursor. Preliminary studies would suggest that both IL-1 and Ia antigen have roles in preparing the T helper precursor for a more effective interaction with antigen. Finally, soluble mediators such as CSF and gamma interferon may have important roles in regulating macrophage expression of Ia antigen and IL-1 secretion.

Several soluble protein factors appear to have key roles in the generation of cytotoxic T cells. These physiologically active proteins essentially meet the criteria for a hormone, since they are produced by one cell type and can deliver their signal to another, distantly-located cell. Four such proteins have been implicated to varying extents in the process of CTL induction. IL-1, of macrophage origin, was discussed in detail above, and appears to render T helper cells more susceptible to antigen stimulation. Interleukin-2, of T helper cell origin, drives antigen stimulated cytotoxic T lymphocyte precursors into rapid

proliferation (2, 15, 32, 108, 116, 183, 186). T cell differentiation factor, which has been only recently described (131, 183), is of unknown origin and stimulates antigen-primed CTL precursors to differentiate into active killers. Several reports have also shown that gamma interferon, of T helper lymphocyte origin, also has a role in the activation of CTL (45, 47, 159).

A number of reports have demonstrated that cell-free supernatant fluids from lectin or alloantigen stimulated splenocytes contain soluble factors which are capable of by-passing the requirement for accessory cells in the CTL response (59, 111, 116, 117, 139, 162, 186). The cytotoxic T lymphocyte precursor has no receptor for IL-2 prior to antigen stimulation, as measured by its inability to absorb IL-2 activity from culture supernatants (15, 94). Rather, effective antigen interaction with the CTL precursor induces the manufacture and surface expression of the IL-2 receptor on the surface of CTL. Once bound, IL-2 instructs the CTL to proliferate. This property has opened the way for the continuous long term culture and cloning of cytotoxic T lymphocytes with defined antigen specificities (13, 16, 19, 143). This has enabled investigators to work for the first time with homogeneous populations of cytotoxic T cells.

IL-2 is produced by $\text{Lyt } 1^{+}2^{-}$ T helper cells which have been antigen-activated and stimulated by IL-1 (82, 116, 117, 158, 162, 186). The IL-2 dependence of cloned T cells and the desire to biochemically purify and characterize the molecule have initiated a search for more convenient sources of IL-2, such as tumor cell lines and T cell

hybridomas (82, 158). A simple but consistent method of assay for IL-2 was developed by Gillis and coworkers (57). The assay measures proliferation of an IL-2 dependent cell line (CTL) via tritiated-thymidine incorporation. As a result, the screening process for IL-2 measured proliferation but not cytotoxic T cell lytic activity. Certain sources of IL-2 (e.g. AOF5-2 and EL-4 cell lines derived IL-2) had proliferative activity but failed to direct the differentiation of antigen-primed CTL precursors into active killer cells (131, 183). This led to a postulation that two distinct factors were required for the proliferation and differentiation of CTL (131, 183).

Two groups, Wagner et al. (183), and Raulet and Bevan (131) have recently provided preliminary evidence for the existence of the differentiation factor, T cell differentiation factor (TCDF). By absorbing out the proliferative (IL-2) activity of crude concanavalin A-stimulated splenocyte supernatant fluids using IL-2 dependent cell lines as an absorbant, it was possible to demonstrate the development of CTL activity in the absence of proliferation (131, 183).

Several reports have implicated immune (gamma) interferon in the induction process for CTL (45, 47, 159). In 1979, Simon et al. (159) characterized a factor obtained from alloantigen-stimulated mouse spleen cell culture medium which could enhance the generation of CTL. Biochemical analysis revealed that the factor was indistinguishable from gamma interferon. Farrar and coworkers (45) demonstrated that anti-gamma interferon antibody could inhibit the response of CTL to alloantigen.

Since two of the factors discussed above (IL-2 and gamma interferon) and probably also TCDF are the products of $\text{Thy } 1^+$, $\text{Lyt } 1^{+23-}$ T helper

lymphocytes, a critical role for these cells in the generation of cytotoxic T lymphocytes is implied. More direct evidence for the participation of helper cells in CTL induction has come from a number of studies.

Pfizenmaier et al. (123) used double chambers to demonstrate that antigen-activated T helpers could deliver help across cell-impermeable membranes. The CTL responses to alloantigen, TNP-modified self, and Sendai virus were all studied. They determined that the helpers were induced in an antigen-specific fashion, since in all cases in vivo primed cells needed to be restimulated in vitro with the same antigen to serve effectively as helper cells.

In 1977, Pilarski (125) showed that primary CTL responses to glutaraldehyde-fixed allogeneic cells could be generated if cultures were supplemented with primed, irradiated T helper cells. This suggested that the failure to generate effective in vitro primary responses to a number of antigens, most notably viruses, might reflect inadequate helper cell activity.

The Pilarski system was soon extended to viral systems (3, 133). Ashman and Mullbacher (3) used in vivo influenza A or Sendai virus primed splenocytes as a source of T helper cells for in vitro primary CTL responses. It was shown that, in order to obtain primary responses from normal cells, Sendai-primed helpers need to be restimulated with Sendai virus, and the same was true for influenza A CTL responses. The helper cells were sensitive to treatment with anti-Ly1 plus complement. Similar results were obtained with primary influenza A CTL responses by Reiss and Burakoff (133).

In the present report, a requirement for T helpers in the CTL response to herpes virus will be demonstrated in a similar fashion. In addition, antigen-primed irradiated splenocytes will be shown capable of restoring the responsiveness of Lyt 1-depleted primed splenocytes.

The activation of CTL (as well as the humoral response and many nonspecific immune activities) therefore requires the sequential interaction of antigen, several cell types, and/or their soluble products.

CTL activity can also be negatively regulated. Suppressor T lymphocytes, which have the phenotype Thy1^+ , $\text{Lyt 1}^-, 23^+$ or Thy1^+ , $\text{Lyt 1}^+, 23^+$, can be stimulated by antigen to synthesize and secrete soluble proteins which prevent CTL activity either by shutting down T cell help (135) or by directly inhibiting the effector CTL (109, 135). The suppressor proteins may act in an antigen-specific (109, 134, 151) or nonspecific (83, 151, 178) fashion. Antigen-specific factors have been described for the CTL response, but have not yet been well-characterized (109, 134, 151). If they are analogous to antigen-specific suppressor factors described for the antibody response, the participation of two cell types can be anticipated (128). The suppressor inducer T lymphocyte is $\text{Lyt 1}^+, 23^-$, I-J^+ , and is the cell that interacts directly with antigen. This cell produces a factor which is antigen-binding and bears I-J region determinants (128). The I-J products are coded for in a subregion of the H-2I region of the mouse, and are involved in the regulation of antigen-specific suppressor

signals. Both the antigen-binding portion and the I-J determinant must be present for the inducer molecule to be functional, and suppression is mediated in an H-2 restricted fashion (128). The inducer molecule binds to the subset of suppressor T lymphocytes bearing receptors that resemble antigen. The activated suppressor then produces a factor bearing an idiotype which mimics antigen, binding to the antigen-specific subsets of T helper lymphocytes or CTL, and specifically inactivates them.

Antigen-specific and/or H-2 restricted suppressor activities have been described by several groups (109, 134, 151). Rich et al. (134) reported a soluble factor which suppressed mixed leukocyte cultures in an H-2 restricted manner. For a responder cell to be suppressed by this factor, it must have I region compatibility, but not K, D, or S region compatibility with the cell which produced the factor. The antigen-specific suppressor cell (TsS) isolated by Schwartz et al. (151) suppresses only the CTL response directed against the alloantigen used to induce suppression. Miller (109) has shown that a subpopulation of cells from the spleen of athymic nude mice can directly inactivate CTL precursors capable of recognizing either the H-2 antigens or TNP modifications of the nude splenocytes.

Nonspecific suppression of the CTL response has also been described (83, 151, 178). Truitt et al. (178), have characterized a soluble factor which nonspecifically suppressed mixed leukocyte reactions. They determined that the factor is not H-2 restricted, and also that its activity can be abrogated by the addition of the reducing agent,

2-mercaptoethanol. Schwartz and coworkers (151) have identified a suppressor cell (TsN), which nonspecifically prevents the generation of new CTL. When these cells are transferred to a fresh mixed leukocyte culture, they suppress the generation of CTL activity without regard to the haplotype used to stimulate. In addition, Kedar and Schwartzbach (83) have shown that the preincubation of normal splenocyte for 3 days or more with fetal calf serum results in the induction of a nonspecific suppressor cell population. The products of these cells suppress both humoral and CTL responses.

An unusual form of antigen-specific suppression at the level of the antigen presenting cell has recently been described by Ptak and Gershon (127). In this system, TNP-labeled peritoneal exudate cells were coinjected with either suppressor T cells or suppressor factor. The suppressor factor was able to block the ability of TNP-labeled PEC to activate a contact sensitivity response, and also blocked their ability to activate suppressor cells. On their own, TNP-labeled PEC can activate both suppressor cells and the cells responsible for contact sensitivity. Thus, T suppressor cells or their factors can guide antigen-bearing accessory cells to bypass the immunological apparatus, such that there is no evidence that the antigen was ever present in the system. The phenomenon has been termed immunological agnosis, since the cells involved seem to have "forgotten" that they have seen specific antigen.

A major obstacle to the study of suppressor systems in the CTL response is the expression by both suppressor cells and CTL of very

similar phenotypes, precluding a straightforward scheme for separating the participating cell types. Recently, Schwartz and coworkers (151) have described a system for the dissection of the CTL suppressor activities by selectively inhibiting either the antigen-specific or the nonspecific suppressor cells. Responder cells were taken from mice which differed from the suppressor cell population only at the Thy1 locus. The responders express the Thy 1.1 allele, and the suppressors express the Thy 1.2 marker. At the time of assay, cultures were treated with anti Thy 1.2 to eliminate any possible contribution by that population to measured CTL activity. It was determined that the nonspecific suppressor cell was extremely sensitive to gamma-irradiation, or to treatment with mitomycin C. The antigen-specific suppressor was resistant to all of these treatments. In contrast, antigen-specific suppression was sensitive to pretreatment with the histamine receptor antagonist, pyrilamine, but nonspecific suppression was unaffected. Thus, it is now feasible to study the activities of both antigen-specific and nonspecific suppressor cells independently of one another.

The existence of specifically-induced antagonistic influences on immune responsiveness suggests that the immune system consists of a network of interacting cells which has as its purpose the maintenance of stability or homeostasis of the entire system of blood cells. The introduction of foreign antigen into the system shifts it into temporary imbalance, inducing the activation of those cells which serve to eliminate the antigen. Once antigen has been dealt with, the new imbalance imposed on the system by the proliferation of

antigen-obliterating cells must be counteracted by a negative signal which then restores the original balance.

Obviously, in a system which is so complicated and large, it is impractical for an investigator to study more than one small portion of it at any one time. The purpose of the results which are reported in this dissertation have as their aim the characterization of those cellular interactions required for the induction of cytotoxic T lymphocytes.

Piecing together information from the alloantigen, tumor cell, and virus systems, the activation of CTL can be considered to take place after the following sequence of events has occurred. Specific antigen is accumulated and possibly processed on the surface of an Ia⁺ accessory cell, which is now actively secreting IL-1. IL-1 interacts with the T helper cell precursor, and antigen is also physically presented to the T helper cell precursor in the context of the Ia antigen. When both of these interactions have taken place, the antigen-specific T helper cells proliferate and begin to secrete a variety of soluble protein factors, including IL-2, gamma interferon and possibly TCDF (Figure I).

Simultaneously, antigen interacts with antigen-specific CTL precursors, directing the synthesis and surface expression of the receptors for IL-2 and TCDF. As these proliferative and differentiative factors become available, they bind to their respective receptors, stimulating the expansion and final differentiation of the antigen-specific subset of CTL to its fully-activated state. These cells are then capable of lysing any cell which expresses the appropriate self histocompatibility antigens as well as the relevant nominal antigen.

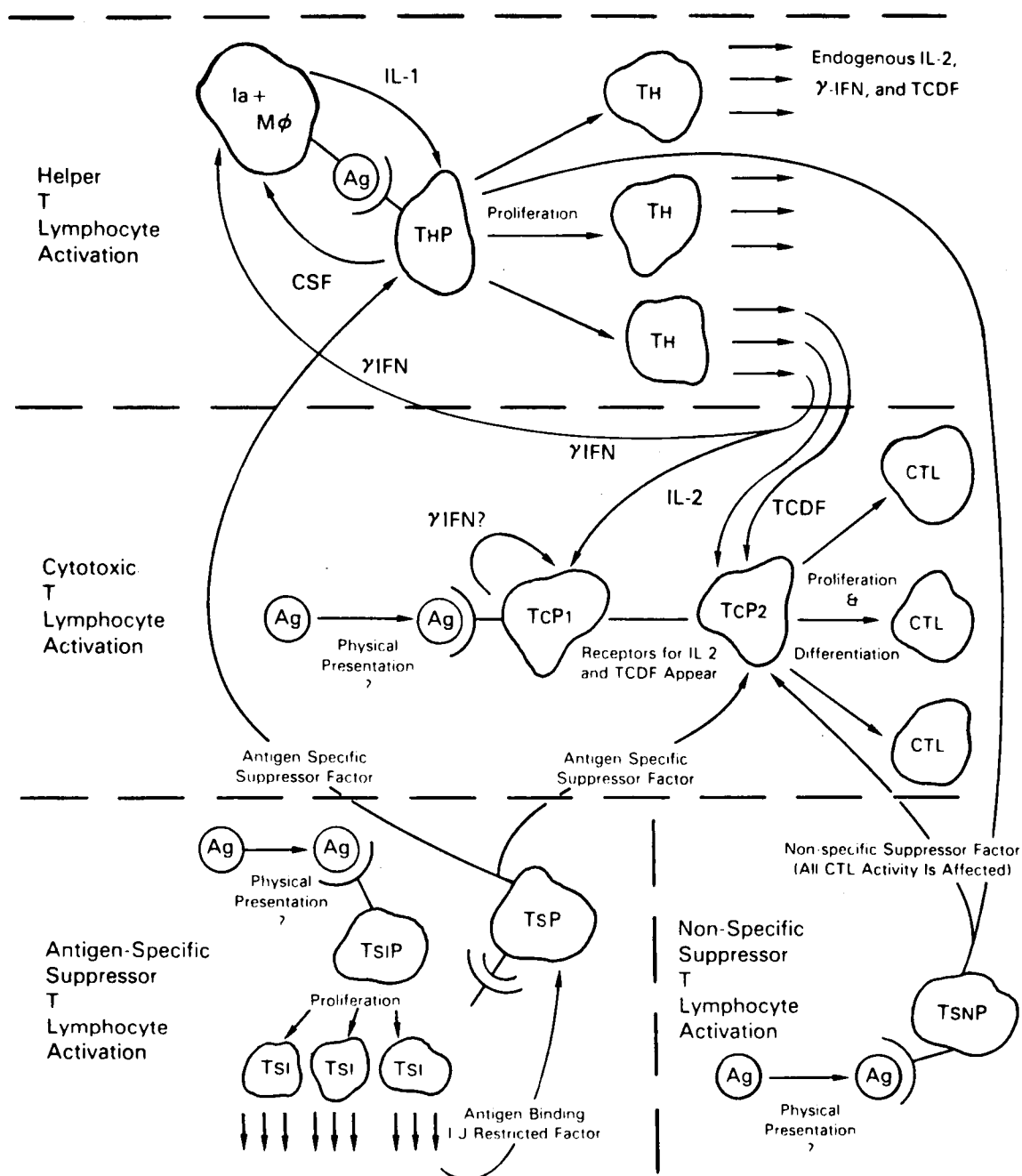


Figure I. Proposed Scheme for the Cellular Interaction Required for the Induction and Suppression of Cytotoxic T Lymphocyte Responses.

The precise conditions under which suppressor cell circuits are engaged or become effective are not well-defined. Certainly the suppressor cell circuit has a role in preventing immune responses directed against self antigens, as evidenced by studies of neonatal mice (25, 64, 74, 171). If antigen is administered repeatedly beginning at birth to mice, they are unable to respond to that antigen (25, 64, 74, 171). The condition of immunological tolerance, which is at least partly dependent upon activated antigen-specific suppressor cells (74, 109), is therefore maintained by the continuous presence of antigen. Under most other circumstances, it would be undesirable to suppress the immune response before the antigen is virtually eliminated, since this would prevent the engagement of immune responses at the point in time when they are most required. Whether an antigen will induce suppressor activity or anti-antigen activity is apparently dependent upon the way in which antigen is presented to the immune system (25, 24). Neonatal mice could be rendered tolerant to TNP if the antigen was repeatedly administered in the form of water soluble TNBS given by i.p. injection, or in the form of TNP-modified red blood cells (25). TNP-modified lymphoid cells did not induce tolerance, and in fact enhanced the generation of hapten-specific CTL. Thus, which arm of the immune response gets activated is perhaps a combined function of the form and quantity of available antigen, as well as the point in time during ontogeny that the antigen is present.

The Nature of Effector CTL: Target Cell Interaction

Numerous studies have been conducted to determine the requirements for a target cell to be recognized and lysed by an antigen-specific CTL. The generation of alloantigen reactive CTL represents a special case, since these CTL are not restricted to self. The most straightforward explanation for alloreactivity is that the foreign H-2 antigens represent both the restricting element and the nominal antigen. Since alloreactive CTL can be generated against I-region determinants or against L region antigens which are not restricted to K or D region antigens, alloreactive CTL do not obey the normal functional division of the MHC complex (36, 68, 85, 112, 181). These disparities in the alloantigen response have made it somewhat difficult to explain alloreactivity in terms of the dual recognition and division of labor within the MHC which has been observed in syngeneic reactions.

Two speculative models have emerged to explain the nature of T cell interactions with other cells: (1) that there are two recognition sites that are specific for the restricting self antigen and the nominal antigen, known as the "dual recognition" model; (2) a single receptor site specific for a neoantigenic determinant is generated by the physical interaction of the self and nominal antigens in the cell membrane, known as the "altered self" model. A clear distinction between these two models is still not possible, and will very likely not be resolved until the T cell receptor molecules are isolated and characterized.

Alloreactivity can be simply explained in terms of the altered self model. The foreign histocompatibility antigens would be recognized as an altered self-histocompatibility antigen by a single T cell receptor.

An explanation of alloreactivity in terms of a two receptor model is possible if one considers the positive and negative selection processes that occur during the establishment of the T cell repertoire of an animal (5, 12, 58, 78, 79). One can postulate that the genetic information of an individual codes for a wide variety of T cell receptors, including those which have a high affinity for self. The presumption is made that, for T cells to be activated, they must have either one receptor engaged at high affinity, or alternatively both receptors engaged at low affinity. The body positively selects those T cells which have measurable, but subthreshold, affinity for self in one of the receptors, and negatively selects those cells which have a high affinity for self or moderate affinity in both receptors. In an individual having a different MHC profile, a different pattern of selection occurs, leaving cells which would react strongly in response to the MHC antigens of another individual.

As with CTL induction, although a number of proteins may be expressed in the cell membrane during viral infection, frequently the CTL response is directed against only one or a few of them.

The CTL activity generated against reovirus-infected cells is all directed against the hemagglutinin molecule, as shown by studies of recombinant viruses (50). For the influenza viruses, both strain specific and cross-reactive CTL activity seems to be predominantly directed against the hemagglutinin and neuraminidase molecules (66).

Although a number of proteins may be expressed in cell membranes during herpes simplex virus infection, virutally all CTL activity is

directed against the viral glycoproteins (97). Target cells which have been infected with HSV-1 in the presence of either tunicamycin or 2-deoxy-D-glucose, both of which are known to inhibit glycoprotein synthesis (31, 99), were no longer susceptible to lysis by herpes-specific CTL (97). Additionally, studies with temperature sensitive HSV-1 mutants, which expressed diminished levels of some glycoproteins at the nonpermissive temperature, have indicated that the HSV-1 viral protein complex VP123 is the most important in the generation of herpes-specific CTL (97).

As in the influenza system, both type-specific and type common CTL have been detected in herpes virus stimulated populations (41, 155). CTL generated against either HSV-1 or HSV-2 contain a substantial subpopulation of effector cells directed against the heterologous type. While the determination of which glycoproteins are involved in cross-reactive and those in type-specific CTL stimulation remains in the preliminary stages, it is considered that viral glycoproteins gC and gAB are responsible for the generation of type-specific CTL. gD is considered the best candidate for the induction of cross-reactive CTL (41).

The question of whether or not viral protein synthesis is required to render a target cell susceptible to CTL lysis remains controversial. Studies of influenza A and ectromelia virus have demonstrated a requirement for viral protein synthesis in the target cell (1, 17, 70, 149). Certain viruses having fusion capacity, such as Sendai virus, can make target cells susceptible to lysis by CTL even though inactivated (149).

Even under conditions where viral fusion with the target cell has been severely impaired, at least one group has been able to demonstrate that targets can still be rendered susceptible to lysis (70). Target cells were glutaraldehyde-fixed prior to exposure to vaccinia virus, so that the viral particles were free to attach, but not to fuse with, target cell membranes. Such targets could still be lysed by vaccinia-specific CTL.

However, the majority of data in all systems tested suggest that the virus must either be infectious or have active fusion capacity to render cells vulnerable to attack (1, 17, 67, 70, 91, 149). For example, Hale and coworkers (67) have done elegant liposome studies which demonstrate a definite role for active fusion protein in the process of making targets susceptible to lysis. Liposomes containing purified hemagglutinin-neuraminidase and fusion glycoproteins of Sendai plus purified H-2K^k were constructed. Liposomes containing no fusion proteins or inactive fusion protein were not effective at rendering uninfected, H-2 negative cells lysable by antigen-specific CTL, whereas liposomes containing active fusion protein made target cells susceptible to CTL mediated lysis. Similar experiments have been conducted with influenza virus liposomes (91), with comparable results.

It has been known for some time that the interactions between virus-specific CTL effectors and the target cell involves both viral antigens and class I antigens of the major histocompatibility complex. Viral systems have been used as a preliminary approach to the resolution of the question of altered self versus dual recognition (8, 56, 62, 71,

148, 154, 194, 195). The altered self hypothesis requires that the H-2 molecule form an association with nominal antigen. Much effort has been made to establish whether or not such complexes are formed in the membrane of target cells. The demonstration of such complexes would not serve as a confirmation of altered self, since the complexed antigens could still be independently recognized by discrete T cell receptors. On the other hand, the demonstration that the complexes are not required for target cell recognition and lysis by CTL would argue in favor of dual recognition.

Cocapping studies have produced variable results. Studies of vaccinia virus antigens (62), Sendai virus (144), and certain leukemia antigens (148, 194) have indicated that the nominal antigens and H-2 antigens of these systems cap simultaneously.

Senik and Neauport-Sautes (91) used a double immunofluorescence labeling approach (fluorescein and rhodamine) to show that when capping of H-2 molecules was induced on a vaccinia virus-infected target cell, the viral antigens also cap. Other antigens on the cell surface, such as Thy 1.2 and FMR failed to cocap, suggesting a special association between viral antigens and H-2. It is possible, however, that the cocapping phenomenon is mere coincidence, the result of both antigens being linked to the same cytoskeletal components.

A number of studies have failed to demonstrate the cocapping of nominal antigen and H-2 antigens. For example, experiments with target cells infected with influenza virus (8), and measles virus (71) failed to show any evidence of cocapping. Murine oncornavirus-induced tumor

antigens (62) and male-specific H-Y antigens (56) also failed to cocap with H-2 antigens.

Zarling et al. (195) used a different approach to determine whether or not Sendai virus proteins associate with H-2 molecules in infected cells. N-succinimidyl-4-azido-phenyl-1,3'-dithiopropionate (SADP) can be used to cross-link adjacent proteins in a cell membrane. Cross-linked molecules were isolated from membranes and immunoprecipitated with antibodies directed against Sendai virus proteins. The cross-linked proteins were dissociated and characterized. H-2 molecules were not found to be associated with the viral antigens.

Thus, while some systems demonstrate an apparent association between nominal antigen and H-2 antigens, the existence of systems in which no such association occurs serve as evidence favoring the two receptor model for T cell recognition.

The Nature of the Cytotoxic T Cell-Mediated Lytic Event

The precise nature of biochemical process by which CTL effect target cell lysis remains a mystery. Still, a number of things are known about the requirements. The CTL effector must directly contact the target cell and must be living (29, 76). There is a requirement for Ca^{++} and Mg^{++} (29). Seraly et al. (153) demonstrated that CTL activity occurs equally well regardless of the phase of the growth cycle. The lytic event is temperature dependent, occurring most efficiently at temperatures of between 29°C and 37°C (4).

Electron microscopic studies have revealed several points of interest regarding the CTL lytic event (145). The electron microscope

distinguishes two subpopulations of T lymphocytes, pale T cells and dark T cells. The pale T cell population is clearly the population responsible for mediating target cell destruction. Although dark cells form contacts with targets, they tend to be less extensive than those formed by pale T cells, and do not result in target cell lysis. The pale cells project pseudopodia into the target cells, invaginating but not breaching their membrane. In addition, the target cells can be seen to go into a state of zeiosis, the formation of cytoplasmic membrane blebs, which give the appearance in time lapse studies of a bubbling membrane. After zeiosis the target becomes quiescent, the nucleus breaks down and then finally the entire cell.

Although early studies attempted to draw parallels between CTL mediated lysis and antibody mediated lysis (by complement fixation) (73), the electron microscopic studies of Sanderson and Galvert (145), and several more recent studies by Russell and coworkers (140, 141, 142) have shown that the CTL mechanism is distinct. Russell et al. (141) double-labelled cells with chromium-51 and iodine 125, with the latter isotope serving as a nuclear label. With antibody + complement-mediated lysis, ⁵¹Cr-release was nearly immediate, with no release of the nuclear label. When the same doubly labelled targets were lysed by CTL, simultaneous release of nuclear and cytoplasmic label was observed. Later investigations (142) revealed that nuclear disintegration occurs quite rapidly, so that the time between the initiation of the lesion and its expression is only 15 minutes. In contrast, 90-120 minutes are required for the appearance of the cytoplasmic membrane lesion. Isolated nuclei

are not susceptible to CTL lysis. Thus, the CTL must associate directly with the cytoplasmic membrane to initiate a process that first manifests itself in the nuclear membrane. The mechanism is now considered to involve either the injection of degradative substances by CTL or activation of a degradative process conducted by the target cell itself, culminating in its own destruction (142).

The In Vivo Relevance of Cytotoxic T Lymphocytes

The relevance of CTL in recovery from viral infections is difficult to demonstrate in a fashion that satisfactorily identifies the cytotoxic T lymphocyte as the effector cell. Nevertheless, there is good evidence to suggest that CTL play an important role in vivo.

Primary in vivo responses to viruses are generally demonstrable in mice, as measured by in vitro assay of cells removed 5-9 days following infection (38, 55, 88, 98, 107, 141). The point in time following infection at which the peak level of CTL activity occurs varies slightly depending on the type of virus injected, the route of injection, and the strain of mouse which is used. If animals are treated with cyclophosphamide, primary CTL responses to HSV-1 can be demonstrated in mice (98). If C3H/HeJ mice are infected i.p. with HSV-1, lower levels of virus-specific, H-2 restricted CTL activity can be detected that peaks 6-8 days post infection.

Two approaches have been taken for inducing secondary CTL responses in vivo. The first method consists of challenging mice with the same virus used in the primary infection 2 to 4 weeks after the first

stimulation. This technique usually results in the rapid generation of CTL activity which is relatively lower than that measured in the primary response (1, 40, 54). The second approach is to transfer primed lymphocytes into an infected, lethally-irradiated recipient, a technique that can result in activities comparable to those obtained for in vitro secondary response (40). The reason for the observation of limited secondary CTL responses in vivo has not been determined. However, it is thought to either reflect the concurrent presence of neutralizing or blocking antibody, or else a more rapid elimination of antigen by cytotoxic T cells. An additional explanation may be that suppressive mechanisms are engaged during secondary in vivo stimulation, which specifically prevent the generation of CTL.

The adoptive transfer of in vitro stimulated cell populations into recipient mice which have been given a lethal dose of virus has been a valuable approach to assessing the role of CTL in recovery, although care must be taken to define the subpopulation of cells transferred. Blanden showed that recovery of mice from mousepox infection could be enhanced by the adoptive transfer of immune cells to infected animals. The enhancement was abrogated if transferred populations were treated with anti-thymocyte antiserum plus complement (9). With the advent of continuously maintained virus-specific H-2-restricted CTL clones, it has become possible to conduct adoptive transfer experiments employing homogeneous populations of cells having exquisitely-defined activities. Lin and Askonas (103) showed that if mice were injected with cloned anti-influenza A virus specific CTL and transferred into mice 24 hours

post-infection, the recipient mice were protected from a lethal dose of virus.

As discussed earlier, considerable cross-reactivity develops against all influenza A viruses when cells are primed to any given influenza A strain (42, 43, 66, 86, 180). When influenza A-specific CTL are transferred to a mouse which has been infected with a different influenza A strain, lung titers of virus are lowered (192). In addition, mice which have been primed with one strain of influenza A, are more resistant to infection by another influenza A strain. It therefore seems likely that cross-reactive CTL develop in vivo as well, and that they have a role in protection against influenza virus infections.

Cytotoxic T lymphocytes also appear to assist in the recovery of mice from herpes virus infection. The adoptive transfer of HSV-1 activated CTL which have been enriched for $\text{Lyt } 1^{-}23^{+}$ CTL can protect mice from a lethal dose of HSV-1 given simultaneously intravenously (93).

Thus, provided that adequate numbers of CTL can be generated, they appear to be effective at improving an infected animal's ability to recover from viral diseases. The ability to induce primary and secondary responses to viruses in vivo, coupled with the observation that adoptively transferred CTL confer protection, serves as good evidence that CTL play an important role in eliminating virus from infected cells. However, since the measured levels of in vivo induced CTL activity are generally low, the relative contribution of CTL to recovery from natural infections is still largely speculative.

Rationale for the Present Study

Numerous studies have shown that the induction of CTL responses depends upon the interaction of several cell types, including the CTL precursor, T helper lymphocytes, and adherent accessory cells (49, 100, 122, 133, 177, 186, 188-190). The explanation for the low CTL responses observed in vivo may involve regulatory mechanisms, such as active suppression or a failure of T cell help. It is possible in many cases to generate high levels of anti-viral CTL activity in vitro (6, 17, 43, 50, 87, 139, 180), suggesting that under the appropriate conditions of stimulation, regulatory activity which favors the generation of CTL can be induced. It therefore became important to better understand the activation requirements, not only of the CTL precursor, but also of the cells involved in regulating its activation. The work presented in this dissertation was designed to determine which interactions of cells and cell products are required to induce CTL responses against HSV-1 in vitro. Some of the properties of an adherent accessory cell, which is absolutely required for the generation of anti-HSV CTL (139), were determined. In addition, the roles of the adherent accessory cell in the CTL activation process were studied. Finally, we studied the characteristics, role, and induction requirements for helper T lymphocytes.

The ultimate importance of this work perhaps lies in its potential to lead to the development of vaccines which are better capable of stimulating CTL activity. Previous studies have indicated that infectious HSV-1 is able to generate potent secondary in vitro responses

(98, 139), whereas inactivated viral preparations (139), or purified HSV-1 glycoproteins incorporated into liposomes (113) are not. The present work will show that the failure of inactivated HSV-1 preparations to induce CTL activity may be due to inadequate stimulation of T cell help. This possibility had been suggested in earlier work, which had shown that heat-inactivated HSV-1 preparations were stimulatory for secondary in vitro CTL responses if helper cell factors were added to cultures (139). It is therefore possible that some viral antigen preparations which are ineffective at generating CTL on their own in vivo, may be made more efficacious by the coadministration of purified, helper cell-derived regulatory molecules.

II. SPECIFIC AIMS

1. The determination of the cellular requirements of secondary in vitro cytotoxic T lymphocyte responses to herpes simplex virus type 1.
2. The determination of the surface antigen profiles of the cell types which participate in the anti-HSV CTL response.
3. Analysis of the role of nonspecific soluble mediators in the generation of CTL.
4. Analysis of the requirements for the stimulation of nonspecific protein mediator production.
5. The characterization of the sequential pattern of interactions of the cellular and molecular participants in the CTL response to HSV-1.

III. THE ROLE OF ACCESSORY CELLS AND T CELL
GROWTH FACTOR IN INDUCTION OF CYTOTOXIC T
LYMPHOCYTES AGAINST HERPES SIMPLEX
VIRUS ANTIGENS

List of Abbreviations

TCGF: T cell growth factor
HSV: herpes simplex virus type 1
CTL: cytotoxic T lymphocyte
FCS: fetal calf serum
PFU: plaque-forming unit
MOI: multiplicity of infection
PC: peritoneal cells

Introduction

It is now apparent from studies in a variety of systems, that the generation of cytotoxic T lymphocyte (CTL) responses involves the essential interaction of a variety of cell types and cell products now fashionably known as interleukins (21, 34, 161, 185). We have begun the analysis of the in vitro induction requirements for CTL generation in antigen primed splenocytes against herpes simplex virus type 1 (HSV) and have shown that responses require the presence of accessory cells or the addition of T cell growth factor (TCGF) to splenocytes depleted of accessory cells (98, 139). In the present communication, we further analyze the role of accessory cells and TCGF for the induction of

secondary CTL responses against HSV and show that accessory cells and TCGF are required at different stages of induction. Our data are interpreted to indicate that accessory cells are essential for the response of helper cells to virus and that CTL precursors can respond optimally to free viral antigens providing TCGF is additionally present.

Materials and Methods

Cells and Viruses Used

Strain L929 cells ($H-2^k$) and strain BALB/c 3T3 clone A31 (abbreviated A31) were grown in McCoy 5A medium supplemented with 5% donor calf serum. Cell strain HEp-2, was cultured in McCoy's 5A plus 10% donor calf serum. HSV-1 strain KOS was grown in HEp-2 cells by infecting cells at low multiplicity and harvesting cell-associated virus as described previously (98).

Mouse Immunization and Preparation of Spleen Cells

Young C3H/HEJ mice ($H-2^k$) and BALB/c mice ($H-2^d$) were obtained from The University of Tennessee Medical Research Center or from Cumberland View Farms, Clinton, TN. F_1 hybrid (BALB/c $\times$ C3H/HEJ) F_1 mice were bred in our own animal facility. Animals were infected intraperitoneally with 0.1 ml inoculum containing 10^7 plaque forming units (pfu) of infectious HSV-1 KOS. At least 4 weeks following infection, mice were killed by cervical dislocation and their spleens were removed aseptically for preparation of single-cell suspensions as described elsewhere (98). To measure cytotoxicity, spleen cells were adjusted to

various concentrations in RPMI 1640 containing 5% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml) and buffered to pH 7.2 with Hepes (RPMI-HEPES). For in vitro culture, spleen cells were adjusted to 2×10^6 /ml in RPMI 1640 containing 10% FCS, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 5×10^{-5} M 2-mercaptoethanol and added to plastic tissue culture plates at a density of 10^6 per cm² surface area. The spleen cell cultures were incubated in a humidified CO₂ (5%) incubator for 5 days, after which time, cells were harvested, washed twice in medium, and adjusted to the desired concentration for cytotoxicity in RPMI-HEPES. For in vitro stimulation, either viable HSV-1 or heat-inactivated (60 min at 56°C) virus was added directly to culture dishes containing spleen cells.

Anti-Thy 1.2 antiserum was provided by Dr. Jon Sprent (Wistar Institute, Philadelphia) and rabbit low mouse toxicity complement from Cedarline Laboratories (Hicksville, NY). Cells were incubated for 45 min at room temperature with a 1:256 dilution of antiserum, followed by 30 min at 37° with complement.

Preparation of T Cell Growth Factor (TCGF) Containing Supernatant Fluid

Spleen cells were isolated from Lewis rats (Charles River, Wilmington, MA) and suspended at a concentration of 1.25×10^6 /ml in RPMI 1640, 10% FCS. Aliquots of 20 ml suspension were placed in plastic 100 mm petri dishes and stimulated with 3.0 g/ml of concanavalin A (Con A Pharmacia, Uppsala, Sweden). Cells were cultured for 24 hr at 37°C in a humidified CO₂ incubator, after which the supernatant fluids

were harvested and freed of cells and cellular debris by centrifugation at $2,000 \times g$ for 30 min. Con A was neutralized by adding 0.1 mg/ml of -methyl mannoside, or removed by absorption to Sephadex G-25 (60). Supernatant fluids were then sterilized by filtration through a 0.2 micrometer membrane filter (Nalgene), aliquoted and stored at -70°C .

Isolation of Resident Peritoneal Cells (PC)

Mice were killed by cervical dislocation and 10 ml of chilled phosphate-buffered saline (PBS) were injected into the peritoneal cavity. Cells were removed and pelleted by centrifugation at $400 \times g$ for 4 min then suspended in culture medium and added to cultures of splenocytes in various numbers. In some experiments, PC were incubated for one hour at 22°C with infectious HSV-1 at a multiplicity of infection (MOI) of ten, washed twice with HBSS and then added to cultures in graded numbers.

Removal of Adherent Cells on Sephadex G-10

This was done as described by Ting and Rodrigues (177). Briefly, sterile Sephadex G-10 was hydrated with RPMI 1640, 10% FCS and packed to a volume of 20 ml in syringes which had been plugged with a small piece of glass wool. The columns were adjusted to 37°C . Intact spleen cells were thoroughly mixed into the G10 by pipetting and incubated for 1 hour at 37°C , with occasional remixing. Nonadherent cells were eluted with 20-25 ml of warm medium and pelleted by centrifugation at $400 \times g$ for 8 min. These cells were suspended in new medium and subjected to a second passage over Sephadex G-10.

Cytotoxicity Assays

The details of these assays were published previously (98). Briefly, L or A31 cells suspended in McCoy 5A medium were infected with HSV-1 at a MOI of 10 for 60 min at 37°C then washed and 5×10^6 cells in 1.0 ml labelled for 1 hr with 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear Corp., Boston, MA). After labelling, cells were washed three times with medium, suspended at 10^5 /ml in RPMI-HEPES and 100 μ l volumes added to effector cells in U shaped microtiter plates. Cytotoxicity assays were begun 5 hr after the initiation of infection with HSV-1 and were carried out for 5 hr in a 5% CO_2 humidified incubator. Standard errors were low (<4%), and results were reported as mean percent specific release values computed according to the following formula:

$$\text{percent specific } ^{51}\text{Cr release} = \frac{(\text{effector cell release} - \text{medium control release})}{(\text{total releasable } ^{51}\text{Cr} - \text{medium control})} \times 100$$

The total releasable ^{51}Cr was obtained by exposing target cells to 1% triton $\times 100$.

Results

Requirement for Syngeneic Accessory Cells during Induction of Anti HSV CTL

As reported previously (139), when intact spleen cells from mice infected 4-6 weeks previously with HSV-1 were cultured in vitro with infectious virus, specific cytotoxic activity was generated which was presumably T cell mediated, since the activity was H-2 restricted and

was destroyed by treatment with anti-thy 1 serum plus complement (Table I). However, CTL induction did not occur upon prior removal of adherent cells from the primed splenocytes. These depleted cell populations could be restored to full activity by the addition of syngeneic, but not allogeneic, peritoneal cells (PC) to a concentration of 2.5% of the total cell population (an accessory cell number found to be optimal). To establish at which stage in the induction process the accessory cells were required, experiments were performed in which G10 depleted primed splenocytes were placed in culture, virus added, after which optimal numbers of syngeneic PC were added at different intervals. Cultures were maintained for 5 days and the levels of cytotoxicity measured at 5 days. As can be seen, the addition of PC at culture initiation or at 24 hr, permitted full restoration of activity but when the addition was delayed to 48 hr or later, little or no cytotoxicity was generated (Table I). However, it is also apparent from Table I that when free virus was removed from depleted cells prior to the addition of PC at 24 hr, then the cytotoxic response was markedly reduced.

Not shown in Table I is the observation that when additional infectious virus was added at the same time as PC at 48 hr or 72 hr and the cultures permitted to proceed for a further 5 days, virtually full cytotoxic activity occurred. Thus, the depleted cells were still viable and responsive to induction after 2 days or more in culture if stimulated by virus presented by an accessory cell.

Our results thus far indicate the requirement for an adherent accessory cell, presumably a macrophage, and that this accessory cell is

TABLE I

ACCESSORY CELL REQUIREMENT FOR INDUCTION OF CYTOTOXIC T LYMPHOCYTE
RESPONSES IN HSV PRIMED SPLENOCYTES¹

Responder Population	Accessory Cells	Time Added	HSV-1 Virus	Percent Specific Lysis ²		
				L-HSV (H-2 ^k)	A31-HSV (H-2 ^d)	L(H-2 ^k)
Undepleted	—	—	+	81.7	17.3	5.1
Undepleted	—	—	—	8.0	0.7	1.7
Undepleted ³	—	—	+	7.2	1.2	1.9
Depleted	—	—	+	3.1	1.3	0.8
Depleted	H-2 ^d	0 hr	+	16.4	2.5	2.0
Depleted	H-2 ^k	0 hr	+	82.4	12.9	4.8
Depleted	H-2 ^k	24 hr	+	78.0	ND	8.1
Depleted	H-2 ^k	48 hr	+	15.3	ND	4.2
Depleted	H-2 ^k	72 hr	+	3.2	ND	1.2
Depleted	H-2 ^k	96 hr	+	1.8	ND	0.2
Depleted	H-2 ^k	24 hr	+4	32.1	ND	3.8

¹Spleen cells from C3H/HEJ (H-2^k) mice were cultured for 5 days intact or depleted of adherent cells by 2 consecutive passages over sephadex G10 at a cell density of 10⁷ cells in

TABLE I (continued)

5 ml culture fluid. Peritoneal cells from C3H or BALB/c (H-2^d) were added at different time intervals at cell concentrations of 2.5×10^5 per culture---a concentration found previously to be optimal (139).

²The values shown represent means of quadruplicate cultures done at effector to target cells ratios of 25:1. The standard errors for the 6 hr assays were always below 4%. The H-2^k targets were L929 cells and the H-2^d targets clone A31. Three repeat experiments gave similar pattern of results.

³Effector cells treated with anti thy 1.2 serum plus complement prior to measurement of cytotoxicity.

⁴In this group, the depleted cells were washed three times to remove free virus prior to the addition of PC at 24 hr.

required early during induction and must be syngeneic with the responding splenocytes to perform the accessory function. To further investigate the requirement for genetic restriction during induction of anti-HSV CTL responses, experiments were performed in which HSV-1 primed (BALB/c $\times$ C3H) F_1 splenocytes were depleted of adherent cells and their responses to virus presented in the context of parental PC determined. In these experiments, the PC were reacted with virus at 37°C for 60 min and washed twice prior to addition to depleted cultures. After 5 days of culture, the cytotoxic activity of surviving cells was measured against infected target cells of both parental genotypes. The results, given in Table II, clearly indicate a preferential restoration of CTL activity against the infected target sharing H-2 identity with the accessory cell used for restoration. Thus, the accessory cell—T cell interaction showed H-2 restriction with presumably only those virus specific T cells in an F_1 population committed to one of the two haplotypes being activated when presented with virus. In some instances, when large numbers of PC were added, some suppression of the maximum response was observed. Such suppression has been observed by others (177) and may reflect a normal regulatory function of accessory cells. The results of these experiments suggest that accessory cells were playing an antigen presentation role rather than simply providing a feeder effect, since if the latter was occurring, parental type PC should fully restore the response of F_1 cells against both target haplotypes.

TABLE II

RECONSTITUTION OF DEPLETED HSV PRIMED (BALB/c × C3H)F₁
SPLENOCYTES WITH PARENTAL TYPE ACCESSORY CELLS

Responder Population	Accessory No.	Cell Strain	Viral Antigen	Percent Specific Lysis ²	
				L-HSV (H-2 ^k)	A31-HSV (H-2 ^d)
Undepleted	—		+	81.2	76.2
Undepleted	—		-	2.7	1.5
Depleted ¹	—		+	1.8	3.4
Depleted	10 ⁵	H-2 ^d	+	2.8	7.1
Depleted	2.5 × 10 ⁵	H-2 ^d	+	7.8	25.6
Depleted	5 × 10 ⁵	H-2 ^d	+	14.5	55.2
Depleted	10 ⁶	H-2 ^d	+	12.6	47.5
Depleted	10 ⁵	H-2 ^k	+	9.7	3.4
Depleted	2.5 × 10 ⁵	H-2 ^k	+	36.0	14.1
Depleted	5 × 10 ⁵	H-2 ^k	+	74.7	29.1
Depleted	10 ⁶	H-2 ^k	+	61.2	17.3
Depleted	5 × 10 ⁵	F ₁	+	68.5	60.1

¹Cells from F₁ mice were depleted as described in footnotes to Table I. The accessory cells were peritoneal cells for C3H (H-2^k), BALB/c (H-2^d) or F₁ mice. These cells were reacted with HSV-1 at a multiplicity of infection of 10 for 60 min at 37°, washed twice then added to cultures.

²The chromium release assay was performed for 6 hr at an effector to target cell ratio of 25:1. Standard errors ranged between 1-4%. Two repeat experiments gave similar results.

Role of TCGF in Induction of Anti-HSV
Cytotoxic T Lymphocytes

It is apparent from the results in several systems (116, 162, 186) including induction of anti-HSV CTL (139) that the requirement for accessory cells during induction may be replaced by certain soluble cell products. In Table III, the results of an experiment designed to investigate the requirement for TCGF for induction of anti-HSV CTL are presented. In the experiment, primed splenocytes depleted on Sephadex G-10 were incubated with virus with TCGF added at varying intervals. The cultures were maintained for 5 days. Such experiments were done concurrently with the types of experiments recorded in Table I. It is apparent that the addition of TCGF permitted the induction of substantial CTL responses when added either at the time of culture initiation or when added as late as 48 hr. Even when added at 72 hr, a partial CTL response occurred. Furthermore, if the virus stimulated depleted cells were washed to remove any free virus prior to the addition of TCGF at 24 and 48 hr, then the response of these cultures was comparable to unwashed cultures. Thus, TCGF can be added later than accessory cells and still permit the generation of a CTL response indicating that TCGF may act later in the induction process than do accessory cells.

TCGF is known to act nonspecifically in other systems studied (160, 185); and it was therefore no surprise to find that upon addition of TCGF to F_1 depleted splenocytes, CTL responses active against both parental haplotypes were generated. This proved to be the case as indicated in Table IV, with the responses to both $H-2^d$ and $H-2^k$ virus infected targets being substantially restored by TCGF. It should also

TABLE III
INDUCTION OF CYTOTOXIC T CELL RESPONSES IN ADHERENT
CELL DEPLETED HSV PRIMED SPLENOCYTES BY ADDITION
OF T CELL GROWTH FACTOR

Responder Population	Viral Antigen	TCGF and time added	Percent Specific Lysis		
			L-HSV (H-2 ^k)	A31 (H-2 ^d)	L(H-2 ^k)
Undepleted	+	—	83.5	13.5	4.9
Undepleted	-	—	9.8	ND	2.1
Depleted	+	—	3.7	1.5	1.0
Depleted	+	0 hr	88.3	14.2	8.6
Depleted	+	24 hr	85.6	ND	9.7
Depleted	+	48 hr	63.0	ND	7.9
Depleted	+	74 hr	23.6	ND	2.3
Depleted	+	96 hr	3.2	ND	0.8
Depleted	+2	24 hr	78.3	ND	5.6
Depleted	+2	48 hr	59.1	ND	3.2

¹Experimental design is described in Table I (page 43) except that TCGF was added to a final concentration of 50% instead of the addition of accessory cells. The TCGF was prepared by stimulating rat splenocytes with Con A for 48 hr. Experiment repeated 4 times with similar results.

²In these groups the virus depleted cells were washed three times to remove free virus prior to the addition of TCGF.

TABLE IV
RESTORATION OF CYTOTOXIC RESPONSE OF ADHERENT CELL
DEPLETED (BALB/c × C3H)_{F1} HSV PRIMED
SPLENOCYTES WITH CELL GROWTH FACTOR

Responder Population	Viral Antigen	TCGF and time added	Percent Specific Lysis	
			L-HSV (H-2 ^k)	A31-HSV (H-2 ^d)
Undepleted	+	not added	74.4	42.6
Undepleted	-	not added	13.6	8.2
Depleted	+	not added	6.4	3.0
Depleted	+	0 hr	66.4	36.3
Depleted	+	24 hr	67.5	33.0
Depleted	+	48 hr	58.3	21.6
Depleted	+	72 hr	14.7	9.7
Depleted	+	96 hr	6.9	3.6

¹Experimental design as described in footnotes to Tables II and III. Experiment repeated 3 times with similar results.

be noted that the addition of TCGF to depleted F_1 splenocytes at different times showed a pattern similar to that observed in the responses of C3H depleted splenocytes to TCGF.

Response of Intact and Depleted Primed
Splenocytes to Heat Inactivated Virus

We have observed that whereas infectious or UV inactivated HSV will induce a CTL response in undepleted primed splenocytes, heat inactivated (HI) virus fails to induce a response unless TCGF is added to the cultures (98). As shown in Table V, the addition of TCGF to virus stimulated adherent cell depleted primed splenocytes permitted the generation of a substantial CTL response to HI virus indicating that CTL precursors can respond to such virus in the absence of accessory cells. The addition of TCGF at different times to HI stimulated depleted primed splenocytes showed similar results to those recorded in Table II (data not shown). Whereas the addition of TCGF to intact or depleted cultures permitted the response to HI virus, the addition of additional accessory cells failed to restore a response (data not shown).

Discussion

The intent of the present communication was to investigate and compare the requirements for accessory cells and TCGF in secondary in vitro CTL responses of spleen cells to HSV antigens. We demonstrated that CTL induction did not occur if adherent cells were removed from HSV primed spleen cells prior to antigen stimulation. Thus, secondary CTL responses to herpes antigens requires the participation of accessory

TABLE V
FAILURE OF INDUCTION OF CYTOTOXIC RESPONSES
IN HSV PRIMED SPLENOCYTES BY STIMULATION
WITH HEAT INACTIVATED VIRUS

Responder Population	Viral Antigen	TCGF addition	Percent Specific Lysis	
			L-HSV	L
Undepleted	infectious ¹	none	83.3	5.9
Undepleted	HI	none	12.1	2.6
Undepleted	none	+	13.4	12.4
Undepleted	HI	+	69.5	9.8
Undepleted	none	none	10.2	3.4
Depleted	infectious	none	14.8	4.1
Depleted	HI	none	4.7	6.1
Depleted	HI	+	50.2	3.6
Depleted	infectious	+	63.6	4.2

¹10⁷ pfu virus added to cultures of 10⁷ cells. The same viral stock was heated at 56°C for 60 min to produce the heat inactivated virus.

cells as has been shown for the induction of CTL to subcellular (35, 188) and tumor antigens (53, 61, 173, 190). Although few studies deal with CTL induction against viral antigens, the role of accessory cells is becoming identified, since under certain conditions they may or may not be required. Apparently viruses that fuse with cell membranes (e.g. Sendai virus) do not need accessory cells (87). In the present report, two ways were found to restore induction of anti-herpes CTL in adherent cell depleted splenocytes: the addition of normal peritoneal cells (PC) as accessory cells, or TCGF. In both cases, full restoration could be achieved under appropriate conditions. In the case of accessory cells, they must comprise 2.5% to 5% of the total cell population. In the present communication, the identity of the accessory cell responsible for restoration was not examined but if parallels can be drawn with other systems (34, 185, 190), we assume that macrophages in the PC population were involved.

Our experiments were directed at comparing the restorative effects of accessory cells and TCGF. Clear differences were noted in the time at which it was necessary to add these components in order to achieve optimal CTL induction. For instance, to obtain optimal induction by restoration with accessory cells, the cells needed to be added soon after culture initiation and antigen stimulation. It was possible to delay the addition for 24 hr and still obtain optimal induction provided free virus was added to stimulate cultures at time zero. However, when efforts were made to remove free virus from depleted cells before adding PC, only a suboptimal CTL response was obtained. These experiments indicate that one phase of the anti-herpes induction process requires

that the virus be presentation of virus on accessory cells. Furthermore this viral presentation had to be made by a genetically compatible accessory cell. Induction did not occur upon restoration with allogeneic PC. Furthermore, after the addition of PC of one parental haplotype to F_1 depleted cells, CTL were induced that were cytotoxic only to target cells of the same haplotype as used in restoration. As shown initially by Rosenthal and Shevach (136) and subsequently confirmed by others in different systems (44, 151, 173), there appears to be two clones of antigen reactive T cell subsets in F_1 animals, each reactive with antigen in the context of only one of the two MHC haplotypes.

Our observations that induction in virus stimulated cultures could be achieved by the addition of TCGF implies that accessory cells are not essential for all phases of the CTL response. Indeed, in contrast to restoration with accessory cells, TCGF could be added as late as 48 hr after culture initiation and still induce optimal CTL responses. Furthermore, virus treated depleted cells washed to remove unadsorbed virus, showed a similar pattern of response to TCGF addition. This response was not seen with accessory cell supplementation (vide supra). Attesting to the nonspecific activity of TCGF function was the observation that the addition of TCGF to F_1 depleted splenocytes permitted the generation of CTL able to kill virus infected targets of both haplotypes. We interpret the results of our present experiments to imply that the H-2 restricted accessory cell requirement for anti-herpes CTL induction is principally at the level of the antigen specific cell that generates TCGF rather than the primed CTL. Others have, in fact,

indicated that TCGF is produced by helper cells (117, 157, 186) that are T cell subsets distinct from CTL precursors and distinguishable by their expression of Ly alloantigens (28, 186). In order for helper cells to produce TCGF, they require stimulation by mitogen and presumably antigen in the context of a genetically compatible macrophage (172). It appears likely that the requirement for H-2 restriction with accessory cells in anti-herpes CTL induction is principally at the level of helper cells. Helper cells then function to produce TCGF which, in turn, trigger CTL precursors that have bound antigen to differentiate into CTL effectors in the absence of macrophages. Whereas the entire CTL induction takes 5 days, TCGF can be provided at least as late as 48 hrs and still be fully effective. Presumably during the first 48 hr of culture TCGF is being generated by herpes virus specific helper cells.

The hypothesis that CTL precursors have different antigen presentation requirements than helper cells may also be the explanation for our observation that HI virus failed to induce CTL. However, additional induction occurred upon the addition of TCGF but not by adding accessory cells. We presume that heated virus, perhaps because it fails to be processed effectively by macrophages, cannot trigger helper T cells. Experiments to verify this hypothesis are in progress.

The observation of H-2 restricted restoration of F_1 depleted cells with parental PC, might be taken as an argument that the CTL precursor as well as the helper cells must recognize antigen in the context of a genetically compatible accessory cell. Thus, if helper cells react with antigen and produce TCGF, since this acts nonspecifically, both subsets

of antigen specific T cells in the F_1 population should be triggered to become cytotoxic. However, in these experiments the only source of antigen was in the membrane of the PC and hence it is possible that H-2 restriction was observed because these cells only make effective interaction with those F_1 T cell population reactive with the compatible haplotype. Indeed, we have observed that if noncell bound antigen was added to these cultures, some breakdown in restriction occurred implying that when virus is available other than in an accessory cell membrane CTL precursors may become triggered.

An alternative explanation could be that although the helper cells show H-2 restriction for their stimulation, the amount of IL-2 produced in cultures in response to antigen may be limited so that it is bound only by closely adjacent cells which are CTL of the same haplotype. We are currently pursuing experimental approaches designed to further substantiate our notion that CTL precursors, but not helper cells, specific to herpes virus antigens, can respond to antigen in a non-H-2 restricted mode. We are also investigating the putative role of antiherpes CTL in host defense in vivo.

IV. ROLE OF Ia ANTIGEN EXPRESSION AND SECRETORY
FUNCTION OF ACCESSORY CELLS IN THE INDUCTION OF
CYTOTOXIC T LYMPHOCYTE RESPONSES AGAINST
HERPES SIMPLEX VIRUS

List of Abbreviations

CTL:	cytotoxic T lymphocytes
HSV-1:	herpes simplex virus, type 1
SAC:	splenic adherent cells
PC:	resident peritoneal cells
IL-1:	interleukin 1
IL-2:	interleukin 2
FCS:	fetal calf serum
NCS:	newborn calf serum
ConA:	concanavalin A
PMA:	phorbol myristic acetate

Introduction

It is apparent from numerous studies on the induction of primary and secondary cytotoxic T cell (CTL) responses to a variety of antigens, that histocompatible accessory cells are an absolute requirement (35, 49, 98, 100, 115, 139, 182, 184, 188, 189, 190). However, exactly how accessory cells subserve their function has not been clarified. Experiments primarily in the alloantigen system have indicated that one requirement for accessory cell activity is that the cells express Ia alloantigens

(188, 189). However, from studies employing alloantigen incorporated into liposomes it seems that at least some subsets of antigen specific T cells required for CTL induction do not require antigen expression in the context of Ia (188). The role of Ia antigen expression for the induction of antiviral CTL responses has not been clarified. In a poxvirus system, Ia antigen expression on accessory cells is apparently not required (70), whereas recent studies on the induction of CTL to reovirus indicated a requirement for Ia expression (100). The nature of accessory cells, particularly the part played by Ia expression, for induction of CTL against herpesvirus antigens has not been reported.

In addition to Ia antigen expression, accessory cells may be required to participate in other functions, such as antigen processing and the production of factors such as IL-1 (46, 92, 189). It has been shown that Ia positive cells can no longer act as accessory cells if their functional activity is impaired by UV irradiation (189). The activity of the cells can, however, be restored by the addition of IL-1 containing supernatant fluids.

In the present communication, the role of Ia antigen expression and other properties of accessory cells for the induction of CTL against herpes simplex virus type 1 (HSV-1) is investigated. We show that the expression of Ia antigen on the surface of accessory cells is essential, but not in itself sufficient, for the performance of accessory cell functions in the generation of CTL against HSV. The participation of IL-1 is also required. The possibility of additional requirements is also indicated.

Materials and Methods

Cells and viruses used: Strain L929 cells (H-2^k) were obtained from Flow Laboratories. Strain BALB/c 3T3 clone A31 (H-2^d) were obtained from Dr. Ray Tennant, Biology Division, Oak Ridge National Laboratory. Strain EL-4 Thymoma (H-2^b) and P388D₁ cells (H-2^d) were obtained from Dr. John Farrar, National Institute of Dental Research. L929 cells and A31 cells were both grown in McCoy's 5A medium supplemented with 5% newborn calf serum. EL-4 cells and P388D₁ cells were grown in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum. Herpes simplex virus type 1, strain KOS (HSV-1) was propagated in HEp-2 cells as described by Bone and Courtney (14). The viral stocks used had infectivity titers ranging from 10^8 to 3×10^8 plaque-forming units (PFU) per milliliter.

Mouse immunization and preparation of spleen cells: Details of these procedures were described elsewhere (98). Briefly, spleen cell cultures were established from mice infected i.p. 6 weeks or more previously with 10^6 PFU of HSV-1. To obtain cell populations depleted of accessory cells, unfractionated spleen cells were incubated in 100 mm culture dishes (Corning) at 37°C for 2 hrs (10^8 cells/plate), followed by incubation on a 20 ml column of Sephadex G-10 according to the method of Ly and Mishell (106), and then incubated on nylon wool using the method of Julius et al. (81). In some experiments, the Sephadex G-10 column passage was replaced by a second incubation on nylon wool.

Depleted (10^7) splenocytes or whole spleen cells were added to 6 well cluster plate wells (35 mm Costar) in a final culture volume of

5.0 ml. Cultures were incubated for 5 days at 37°C, 5% CO₂, and then assayed for cytotoxic T cell activity by measuring percent specific ⁵¹CR release.

Preparation of accessory cell populations: Resident peritoneal cells (PC), were collected from adult C3H, BALB/c, or (C3H/HeJ × BALB/c)F₁ mice. Animals were killed by cervical dislocation, and PC were obtained by lavage with chilled phosphate-buffered saline. The cells were washed once with cold RPMI 1640 medium, enumerated, and added at varying cell concentrations to spleen cell cultures. The same population was used to prepare cultured PC. For predominantly Ia⁻ cultured PC, cells were cultured at least 48 hrs prior to use in RPMI 1640, 10% FCS. For high percentage Ia⁺ cultured PC, cells which had been cultured for 24-28 hrs in the absence of ConA supernatant were incubated a further 48 hrs with ConA supernatant (represented as 12.5% of the final culture volume).

Splenic adherent cells (SAC) were obtained by the method of Cowing et al. (33). 5×10^7 spleen cells/plate were incubated in 100 mm glass petri dishes for 2-3 hrs at 37°C, at which time the nonadherent cells were gently washed off. Adherent cells were removed by incubating them at 37°C with several milliliters of a 1:5000 dilution of EDTA buffer for 15 minutes.

Populations enriched with dendritic cells were obtained using a modification of the method of Steinman and Witmer (169). 10^8 splenocytes/plate were incubated for 3 hrs on 100 mm glass petri dishes at 37°C in RPMI 1640, 10% FCS. Nonadherent cells were gently washed away and the adherent cells incubated a further 24 hours in

RPMI 1640, 10% FCS. Cells which had detached during the second incubation were removed and pooled as dendritic cells.

Populations were enriched for B lymphocytes by incubating unfractionated splenocytes for 1 hour at 37°C on glass wool columns which had been saturated with RPMI 1640, 10% FCS. The eluted population was then treated with anti-Thy 1.2 + C. Cell populations were then centrifuged for 20 minutes on density gradients (Lymphocyte M, Cedarline Labs) to remove dead cells and debris. T lymphocytes were prepared by incubating whole splenocytes on nylon wool for 1 hr at 37°C, followed by treatment with anti-mouse Ig + C. Cells were centrifuged on Lymphocyte M gradients to remove dead cells and debris.

L cells, A31 cells, P388D₁ cells, B and T lymphocytes, when employed as potential surrogate accessory cells, were infected at a high multiplicity of infection (m.o.i. = 10) for 6-7 hrs prior to their addition to cultures. When PC, dendritic cells, or SAC were used, virus was either added directly to culture or incubated for 1 hr at 37°C on the cells prior to addition to culture. Cells cultured in ConA supernatants were thoroughly washed prior to addition to cultures.

Glutaraldehyde fixation of cells: Cells were infected with HSV-1 (m.o.i. = 10) 6-8 hrs prior to glutaraldehyde-fixation to ensure the expression of viral glycoproteins on the cell surface. Following this, 1.0 ml of a 0.1% glutaraldehyde solution (diluted in serum-free HBSS) was added to the cell pellet and mixed continuously for 15 seconds. Immediately, the glutaraldehyde suspension of cells was transferred to 50 ml of HBSS and mixed thoroughly. Cells were washed 4-5 times in HBSS.

Preparation of IL-1: This was done according to the method of Mizel and Mizel (110). Briefly, 2×10^6 cells/ml P388D₁ cells in RPMI 1640 (1% FCS) were plated in 16 mm 24 well cluster plates (Costar) at 1.0 ml/well. Cells were incubated one hour to permit adherence. The medium was replaced with RPMI 1640 (1% FCS) + 10 µg/ml PMA, 10 µg/ml cyclohexamide, and 2 mM sodium butyrate. Plates were incubated 4 hours at 37°C and washed to remove the inhibitor. RPMI 1640 (1% FCS) + 10 µg/ml actinomycin D was added and the cells incubated for 1 hour. Plates were then washed 2 times, replenished with RPMI 1640 (1% FCS) + 2 mM sodium butyrate and incubated for 24 hours. Supernatant fluids were collected and centrifuged to remove any cells, whereupon they were precipitated with 65% (NH₄)₂SO₄ at 4°C, redissolved in HBSS, and dialyzed against PBS. The supernatant fluid was clarified by centrifugation at $10,000 \times g$ and filter sterilized. The IL-1 activity was assayed by measuring the ability of preparations, serially diluted, to permit thymocyte proliferative responses in the presence of ConA, as described by Mizel and Mizel (110). In the experiments, 4 units of IL-1 were routinely used, a unit being defined as the highest dilution having detectable activity.

Antisera and indirect immunofluorescence: A.TH anti-A.T1 was obtained from Cedarline Laboratories, Inc., Homby, Ontario. To deplete cultures of Ia⁺ cells, 10^7 cells were incubated with a 1:40 dilution of antiserum, followed by incubation with rabbit (low tox mouse) complement (Cedarline Labs) at a 1:6 dilution. In some experiments, cells were incubated with antiserum alone, in the absence of complement.

Indirect immunofluorescence was carried out in the following fashion. If cells were capable of adherence, they were grown in monolayer on coverslips. If nonadherent, they were incubated in an equivalent volume of antiserum with occasional shaking. Cell suspension or coverslips containing adherent cells were incubated with a 1:40 dilution of A.TH anti-A.TL antiserum for 30 minutes at 4°C. Cells incubated with a 1:10 dilution of normal mouse serum (C3H/HeJ) were used as a control. Cells were then washed and similarly incubated with fluorescein-labeled F(ab)₂ anti-mouse IgG diluted 1:50 (1 ml of dilution for each coverslip). Coverslips were then washed and mounted in glycerol.

Monoclonal anti-thy 1.2 was obtained from Dr. John Sprent, Wistar Institute.

Preparation of Concanavalin A-stimulated spleen cell supernatant fluids: This was prepared according to the method of Steeg et al. (167). 10⁸ splenocytes were incubated in 35 mm culture dishes in a volume of 6.5 ml/culture of RPMI 1640 (2% FCS) with 3 µg/ml ConA (Pharmacia) at 37°C for 48 hrs under 5% CO₂. Cells and cell debris were removed by centrifugation at 2,000 × g for 15 minutes. Supernatant fluids were absorbed with Sephadex G-25 to remove ConA, filter-sterilized through 0.45 µ membrane filters (Nalgene), aliquoted and stored at -70°C.

Cytotoxicity assay: Cytotoxic T cell activity was measured by determining the percent specific release of ⁵¹Cr from labeled, virus-infected target cells. Target cells were prepared by infecting cells

with HSV-1 (m.o.i. = 10) for 2 hours at 37°C, at which point free virus was washed from the cells. 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ solution (New England Nuclear; 1 mCi/ml) was added to the virus-infected cells, which were suspended in 0.9 ml of RPMI 1640, 5% NCS, and labeled at 37°C for 2 hours. Cells were washed 2 times in HBSS, suspended in 5 ml of RPMI 1640, 5% NCS, and incubated 2 more hours at 37°C for a total incubation time of 6 hours. Target cells were washed twice more with HBSS, counted, and adjusted to 10^5 cells/ml in RPMI 1640, 5% NCS.

Effector cells were centrifuged at $400 \times g$ for 8 minutes, washed one time in HBSS, and suspended in RPMI 1640, 5% NCS. Cells were counted, adjusted in RPMI 1640 to the appropriate concentration, and added to 96 well round bottom plates (Costar) in various ratios to 10^4 virus-infected, ^{51}Cr -labeled cells/well. Total volume/well was 200 μ l. Uninfected syngeneic as well as infected allogeneic targets were assayed as controls. Plates were centrifuged at $200 \times g$ for 2 min to pellet the cells, and incubated for 4 hours at 37°C, 5% CO_2 . At the end of the culture period, 100 μ l of supernatant was carefully drawn from each well and transferred to tubes, which were then assayed in a Beckman gamma 4000 spectrometer. All tests were performed in quadruplicate, and % specific ^{51}Cr release was determined using the formula:

$$\% \text{ specific } ^{51}\text{Cr}\text{-release} = \frac{\text{Test release} - \text{background release}}{\text{Triton release} - \text{background release}} \times 100$$

Background medium release was assayed for by incubating 10^4 targets in 200 μ l of medium for the length of the culture period, and Triton

total release was determined by lysing 10^4 cells in 100 μ l of medium with 100 μ l of 3% Triton X-100. Standard errors for the experiments shown were under 4.5%.

Results

Comparison of Different Cell Types to Act as Accessory Cells in Anti-HSV CTL Responses

In previous reports (139, 146), we have shown that in the absence of adherent accessory cells, secondary in vitro CTL responses against HSV cannot be induced. However, responsiveness can readily be restored by the addition of syngeneic, but not allogeneic peritoneal cells (PC), or by the addition of interleukin 2. A further analysis of the nature of the accessory cell type for anti-HSV CTL responses is shown in Table VI, in which varying numbers of different cell types were added to primed spleen cells exhaustively depleted of adherent cells. It is readily apparent that whereas full restoration of anti-HSV CTL responses was achieved with some cell types, others were ineffective. The effective cell types all contained Ia^+ cells and, with the exception of Ia^+ B cells, the ineffective cells were Ia negative. The ineffective cell types included L cell fibroblasts, A31 cells, and syngeneic fibroblasts; cells in which HSV-1 replicates vary effectively and so would be expressing high concentrations of cell surface antigens.

Whereas at high cell numbers of accessory cells (above 2.5%), PC (7-10% Ia^+) were equally as effective as splenic adherent cells (SAC, 35-40% Ia^+), at lower cell numbers the SAC were more effective

TABLE VI
COMPARISON OF DIFFERENT CELL TYPES TO ACT AS ACCESSORY CELLS
IN ANTI-HSV CTL RESPONSES

Cell type added	Percent representation in culture	L(H-2 ^k)	Percent specific ⁵¹ Cr release		
			LHSV (H-2 ^k)	A31 HSV (H-2 ^d)	EL4 HSV (H-2 ^b)
<u>controls</u>					
whole cells + virus	—	6.7	76.8	61.4	9.5
whole cells + no virus	—	1.1 3	10.0	8.4	1.4
depleted cells + virus	—	N.D.	16.2	13.3	2.9
resident PC (F ₁)	.05	4.1	15.7	12.4	1.1
(9% Ia ⁺) ¹	.10	7.8	12.2	10.9	2.6
	.50	6.3	21.3	17.2	2.9
	1.00	7.3	30.4	28.7	6.0
	2.50	2.5	43.7	36.5	8.0
	5.00	4.6	66.2	56.6	3.4
SAC (F ₁)	.05	3.7	16.5	14.3	0.6
(38% Ia ⁺)	.10	2.9	32.7	28.9	1.4
	.50	3.5	49.8	37.4	9.7
	1.00	4.8	67.2	56.9	7.0
	2.50	2.9	66.7	59.7	5.4
	5.00	6.4	63.8	57.2	2.6
Dendritic cells (F ₁)	.50	6.3	42.4	31.7	3.4
	1.00	6.3	62.3	46.4	7.9
(% not determined)	2.50	7.7	77.4	55.8	5.6
	5.00	7.9	75.9	57.6	1.1

TABLE VI (Continued)

Cell type added	Percent representation in culture	L(H-2 ^k)	Percent specific ⁵¹ Cr release		EL4 HSV (H-2b)
			LHSV (H-2 ^k)	A31 HSV (H-2 ^d)	
Cultured PC (F ₁)	.50	6.8	16.5	12.3	8.2
(1.2% Ia ⁺)	1.00	3.1	14.9	11.2	6.9
	2.50	6.0	17.6	11.7	3.9
	5.00	4.9	23.4	17.6	7.5
L cells (H-2 ^k)					
(n.d. Ia) ²	5.00	6.0	14.2	N.D.	1.0
A31 cells (H-2 ^d)					
(n.d. Ia)	5.00	4.3	12.5	N.D.	3.2
Normal fibroblasts (H-2 ^k)					
(n.d. Ia)	5.00	5.5	17.3	N.D.	8.3
B lymphocytes (F ₁)					
(% not determined)	5.00	5.1	14.5	15.3	9.7
T lymphocytes (F ₁)					
(% not determined)	5.00	6.2	16.4	13.7	5.9

¹parentheses indicate percent of Ia positive cells detected by indirect immunofluorescence.

²n.d. Ia = no detectable Ia antigens.

³N.D. = not done.

(C3H/HeJ × BALB/c)F₁ mice were used in these experiments.

Effector cells were assayed for activity at ratios of 25:1 and 5:1 (E:T), and the amount of cytotoxic activity was proportional to the ratio of the effectors used. Values shown are for 25:1.

Assays were conducted for 4 hours using effector populations which had been cultured for 5 days.

TABLE VI (continued)

The ^{51}Cr -targets used were HSV-1 infected L cells (LHSV), uninfected L cells (L), infected 3T3 A31 cells (A31 HSV), and infected EL-4 cells (EL4 HSV). Target cells were infected at least 6 hrs prior to the beginning of the assay.

All cell types were added in at least 4 different ratios to 10^7 depleted splenocytes; wherever all ratios are not shown, the highest number of cells added is represented.

All experiments have been repeated at least 3 times.

than PC. Furthermore, culture of PC for 2 days in vitro, a process that is accompanied by the loss of Ia antigen expression (167), were markedly less effective accessory cells than were uncultured PC. Taken together these results indicate that the expression of Ia antigen is a requirement for cells to act as accessory cells. However, the expression of Ia is not alone sufficient since B cells, in which HSV can replicate, were unable to restore anti-HSV CTL responses in adherent cell depleted splenocytes.

Inhibition of Accessory Cell Function with Anti-Ia Antiserum

To further implicate the role of Ia antigen expression for accessory cell activity, the effect of anti-Ia antiserum (A.TH anti-A.TL) on the response was measured. The first approach, unfractionated C3H/HeJ spleen cells were treated with dilutions of antiserum followed by complement, after which the cells were stimulated for 5 days with virus. As shown in Table VII, the cytotoxic response could be prevented by treatment with anti-Ia serum followed by complement. In the absence of complement, which alone was not inhibitory, the levels of inhibition of the CTL response by the anti-Ia serum were less marked. Viable cell recovery from anti-Ia and complement treated cultures was similar to that from untreated cultures indicating that the antiserum treatment was not nonspecifically toxic.

In a second approach to implicate the importance of Ia antigen expression, resident PC were treated with anti-Ia serum, washed, then treated with complement to kill the Ia positive cells. Control cells

TABLE VII

THE ABROGATION OF ACCESSORY CELL FUNCTION WITH ANTI-Ia
ANTISERUM: TREATMENT OF WHOLE SPLENOCYTES

Antiserum dilution	virus	L	Percent specific ⁵¹ Cr release	
			LHSV	EL4 HSV
no antiserum	+	3.6	56.1	13.6
no antiserum	-	4.5	6.8	7.9
1:20 + C	+	5.2	9.2	8.3
1:40 + C	+	N.D.	8.5	N.D.
1:80 + C	+	N.D.	13.9	4.0
1:160 + C	+	N.D.	32.8	N.D.
1:320 + C	+	N.D.	58.3	N.D.
1:640 + C	+	N.D.	53.6	N.D.
C alone	+	N.D.	53.8	N.D.
1:40; no C	+	3.2	21.3	3.0
1:80; no C	+	4.6	56.8	5.7
1:160; no C	+	1.7	53.4	6.5
1:320; no C	+	2.6	59.3	3.2

A.TH anti-A.TL antiserum dilutions were added directly to culture 1 hour prior to the addition of rabbit (low tox mouse) complement. Both additions were made at the onset of culture.

10^7 unfractionated C3H/HeJ, HSV-primed splenocytes were added to each well.

All other conditions were as described in Table VI.

were treated with complement alone. Both cell populations were added, at optimum viable cell concentrations, to adherent cell-depleted, HSV-primed splenocytes and stimulated with virus. As can be seen, the Ia^- population could no longer act as accessory cells for anti-HSV CTL production (Table VIII).

Enhancement of Accessory Cell Function
Following the Induced Expression of
Ia Antigens

Resident PC freshly obtained by peritoneal lavage, typically include a subpopulation of between 7-10% Ia^+ cells. After 48 hrs of in vitro culturing, the percentage of Ia^+ cells drops, normally to 1% or less. Steeg et al. (167) demonstrated that Ia^- cultured macrophages, as well as Ia^- P388D₁ cells, can be induced to express Ia antigen within 48 hrs if they are cocultured with ConA-stimulated spleen cell supernatant fluids. The relevant mediator in this process is now considered to be gamma interferon (167). This expression of Ia (or lack of it) was detected in all cases by direct immunofluorescence using A.TH anti-A.TL as the primary binding reagent. In these experiments, Ia^- cultured PC and P388D₁ cells were compared with their ConA supernatant induced, Ia^+ counterparts for accessory cell function. All cells were extensively washed prior to virus infection and used as accessory cells to avoid the transfer of soluble factors such as interleukin 2.

It is readily apparent from the data depicted in Table IX, that the conversion of Ia^- cultured PC into Ia^+ cells (15-30%) by the ConA supernatants led to a considerable enhancement of accessory cell function. Thus, whereas cultured PC were virtually without accessory

TABLE VIII
INHIBITION OF ACCESSORY CELL FUNCTION WITH ANTI-Ia
ANTISERUM: PRETREATMENT OF PC

Cell type added	L	Percent specific ⁵¹ Cr release	EL4 HSV
		LSHV	
controls			
whole cells + virus	6.5	56.9	4.6
whole cells + no virus	3.1	8.5	3.4
depleted cells + virus	1.7	11.6	2.3
resident PC	1.3	53.4	8.9
anti Ia 1:40	2.8	13.2	7.3
pretreated PC 1:80	3.6	12.4	3.2
+ C 1:160	2.9	29.3	4.7
1:320	4.3	46.1	5.0
PC pretreated with C alone	1.6	54.0	3.9

PC were pretreated with various dilutions of antiserum in the presence of C, then added to cultures at 5×10^5 viable cells/culture of 10^7 adherent cell depleted, HSV-1-primed splenocytes.

All other conditions of assay were as described in Table VI (page 65).

TABLE IX
ENHANCEMENT OF ACCESSORY CELL FUNCTION FOLLOWING
INDUCED EXPRESSION OF Ia

Cell type added	Percent representation in culture	L	Percent specific ⁵¹ Cr release		
			LHSV	A31 HSV	E14 HSV
controls					
whole cells + virus	—	9.2	65.7	44.6	3.4
whole cells + no virus	—	N.D.	13.9	8.7	6.7
depleted cells + virus	—	N.D.	17.9	9.6	3.9
resident PC (F ₁) (9% Ia ⁺)	0.5	7.2	18.6	8.4	5.6
	1.0	8.4	27.5	12.3	3.4
	2.5	3.7	61.7	29.7	8.2
	5.0	6.8	65.3	37.7	2.5
cultured PC (F ₁) (1% Ia ⁺) ¹	0.5	N.D.	16.3	11.9	N.D.
	1.0	N.D.	13.7	8.8	N.D.
	2.5	5.6	14.0	9.7	12.3
	5.0	N.D.	21.2	15.6	N.D.
supernatant-stimulated PC (F ₁) (21% Ia ⁺) ²	0.5	N.D.	12.9	10.1	4.8
	1.0	N.D.	23.7	16.5	6.4
	2.5	8.4	49.6	29.2	4.7
	5.0	N.D.	61.2	29.9	N.D.
P388D ₁ (H-2 ^d) (<0.5% Ia ⁺)	0.5	9.0	15.7	11.2	7.6
	1.0	11.5	14.6	10.7	4.1
	2.5	10.7	16.7	8.4	3.2
	5.0	6.9	16.2	9.1	3.7
supernatant-stimulated P388D ₁ (H-2 ^d) ² (Ia ⁺) 15%	0.5	10.5	18.8	9.4	9.3
	1.0	7.9	16.5	8.6	7.8
	2.5	12.8	19.4	9.9	10.5
	5.0	12.2	15.2	10.8	3.9

(C3H/HeJ × BALB/c)F₁ mice were used in all experiments.

¹Ia⁻ cultured F₁ PC were incubated in culture at least 48 hrs prior to addition to cultures of splenocytes.

²Ia⁺ cultured PC and P388D₁ cells were incubated with ConA supernatants (present as about 12.5% of total culture volume) for at least 48 hrs prior to addition to culture.

TABLE IX (continued)

All cell types were routinely assayed for Ia antigen expression.

All other conditions of assay were as described in Table VI (page 65).

cell activity, converted PC were as effective as fresh PC and even SAC (data not shown). In contrast, neither Ia^- P388D₁ cells nor Ia^+ converted cells provided accessory cell function. Explanations for the failure of Ia^+ P388D₁ to provide accessory cell function could include insufficient Ia antigen expression, failure to express viral antigen and failure to provide other functional activities such as the secretion of required biological activities. The first explanation of quantity of Ia antigen could not be assessed but the second explanation of viral antigen was considered unlikely since P388D₁ cells do express HSV antigens following their infection with virus (data not shown). Some data on the possible relevance of the secretion of biological mediators is presented below.

Requirement of Functionally Active Accessory Cells

To assess the requirement for functional activity of accessory cells in addition to their Ia antigen expression, experiments were performed in which unfixed and glutaraldehyde-fixed cells were compared for their accessory cell activity. In these experiments, cells were infected for 7 hrs to permit viral antigen expression, rapidly fixed, and then washed 4-5 times and added to accessory cell-depleted, HSV-primed splenocytes. As shown in Table X, glutaraldehyde fixation abolished the accessory cell function. However, the addition of the macrophage product IL-1, shown to play an important role in the induction of CTL responses (27, 46, 92), led to the partial restoration of the accessory cell function of the fixed Ia^+ population. The Ia^- cells were not induced to act as accessory cells

TABLE X
REQUIREMENT FOR FUNCTIONALLY ACTIVE ACCESSORY CELLS

Cell type added	Percent representation in culture	IL-1* added	L	Percent specific ⁵¹ Cr release		EL4 HSV
				LHSV	A31 HSV	
<u>controls</u>						
whole cells + virus	—	—	4.6	62.4	49.5	6.4
whole cells + no virus	—	—	N.D.	10.2	11.1	N.D.
depleted cells + virus	—	—	4.3	12.2	8.4	3.9
depleted cells + 5% PC + virus	—	—	2.7	45.9	39.6	3.8
depleted cells + virus	—	+	6.1	13.7	12.3	4.5
virus-infected, glutaraldehyde-fixed Ia ⁺ resident PC						
	0.5	—	2.9	17.7	9.3	6.6
	1.0	—	N.D.	14.8	10.0	3.5
	2.5	—	N.D.	16.0	8.4	N.D.
	5.0	—	3.5	13.5	9.1	4.7
	0.5	+	2.9	15.7	11.2	8.2
	1.0	+	0.6	14.3	10.4	5.3
	2.5	+	1.5	25.8	19.7	4.6
	5.0	+	5.5	39.5	25.3	7.3
virus-infected glutaraldehyde-fixed Ia ⁻ PC						
	2.5	—	N.D.	14.8	11.4	N.D.
	5.0	—	N.D.	15.4	8.9	2.3
	2.5	+	2.8	17.5	9.7	9.2
	5.0	+	3.1	15.6	10.4	7.3
virus-infected glutaraldehyde-fixed L cells						
	2.5	—	N.D.	12.7	9.5	7.2
	5.0	—	N.D.	8.7	10.2	6.5
	2.5	+	N.D.	11.5	12.1	7.8
	5.0	+	N.D.	8.9	7.3	4.9

TABLE X (continued)

Cell type added	Percent representation in culture	IL-1* added	L	Percent specific ⁵¹ Cr release		
				LHSV	A31 HSV	EL4 HSV
virus-infected glutaraldehyde-fixed A31 cells	2.5	-	3.2	11.4	9.8	5.4
	5.0	-	N.D.	9.1	9.3	N.D.
	2.5	+	N.D.	13.4	11.6	4.8
	5.0	+	N.D.	10.0	8.5	5.1

*IL-1 was added to cultures at the rate of 4 units/well as determined by endpoint 2-fold dilution of semi-purified IL-1 preparations assayed for the ability to stimulate the proliferation of ConA treated thymocytes.

All other conditions of assay were as described in Table VI (page 65).

by the addition of IL-1. Furthermore, even the addition of IL-1 to the unfixed Ia^- cultured PC failed to enhance the accessory cell function. These results imply that IL-1 secretion, in addition to Ia antigen expression, is required for cells to act as accessory cells.

To assess the notion that Ia^+ P388D₁ cells are unable to act as accessory cells because of insufficient secretion of a functional molecule such as IL-1, experiments were done in which the accessory cell activity of Ia^+ P388D₁ cells were compared to that of the same cells supplemented with IL-1. As shown in Table XI, the addition of IL-1 to Ia^+ P388D₁ cells increased their accessory cell function, although not to the level expressed by resident PC. In two further experiments the level of supplementation provided by IL-1 to P388D₁ cells was only marginal. The addition of IL-1 to B-cells (Ia^+) did not result in accessory cell function, indicating that B cells lack still another as yet undetermined characteristic of accessory cells (data not shown).

Discussion

In this communication we have analyzed the nature of accessory cells required for the in vitro induction of secondary CTL against HSV-1. Thus as shown previously by our group (139, 146) as well as others (124, 156), primary CTL responses cannot be demonstrated in vitro against HSV. However, splenocytes from animals previously infected in vivo with infectious virus and stimulated in vitro for 5 days, potent H-2 restricted CTL responses are demonstrable. Such responses only occur,

TABLE XI

REQUIREMENT FOR FUNCTIONALLY ACTIVE ACCESSORY CELLS: EFFECT OF IL-1
ON Ia⁻ CULTURED PC AND P388D₁ CELLS

Cell type added	Percent representation in culture	IL-1 added	Percent specific ⁵¹ Cr release		
			A31	A31	EL4 HSV
<u>controls</u>					
whole cells + virus	—	—	11.4	73.4	7.2
whole cells + no virus	—	—	12.1	16.9	6.4
depleted cells + virus	—	—	11.1	21.5	3.9
depleted cells + virus	—	+	14.7	22.6	4.7
resident PC	5.0	—	7.3	71.3	8.5
Ia ⁻ P388D ₁	0.5	+	8.3	17.8	9.3
	1.0	+	8.1	15.9	10.1
	2.5	+	9.7	20.3	7.5
	5.0	+	10.5	18.8	9.4
Ia ⁺ supernatant	5.0	—	6.7	14.3	7.2
stimulated P388D ₁	0.5	+	7.7	16.5	3.5
	1.0	+	8.1	20.0	6.4
	2.5	+	8.3	29.6	8.1
	5.0	+	10.6	34.7	6.2
Ia ⁻ cultured PC	0.5	+	7.5	21.3	10.5
	1.0	+	9.4	23.1	8.8
	2.5	+	N.D.	19.7	6.9
	5.0	+	N.D.	20.5	8.4

IL-1 was added to cultures at the rate of 4 units/well. BALB/c mice and PC were used in these experiments. All other assay conditions were as described in Table VI (page 65).

however, when undepleted splenocytes are stimulated with virus. If adherent cells are removed from the splenocytes by passage over Sephadex G-10 and nylon wool, the nonadherent fraction remains unresponsive. Previously we had demonstrated that the response of the nonadherent cells to virus could be restored either by adding syngeneic adherent cells (presumably macrophages), or by the addition of the helper cell lymphokine IL-2 (139, 146). The results presented herein indicate that the cell type that acts as an accessory cell must express the alloantigen Ia. Thus, our results extend to a viral system, an observation made with other antigens, that cells serving to present antigen for in vitro (100, 169, 188, 189, 190), and presumably in vivo (164) CTL responses are Ia positive. In addition, however, our results demonstrate that cells must not only express Ia, but need to be functionally active and capable of producing soluble factors such as IL-1.

The necessary expression of Ia for accessory cell function was deduced from the results of three complimentary approaches. Firstly, in experiments designed to compare the ability of various syngeneic cell populations to permit the induction of CTL in nonadherent primed splenocytes, only populations containing Ia⁺ cells were shown effective. Syngeneic Ia⁻ fibroblasts and L cells, both cell types in which HSV replicates to a high titer, failed to provide accessory cell function. In contrast, resident PC (7-10% Ia⁺), SAC (35-40% Ia⁺), and populations enriched for Ia⁺ dendritic cells were highly effective. Although resident PC are only 7-10% Ia⁺, this Ia⁺ subpopulation was assumed to

provide accessory function since after 48 hrs of in vitro culture, a procedure that is accompanied by the loss of Ia antigen expression (160, 167), but not the loss of susceptibility to infection, the cells were almost inactive as accessory cells. Furthermore, on a cell per cell basis SAC were shown to be more effective accessory cells than were the resident PC.

Further substantiation for the necessary expression of Ia antigen came from experiments which exploited the recent findings of Steeg et al. (166, 167) that Ia negative cells could be rendered Ia positive following their culture in vitro with culture supernatant fluids from ConA stimulated splenocytes. Although such cultured supernatant fluids contain multiple biologically active molecules, the active agent is now suspected to be gamma interferon (167). Using their approach, cultured PC (1-2% Ia⁺) were stimulated with supernatant fluids for 48 hrs, after which the cells became 15-30% Ia positive. Whereas cultured PC were inactive as accessory cells, the converted populations were highly effective, clearly implying a role for Ia antigen expression. However, in similar experiments performed with the macrophage cell line P388D₁ (<0.5% Ia⁺), the induced expression of Ia by culture cells with ConA supernatant fluids was not accompanied by an acquisition of accessory cell function. Possible reasons for the failure of Ia⁺ P388D₁ cells to act as accessory cells are discussed subsequently.

The third set of observations indicating the requirement for Ia antigen expression for accessory cell function was that CTL responses could be prevented by the presence of anti-Ia antiserum in spleen cell

cultures during the induction process. This inhibitory effect of anti-Ia antiserum, which was more effective in the presence of complement, was considered to result from its effect specifically on Ia antigen access since cells also lost their accessory cell function if pretreated with anti-Ia and complement in vitro prior to their addition to depleted cultures. Therefore in the absence of Ia expression, CTL induction failed to occur. By means of a similar approach, Weinberger et al. (188) demonstrated that the accessory cells required for the induction of anti-H-2^k CTL with liposome-bound antigens also needed to express Ia antigen. Similarly, CTL induction against Sendai virus (49) and reovirus (100) can be inhibited by the presence of anti-Ia serum during culture. Letvin et al. (100) were able to block the C3H/HeJ (H-2^k) CTL response to reovirus using monoclonal anti I-A^k but not anti-Ia^d antibody.

Although our results clearly implicate an essential role for Ia antigen expression for accessory cell function, the mere expression of Ia by syngeneic accessory cells was not sufficient to ensure responsiveness. Thus, neither Ia positive B cells or P388D₁ cells induced to express Ia could act as accessory cells. In both instances, the cells could be infected by virus and expressed viral antigen at their cell surface. This may indicate that P388D₁ cells, which are known to secrete IL-1 under stimulation by PMA, fail to secrete adequate quantities under these conditions of culture.

A possible explanation for the failure of some Ia⁺ cells to act as accessory cells came from recent work in the alloantigen system indicating

that such cells must be capable of elaborating soluble factors (46). Our results add support to this notion. Thus we showed that Ia positive, virus-infected accessory cells could no longer provide accessory cell function following their fixation with glutaraldehyde. However, accessory cell function was restored by the addition of preparations containing the macrophage factor IL-1. Since the IL-1 failed to restore the accessory cell function of Ia negative PC (viable or fixed), the results indicate that both Ia antigen expression and factor production are required in order to allow induction of anti-HSV CTL. Neither characteristic alone will suffice. However, it is possible that still other requirements are necessary. Whereas the IL-1 containing supernatants permitted Ia⁺ fixed PC to provide accessory function, Ia⁺ B cells still failed to play such a role, and the efficacy of Ia⁺ P388D₁ cells was marginal. Further experiments are clearly required to elucidate the exact mechanism by which cells provide accessory cell function for the induction of CTL as well as to define whether or not all interacting specific cells express similar requirements in order to respond to viral antigens.

Finally, it seems likely that the accessory cell functions discussed in this paper are required for the stimulation of virus specific T helper cells rather than for the activation of the cytotoxic T cell precursors. The basis for this hypothesis derives from earlier work performed in this laboratory (139, 146) and others (53, 157, 161), which showed that free antigen added to adherent cell depleted, primed splenocytes could stimulate secondary in vitro CTL responses if IL-2

was supplied. This implies that antigen presentation on adherent accessory cells is not a requirement for the CTL precursor or that the cell type required for antigen presentation to the precursor CTL is different from that required by the IL-2 producing helper cell. Experiments are in progress which may serve to clarify these points.

V. CELLULAR INTERACTIONS IN THE CYTOTOXIC T LYMPHOCYTE

RESPONSE TO HERPES SIMPLEX VIRUS ANTIGEN:

DIFFERENTIAL ANTIGEN ACTIVATION

REQUIREMENTS FOR THE HELPER T

LYMPHOCYTE AND CYTOTOXIC T

LYMPHOCYTE PRECURSORS

List of Abbreviations

HSV-1: herpes simplex virus, type 1

CTL: cytotoxic T lymphocyte

IL-2: interleukin-2

FCS: fetal calf serum

DCS: donor calf serum

HI-HSV: heat-inactivated herpes simplex virus

UV-HSV: ultraviolet light—inactivated herpes simplex virus

ConA SUP: Concanavalin A-stimulated splenocyte supernatant fluid

Introduction

It has been well-established that H-2 restricted, cytotoxic T lymphocytes (CTL) have an important role in the recovery from viral infections (11, 90, 103, 114, 179, 192, 198). The induction of active CTL requires the participation of at least three cell types such as an Ia⁺ accessory cell (89, 100, 147, 190), an Lyt 1⁺ T helper cell (116, 125, 133, 186), and the Lyt 123⁺ and Lyt 23⁺ CTL precursors. Both the CTL precursors and the helper T lymphocyte require specific activation by

nominal antigen (15, 94, 186). Helper cells are stimulated by antigen and a nonspecific macrophage-derived soluble factor, interleukin-1 (46) to proliferate and to synthesize and secrete a variety of soluble protein mediators, among which are the nonspecific proliferative and differentiative signals for the antigen-driven CTL precursor (13, 19). CTL precursors are instructed by antigen to initiate the synthesis and membrane expression of the appropriate receptors for the nonspecific helper cell mediators (15, 94, 183).

Earlier reports from this laboratory indicated that heat-inactivated (HI) herpes simplex virus type 1 (HSV-1) preparations fail to stimulate secondary CTL responses in vitro (139, 147). However, inactivated virus preparations generated strong in vitro secondary CTL responses if cultures were supplemented with a source of nonspecific T helper cell factors (146, 147). These results were interpreted to show that inactivated HSV-1 was unable to activate T helper cells, whereas CTL precursors were effectively stimulated.

These observations prompted us to determine whether or not the failure of inactivated HSV-1 preparations to prime mice for a CTL response in vivo reflected an inability of these preparations to adequately stimulate T cell help. Our results indicate that irradiated splenocytes from mice which were primed in vivo with infectious virus could supply helper activity for helper-deficient responder populations in in vitro cultures which had been stimulated with live virus. However, irradiated populations from mice which had been primed with inactivated viral preparations could not provide help.

These data could be explained as a failure of helper T lymphocytes to recognize heat or UV-inactivated HSV-1, or alternatively inactivated virus may preferentially stimulate suppressor cell activity. No suppressive activity was demonstrated from inactivated virus-primed populations, at least by our methods of assay. Instead, inactivated HSV-1 appears to be incapable of activating T helper cells adequately, suggesting that the helper cells and CTL have different requirements for stimulation by antigen.

Materials and Methods

Cells and Viruses Used

Strain L929 cells (H-2^k) and BALB/c 3T3 clone A31 cells (H-2^d) were grown in McCoy's 5A medium supplemented with 5% donor calf serum (DCS). Strain EL-4 cells (H-2^b) were grown in RPMI 1640 with 10% FCS and cell strain HEp-2 in McCoy's 5A plus 10% DCS. The interleukin-2 dependent cell lines, CL3 and CT6, were kindly supplied by Dr. Thomas R. Hunig, Universitat Wurzburg, Wurzburg, FRG, and Dr. Mary Hilfiker, Cleveland Clinic, Cleveland, OH, respectively. CL3 cells were grown in RPMI 1640 10% FCS supplemented with 10% con A-stimulated spleen cell supernatant fluid and CT6 cells cultured in the same medium but with 25% con A supernatant fluid. HSV-1 strain KOS was produced in HEp-2 cells by infecting cells at low multiplicity of infection (m.o.i.) and harvesting cell-associated virus as described previously (14). Heat-inactivated virus (HI) was prepared by heating virus at 56°C for 60 minutes. UV-inactivation of virus was accomplished by exposing 10⁸ plaque-forming

units (pfu) of virus at a distance of 5 cm to UV light from two G15T8 germicidal bulbs (General Electric Co., Waterford, NY) for 5 minutes. Infectious HSV-1 was partially-inactivated by two minutes exposure to UV light to reduce cytopathic effects during in vitro cultures.

Mouse Immunization and Preparation of Cell Suspensions

Young C3H/HeJ mice (H-2^k) were obtained from Cumberland View Farms, Clinton, Tennessee. Animals from which cells were used as a source of CTL responders were given no virus, or alternatively a single injection intraperitoneally of 10^7 pfu of either HI-HSV or infectious-HSV. Splenocytes from injected mice were removed 2-4 weeks following injection, and used as responders.

Animals from which cells were to be tested for T helper activity were injected with 10^7 pfu of infectious HSV-1 or equivalent amounts of HI or UV-inactivated HSV. At the end of four weeks, mice were given a second i.p. injection of the same virus preparation used to prime animals and the spleens were removed 2 days later.

Mice were killed by cervical dislocation and their spleens, thymus, or popliteal lymph nodes were aseptically removed. Single-cell suspensions were prepared by teasing cells through 80-mesh sieves into Hank's balanced salt solution (HBSS). After one wash, erythrocytes were lysed by brief exposure at room temperature to 0.83% NH_4Cl in .17M Tris buffer (pH = 7.2). Cells were then washed in HBSS twice more, the cell aggregates were removed, and the viable cells were enumerated.

Various prospective helper cell populations, virus-infected feeder cells, and normal filler cells were irradiated at 1500 rads prior to

inclusion in cultures. Irradiated feeder cells were prepared by incubating normal splenocytes at 37°C for two hours in low volume with partially UV-inactivated HSV-1 at a moi of 10 (prior to irradiation).

For in vitro culture, 1×10^6 viable responder cells per well were added to 6 well 35 mm plastic tissue culture dishes (Costar, Cambridge, MA). Irradiated feeder cells were included at a concentration of 3×10^6 /ml cells per well and a combined total of 1×10^6 /ml irradiated helper or naive filler cells were added per well. Each well was adjusted to a final volume of 6 ml with RPMI 1640 containing 10% FCS, 2 mM glutamine, penicillin (100 u/ml), streptomycin (100 µg/ml) and 5×10^{-5} M 2-mercaptoethanol.

Antiserum Treatments

Anti-Lyt 1.1 and anti-Lyt 2.1 were obtained from Cedarlane Laboratories, Westbury, NY. 10^7 splenocytes were incubated for one hour at room temperature with occasional shaking in 2.0 ml of a 1:40 dilution of either antiserum. Cells were subsequently washed and resuspended in 2.0 ml of a 1:10 dilution of rabbit low toxicity mouse complement (Cedarlane Laboratories), and incubated for 45 minutes at 37°C. Dilutions of antiserum and complement were made in cytotoxicity medium (Cedarlane Laboratories).

Preparation of Supernatant Fluids

Young C3H/HeJ mice were injected intraperitoneally with 10^7 pfu of infectious HSV-1 or equivalent amounts of HI or UVI-HSV. Two to four weeks later, spleens were removed and prepared into single cell

suspensions. Cells were then restimulated in vitro with the same virus used to prime, cultured at a concentration of 2×10^6 cells/ml, and incubated at 37°C for 30 hours (5% CO₂, humidified). For some experiments, spleen cells primed with one of the various preparations of HSV-1 were given partially UV-inactivated HSV-1 for the secondary in vitro stimulation.

Suspensions were centrifuged for 15 minutes at $1000 \times g$, and the supernatant fluids collected and concentrated 10-fold on YM10 membrane filters (Amicon, Lexington, MA). Concentrated supernatant fluids were then filter-sterilized through 0.45 micrometer syringe-filtered membrane filters (Gelman Sciences, Inc., Ann Arbor, MI), aliquoted and stored at -20°C.

Concanavalin A-stimulated Lewis rat splenocyte supernatant fluids were prepared by incubating splenocytes at a concentration of 2×10^6 cells/ml in RPMI 1640 10% FCS plus 2.5 µg/ml of Con A for 48 hours at 37°C, 5% CO₂. Supernatant fluids were collected, and the Con A removed by adsorption to Sephadex G-25. Fluids were then filter-sterilized through 0.45 micrometer vacuum-operated 100 ml capacity membrane filtration units (Gelman Sciences, Inc.), aliquoted and stored at -20°C.

Cytotoxicity Assays

The details of these assays were published previously (23). Briefly, L (H-2^k), A31 (H-2^d), or EL-4 (H-2^b) cells suspended in RPMI 1640 were infected with HSV-1 at a m.o.i. of 10 for 120 minutes at 37°C, then washed and 5×10^6 cells in a 1.0 ml labelled for 2 hours

at 37°C with 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear Corp., Boston, MA). After labelling, cells were washed three times with medium, suspended at $10^5/\text{ml}$ in RPMI 1640 plus 5% DCS, and 100 μ l volumes added to effector cells in U-shaped microtiter plates. Cytotoxicity assays were begun 6 hours after the initiation of infection with HSV-1, and were carried out for 4 hours in a 5% CO_2 humidified incubator. Standard errors were low (<4%) and results are reported as mean percentage specific release values computed according to the following formula: percentage specific ^{51}Cr release = [(effector cell release - medium control release)/(total releasable ^{51}Cr - medium control release)] $\times$ 100. The total releasable ^{51}Cr was obtained by exposing target cells to 1.5% Triton $\times$ -100.

Results

Generation of CTL Activity in Naive Murine Cell Populations

We employed a system of cell culture similar to that introduced by Pilarski (125), in which CTL responses could be generated from naive splenocytes if cocultured with a source of antigen-activated helper T lymphocytes. C3H/HeJ mice were primed in vivo with infectious HSV-1 and restimulated 2-4 weeks later in vivo with the same virus. Two days following the second injection, spleen cells were removed, irradiated at 1500 rads to eliminate CTL activity and used as a source of radio-resistant T helper cells. When these cells were added in graded numbers to naive lymphocyte-rich populations along with irradiated virus-infected stimulator cells, low, but detectable levels of H-2 restricted CTL

activity could be demonstrated after 4-5 days of in vitro culture (Table XII). Similar low levels of CTL activity could be detected regardless of whether naive splenocytes, thymocytes, or lymph node cells were used as responder populations. Irradiated, antigen-activated helper cells, which were cultured with virus-infected stimulators and irradiated naive feeder cells for 4-5 days displayed no detectable CTL activity. This indicated that the x-irradiation of the helper cell source was effective at preventing the help required for the CTL precursors within that population from differentiating into active CTL. It also demonstrated that the CTL activity measured in the above experiments arose from the unirradiated naive responder population.

In contrast to the result obtained with primed splenocytes from animals given infectious HSV-1 as a source of T cell help, splenocytes from mice primed and restimulated in vivo with either HI or UV-inactivated HSV-1 were not able to provide help for the generation of CTL from naive splenocytes. In these experiments, the in vitro cultures were antigen-stimulated with the same virus preparation that effectively stimulated helper effects from splenocytes from animals given infectious virus. Thus, the antigen presentation requirements for the CTL precursors should have been adequate, and the failure to obtain a CTL response was attributed to a failure of inactivated virus to prime for adequate levels of helper activity in vivo.

When nonirradiated intact immune spleen cells were stimulated with infectious virus high levels of CTL were induced (Table XII). However as shown previously, HI virus failed to induce CTL in vitro unless

TABLE XII

STIMULATION OF CTL ACTIVITY IN NORMAL CELL POPULATIONS COCULTURED WITH
IRRADIATED, SECONDARY T HELPER CELLS

5 × 10 ⁶ Responders	Virus-Incubated Stimulator Cells	Th Population		Naive Filler Splenocytes	Percent Specific 51Cr Release		
		Source ¹	Number		L	L HSV	EL4-HSV
Immune Spleen	3 × 10 ⁶	—	—	—	9	77	11
Immune Spleen	—	—	—	—	3	11	8
Immune Spleen	Free UV-HSV ³	—	—	5 × 10 ⁶	ND ²	10	7
Immune Spleen	Free UV-HSV	Con A-stimulated Sup	—	5 × 10 ⁶	ND	62	4
Immune Spleen	Free HI-HSV	—	—	5 × 10 ⁶	8	13	6
Immune Spleen	Free HI-HSV	Con A-stimulated Sup	—	5 × 10 ⁶	ND	56	14
Normal Spleen	3 × 10 ⁶	—	—	—	6	8	8
Normal Spleen	3 × 10 ⁶	Infectious	5 × 10 ⁶	—	8	22	7
Normal Spleen	3 × 10 ⁶	Infectious	2.5 × 10 ⁶	2.5 × 10 ⁶	10	13	4
Normal Spleen	3 × 10 ⁶	Infectious	10 ⁶	4 × 10 ⁶	5	9	8
Normal Spleen	3 × 10 ⁶	Infectious	—	5 × 10 ⁶	5	8	6
Normal spleen	3 × 10 ⁶	Con A-stimulated Sup	—	5 × 10 ⁶	7	6	9
Normal Spleen	3 × 10 ⁶	HI-HSV	5 × 10 ⁶	—	6	8	6
Normal Spleen	3 × 10 ⁶	HI-HSV	2.5 × 10 ⁶	2.5 × 10 ⁶	ND	11	5
Normal Spleen	3 × 10 ⁶	HI-HSV	10 ⁶	4 × 10 ⁶	ND	7	3
Normal Spleen	3 × 10 ⁶	UV-HSV	5 × 10 ⁶	—	ND	9	3
Normal Spleen	3 × 10 ⁶	UV-HSV	2.5 × 10 ⁶	2.5 × 10 ⁶	5	11	7
Normal Spleen	3 × 10 ⁶	UV-HSV	10 ⁶	4 × 10 ⁶	ND	9	6

TABLE XII (continued)

5 × 10 ⁶ Responders	Virus-Incubated Stimulator Cells	T _H Population Source ¹	Naive Filler Splenocytes	Number	Percent Specific ⁵¹ Cr Release		
					L	L HSV	EL4-HSV
Normal Thymocytes	3 × 10 ⁶	Infectious	—	5 × 10 ⁶	7	16	5
Normal Lymph Node	3 × 10 ⁶	Infectious	—	5 × 10 ⁶	6	19	3
—	3 × 10 ⁶	Infectious	5 × 10 ⁶	5 × 10 ⁶	7	8	6

¹Source indicates which form of virus was used to generate the putative T_H population.

²N.D. = not done.

³Free virus was added directly to culture (5 × 10⁶ pfu/well) and not preincubated on stimulator cells.

C3H/HeJ strain mice were used in these experiments. Effector cells were assayed for activity at ratios of 25:1 and 5:1 (effector:target), and the amount of cytotoxic activity was proportional to the ratio of the effector cells used. Values shown are for 25:1. Assays were conducted for 4 h with effector cell populations which had been cultured for 4 or 5 days. The ⁵¹Cr target cells used were HSV-1-infected L cells (L HSV), uninfected L cells (L), and infected EL-4 cells (EL-4 HSV). Target cells were infected at least 6 h before the beginning of the assay. All helper cell types were added in at least three different ratios (total of 5 × 10⁶ helper cells plus filler cells per addition) to 5 × 10⁶ responder cells. Wherever all ratios are not shown, the highest number of helper cells added is represented. All experiments were repeated at least three times.

Virus-incubated stimulator splenocytes, T helper (T_H) populations, and naive filler populations were all irradiated at 1500 R. Con A stimulated spleen cell supernatant fluids were prepared by incubating 2 × 10⁶ Lewis rat splenocytes/ml with 2.5 µg/ml Con A at 37°C, 5% CO₂ for 24 hrs. The Con A was removed by adsorption to Sephadex G-25.

HI virus was prepared by heating at 56°C for 60 minutes. UV-inactivation was accomplished by 5 minutes exposure at 5 cm distance to two germicidal lamps.

Con A-stimulated rat spleen cell supernatant fluids, which are rich in T helper cell factors were added. This indicated that inactivated HSV-1 preparations are antigenic in vitro and capable of driving CTL precursors into a state of preparedness for the reception of non-specific helper T cell-derived factors. Despite repeated attempts, we were unable to render naive splenocytes responsive in vitro to any form of HSV-1 with Con A supernatant fluids (data not shown). This possibly suggests that helper activity for the primary CTL response to virus includes some physical interaction between helper and responder cells which is not provided by the cell-free supernatant fluids.

Generation of CTL Activity in HI-Primed
Splenocytes or Lyt 1-Depleted
Immune Splenocytes

Because responses HSV-1 in naive cell cultures were low, attempts were made to find a helper cell deficient population which could be made to generate higher CTL activity against HSV-1. We have observed that splenocytes from mice which have been primed in vivo with HI virus do not respond with detectable levels of CTL activity when restimulated in vitro with infectious HSV-1 (98). Nevertheless, limiting dilution analysis in which naive splenocytes were compared to HI-HSV-primed splenocytes, indicates that higher numbers of CTL precursors are present in the latter population (138). HI-HSV-primed splenocytes were cocultured with graded numbers of putative irradiated helper cells from animals primed with infectious, HI, or UV-inactivated HSV-1 (Table XIII). As before, cultures were stimulated in vitro with infectious HSV-1 that was presented on the surface of $\times$ -irradiated naive splenocytes. In most

TABLE XIII

STIMULATION OF SPLENOCYTES FROM MICE PRIMED WITH HEAT-INACTIVATED VIRUS
AND COCULTURED WITH IRRADIATED, SECONDARY T HELPER CELLS

5 10 ⁶ Responders	Virus Incubated Stimulator Cells	T _H Population		Naive Filler Splenocytes	Percent Specific ⁵¹ Cr Release		
		Source	Number		L	L HSV	EL4-HSV
Immune Spleen	3 × 10 ⁶	—	—	—	11	69	8
Immune Spleen	—	—	—	—	8	13	10
HI-Primed Spleen	3 × 10 ⁶	—	—	—	9	11	7
HI-Primed Spleen	3 × 10 ⁶	—	—	5 × 10 ⁶	8	11	4
HI-Primed Spleen	3 × 10 ⁶	Infectious	5 × 10 ⁶	—	12	31	8
HI-Primed Spleen	3 × 10 ⁶	Infectious	2.5 × 10 ⁶	2.5 × 10 ⁶	9	16	11
HI-Primed Spleen	3 × 10 ⁶	Infectious	10 ⁶	4 × 10 ⁶	6	9	9
HI-Primed Spleen	3 × 10 ⁶	HI-HSV	5 × 10 ⁶	—	10	11	7
HI-Primed Spleen	3 × 10 ⁶	HI-HSV	2.5 × 10 ⁶	2.5 × 10 ⁶	5	12	9
HI-Primed Spleen	3 × 10 ⁶	HI-HSV	10 ⁶	4 × 10 ⁶	6	10	8
HI-Primed Spleen	3 × 10 ⁶	UV-HSV	5 × 10 ⁶	—	7	11	11
HI-Primed Spleen	3 × 10 ⁶	UV-HSV	2.5 × 10 ⁶	2.5 × 10 ⁶	7	10	9
HI-Primed Spleen	3 × 10 ⁶	UV-HSV	10 ⁶	4 × 10 ⁶	6	8	12
Normal Spleen	3 × 10 ⁶	Infectious	5 × 10 ⁶	—	10	25	7
Normal Spleen	3 × 10 ⁶	Infectious	2.5 × 10 ⁶	2.5 × 10 ⁶	12	15	10
—	3 × 10 ⁶	Infectious	5 × 10 ⁶	5 × 10 ⁶	8	9	12

HI-primed splenocytes taken from C3H/HeJ mice which had been given a single i.p. injection of 10^7 pfu of heat-inactivated virus at least two weeks prior to culture.

All other conditions were as described in Table XII.

experiments, the CTL responses generated from HI-HSV-primed responders cocultured with infectious virus-activated T helpers were slightly higher than those generated from naive responders, but these differences were always less than two-fold. Since the level of help provided by the irradiated population should have been roughly identical for both the naive and HI-primed populations in any single experiment, these data suggest that the number of CTL precursors present in the HI-primed population is not sufficiently higher than that of naive splenocytes to result in appreciably higher CTL activity in a 4-5 day culture. As with the naive responders, HI-primed responders failed to generate CTL when cocultured with splenocytes from animals given inactivated virus.

In a further attempt to raise the level of CTL activity obtained in this system, C3H/HeJ splenocytes (which had been primed in vivo with infectious HSV-1) were depleted of Lyt 1⁺ cells using anti-Ly 1.1 plus complement. Since the helper T lymphocyte is Lyt 1⁺2⁻ in other systems investigated (12, 13), this treatment should have removed all helper activity. The depleted cells were then cocultured with irradiated splenocytes which had been stimulated twice by i.p. injection of infectious, HI, or UV-inactivated HSV-1 as well as with virus-infected stimulators for 4-5 days (Table XIV).

The Lyt 1-depleted immune splenocytes failed to respond to in vitro viral stimulation, indicating that in the anti HSV CTL induction system, helper cells are in part required. In further support for the helper cell requirement, if Con A supernatant fluid was added to HSV-stimulated, Lyt 1-depleted cells, high levels of CTL activity were obtained.

TABLE XIV

RESTORATION OF RESPONSIVENESS OF LYT 1⁺-DEPLETED IMMUNE SPLENOCYTES
TO HSV-1 BY THE ADDITION OF HELPER CELL SOURCES

5 × 10 ⁶ Responders	Virus-Incubated Stimulator Cells	Th Population		Naive Filler Splenocytes	Percent Specific 51 Cr Release		
		Source	Number		L	L HSV	EL4-HSV
Untreated	3 × 10 ⁶	—	—	—	15	73	6
Untreated	—	—	—	—	ND	17	7
Lyt 1-treated	3 × 10 ⁶	—	—	—	ND	8	8
Lyt 1-treated	3 × 10 ⁶	Con A-sup	—	—	10	46	11
Lyt 1-treated	3 × 10 ⁶	—	—	5 × 10 ⁶	7	12	3
Lyt 1-treated	3 × 10 ⁶	Infectious	5 × 10 ⁶	—	7	40	7
Lyt 1-treated	3 × 10 ⁶	Infectious	2.5 × 10 ⁶	2.5 × 10 ⁶	ND	23	10
Lyt 1-treated	3 × 10 ⁶	Infectious	10 ⁶	4 × 10 ⁶	9	11	12
Lyt 1-treated	3 × 10 ⁶	HI-HSV	5 × 10 ⁶	—	ND	17	8
Lyt 1-treated	3 × 10 ⁶	HI-HSV	2.5 × 10 ⁶	2.5 × 10 ⁶	ND	14	6
Lyt 1-treated	3 × 10 ⁶	HI-HSV	10 ⁶	4 × 10 ⁶	8	10	9
Lyt 1-treated	3 × 10 ⁶	UV-HSV	5 × 10 ⁶	—	9	15	9
Lyt 1-treated	3 × 10 ⁶	UV-HSV	2.5 × 10 ⁶	2.5 × 10 ⁶	5	13	7
Lyt 1-treated	3 × 10 ⁶	UV-HSV	10 ⁶	4 × 10 ⁶	ND	12	11
—	3 × 10 ⁶	Infectious	5 × 10 ⁶	5 × 10 ⁶	8	10	5

Lyt 1-treated immune C3H/HeJ splenocytes were prepared by incubation of 10⁷ nylon wool nonadherent splenocytes in a 1:40 dilution of antiserum at room temperature for 1 h, followed by a 45 minute incubation at 37°C with a 1:10 dilution of rabbit low toxicity mouse complement.

All other conditions were as described in Table XII.

Substantial, though submacimal levels, of anti-HSV CTL activity occurred when the $\text{Lyt } 1^{-}2^{+}$ immune population was cultured with irradiated, infectious virus-stimulated splenocytes. As before, splenocytes from animals that received two injections of inactivated HSV-1 provided no helper activity to the $\text{Lyt } 1$ -depleted cell population. Thus, from data obtained in three variations of this system, our results suggest that inactivated HSV-1 fails to generate functional levels of T cell help in vivo.

The restoration of CTL responsiveness by both Con A supernatant fluid and by irradiated helper cells were below maximal levels obtained in undepleted immune splenocytes. Since CTL precursors were reported to be a mixture of $\text{Lyt } 123^{+}$ and $\text{Lyt } 1^{-}23^{+}$ cells (20), the lowered response perhaps reflects the elimination of the $\text{Lyt } 123^{+}$ subpopulation with the antiserum treatment.

Definition of Lyt Type of Responder and Helper Population

Because both the responder and putative T helper populations in our experiments consisted of mixed populations of cells, it was necessary to further define the functionally relevant cell types. The effect of pretreatment of either the responder or helper populations with Ly antisera is shown in Table XV. Treatment with anti-Lyt 1.1 plus complement of the helper population from animals which received infectious virus abrogated their ability to provide helper effects. However, treatment with anti-Lyt 2.1 had no effect. Treatment of the responder population from animals which had received live virus with

TABLE XV

EFFECTS OF LY ANTISERA ON STIMULATION OF CTL IN VARIOUS RESPONDER POPULATIONS

Responders	Virus-Incubated Stimulator Cells	T _H Population		Antiserum Treatment	Percent Specific 51Cr Release		
		Source	Number		L	L HSV	EL4-HSV
Immune Spleen	3 × 10 ⁶	—	—	—	3	74	11
Immune Spleen	—	—	—	—	7	13	7
Immune Spleen*	3 × 10 ⁶	—	—	Lyt 1.1	5	8	13
Immune Spleen*	3 × 10 ⁶	—	—	Lyt 2.1	6	10	8
Immune Spleen*	3 × 10 ⁶	Con A	supernatant	Lyt 1.1	10	39	7
Immune Spleen*	3 × 10 ⁶	Con A	supernatant	Lyt 2.1	8	9	11
HI-Primed spleen	3 × 10 ⁶	—	—	—	7	8	9
HI-primed spleen	3 × 10 ⁶	Infectious	5 × 10 ⁶	—	6	28	10
HI-primed spleen	3 × 10 ⁶	Infectious	5 × 10 ⁶ *	Lyt 1.1	8	7	8
HI-primed spleen*	3 × 10 ⁶	Infectious	5 × 10 ⁶	Lyt 2.1	2	9	9
HI-primed spleen*	3 × 10 ⁶	Infectious	5 × 10 ⁶ *	Lyt 2.1	7	24	8

*Denotes which population of cells received the treatment with antiserum.

10⁷ cells were incubated for 1 hour at ambient temperature in 2 ml of a 1:40 dilution of either anti-Lyt 1.1 or anti-Lyt 2.1. Cells were then washed and resuspended in 1 ml of a 1:8 dilution of rabbit low toxicity mouse complement and incubated at 37°C for 45 minutes.

All other conditions of assay were as described in Table XII (page 92).

either Lyt 2.1 or 1.1 prevented the CTL response. However, whereas the CTL responsiveness of Lyt 1.1-depleted immune splenocytes was restored by the addition of Con A supernatant fluid, the Lyt 2.1-depleted population failed to respond to the same treatment. These experiments indicate that the CTL responder must express Lyt 2 antigen on its surface and the relevant cell type in the helper population is an $\text{Lyt } 1^+ 2^-$ cell.

Assay for Suppressor Activity in Helper
Cell Populations and Concentrated
Supernatant Fluids of Antigen-
Stimulated Splenocyte

The observed failure of inactivated HSV-1 to prime helper T cell activity in vivo could be explained in at least two ways. Either the antigens failed to trigger helper cells recognition or they might preferentially induce suppressor mechanisms that acted on the helper cells or the cytotoxic T lymphocytes or their precursors. To evaluate the suppressor cell hypothesis, the three putative helper populations were assayed for their ability to suppress a secondary in vitro response to HSV-1. This consisted of incubating splenocytes from mice exposed in vivo to infectious virus with putative helpers plus infectious virus in vitro. Such cultures generated high levels of H-2 restricted CTL (Table XVI). Upon the addition of graded numbers of irradiated putative helper populations (up to 30% of total cell numbers in cultures) the CTL response was not appreciably suppressed, by any of the 3 helper populations. Because some suppressor cells of the CTL response are known to be radiation-sensitive (151), a second approach was employed

TABLE XVI
ASSAY FOR SUPPRESSIVE ACTIVITY FROM VARIOUS IRRADIATED T_H POPULATIONS

5×10^6 Responders	Virus-Incubated Stimulator Cells	T_H Population		Percent Specific 51Cr Release		
		Source	Number	L	L HSV	EL-4 HSV
Immune Spleen	3×10^6	—	—	6	67	15
Immune Spleen	—	—	—	1	8	10
Immune Spleen	3×10^6	Infectious	5×10^6	6	59	10
Immune Spleen	3×10^6	HI-HSV	5×10^6	7	60	14
Immune Spleen	3×10^6	UV-HSV	5×10^6	8	57	13

Responder cells were splenocytes taken from C3H/HeJ mice which had been primed i.p. with infectious HSV-1 5-8 weeks previously.

All other conditions of assay were as described in Table XII (page 92).

for the detection of suppressor activity. In these experiments, C3H/HeJ mice were primed i.p. with infectious HSV-1, HI, or UV-inactivated virus. Two to four weeks later, the splenocytes were isolated and restimulated in vitro using the same type of virus preparation that had been used to prime the animal. At the end of 30 hours, the supernatant fluids were collected and concentrated 10-fold on Amicon YM-10 filters. The concentrated fluids were then added in graded volumes to cultures of secondarily stimulated immune splenocytes (Table XVII). As with the whole cell transfers, none of the supernatant fluid concentrates was able to suppress in vitro secondary CTL responses to HSV-1. The preparation of supernatant fluids by priming and restimulating with the same virus preparation introduced a second variable into the protocol. Therefore, the suppressive affect of supernatant fluids from splenocytes stimulated with partially UV-inactivated virus on stimulator cells such as was used to induce CTL in vitro was tested. Such supernatant fluids concentrated from cells stimulated in this manner were also incapable of suppressing secondary CTL responses in vitro, regardless of which type of virus was used to prime the mice. Therefore, at least by the methods tested here, heat-inactivated and UV-inactivated preparations of HSV-1 do not appear to preferentially engage suppressor cells or cause the production of suppressor factors.

TABLE XVII

ASSAY FOR SUPPRESSIVE ACTIVITY FROM CONCENTRATED SUPERNATANT FLUIDS FROM SPLENOCYTES
PRIMED AND RESTIMULATED IN VITRO WITH HI, UV, OR INFECTIOUS HSV-1

10 ⁷ Responders	Virus-Incubated Stimulator Splenocytes	Supernatant Preparations		Percent Supernatant in Cultures	Percent Specific 51Cr Release		
		In vivo Stimulation	In vitro Stimulation		L HSV EL-4 HSV		
					L	L HSV	EL-4 HSV
Immune Spleen	3 × 10 ⁶	—	—	—	14	59	7
Immune Spleen	—	—	—	—	9	7	8
Immune Spleen	3 × 10 ⁶	Infectious Infectious Infectious	Infectious	25	11	62	9
Immune Spleen	3 × 10 ⁶		Infectious	12	9	55	8
Immune Spleen	3 × 10 ⁶		Infectious	1	13	56	8
Immune Spleen	3 × 10 ⁶	HI HI HI	HI	25	10	59	7
Immune Spleen	3 × 10 ⁶		HI	12	9	62	10
Immune Spleen	3 × 10 ⁶		HI	1	8	58	9
Immune Spleen	3 × 10 ⁶	UV UV UV	UV	25	13	60	9
Immune Spleen	3 × 10 ⁶		UV	12	13	57	7
Immune Spleen	3 × 10 ⁶		UV	1	9	54	8
Immune Spleen	3 × 10 ⁶	HI HI HI	HI	25	10	53	11
Immune Spleen	3 × 10 ⁶		HI	12	6	61	8
Immune Spleen	3 × 10 ⁶		HI	1	11	57	7
Immune Spleen	3 × 10 ⁶	UV UV UV	UV	25	8	59	10
Immune Spleen	3 × 10 ⁶		UV	12	12	56	11
Immune Spleen	3 × 10 ⁶		UV	1	7	61	9

Splenocyte supernatant fluid concentrates were prepared by collecting the 30 hour medium from C3H/HeJ splenocytes that had been primed in vivo with infectious, UV, or heat-inactivated virus, and restimulated in vitro with the same virus used to prime. The medium was concentrated 10-fold on Amicon YM-10 filters. Some fluids were prepared from splenocytes which were primed with inactivated virus and restimulated in vitro with infectious virus.

All other conditions of assay were as described in Table XII (page 92).

Assay for Helper Activity in Concentrated Supernatant Fluids from Antigen-Activated Splenocytes

Using the in vitro production of interleukin-2 as an indicator of helper cell activity, the supernatant fluid concentrated from cultures of splenocytes primed in vivo with live or inactivated HSV-1 and restimulated in vitro with live virus was tested. CL3 cells and CT6 cells are dependent on the continued presence of interleukin-2, known to be a product of the $Ly\ 1^{+}2^{-}$ helper T lymphocyte and an essential participant in CTL responses (186). In the absence of interleukin-2, both cell lines are unable to proliferate. In its presence, the proliferative activity of CL3 or CT6 cells can be measured via ^3H -thymidine uptake. Concanavalin A-stimulated Lewis rat spleen cell supernatant fluids were used as a positive control (Table XVIII). Little or no IL-2 activity was detectable in concentrated fluids from splenocytes which had been primed and restimulated with either UV or HI virus. Furthermore the same splenocytes stimulated with infectious or partially UV-irradiated virus also failed to produce IL-2. In contrast, supernatant fluids from splenocytes from animals given infectious virus and restimulated in vitro with infectious virus or partially UV-irradiated virus gave high levels of IL-2. However, the same splenocytes stimulated with HI virus failed to generate IL-2. These observations were interpreted to mean that the inactivated forms of HSV-1 fail to generate helper T lymphocytes in vivo that could be subsequently induced in vitro to synthesize and secrete factors such as IL-2 required for the differentiation and proliferation of antigen

TABLE XVIII
ASSAY FOR IL-2 PRESENT IN CONCENTRATED SUPERNATANT FLUIDS

In vivo Stimulation	Supernatant Preparations In vitro Stimulation	Percent Supernatant in Assay Cultures	³ H-Thymidine Uptake (cpm) Experiment 1	Experiment 2
None	None (Splenocytes alone)	10	735	874
None	Con A (rat splenocytes)	10	15,321	20,773
Infectious	Infectious	25	11,797	13,249
Infectious	Infectious	6	11,468	8,865
Infectious	Infectious	1	2,473	3,147
HI	HI	25	2,649	1,833
HI	HI	12	514	756
HI	HI	1	675	779
UV	UV	25	4,221	2,945
UV	UV	12	767	839
UV	UV	1	632	763
HI	Infectious	25	3,103	1,583
HI	Infectious	12	1,432	901
HI	Infectious	1	670	756
UV	Infectious	25	3,698	3,030
UV	Infectious	12	1,592	822
UV	Infectious	1	647	807

Concentrated supernatant fluids were prepared as described in Table XVII.

TABLE XVIII (continued)

CL3 cells were starved for IL-2 for one hour prior to initiation of assay. 4×10^3 CL3 were pipetted into 96 well microtiter culture plates with dilutions of supernatant fluids to a final volume per well of 0.2 ml. Cultures were incubated 24 h at 37°C and pulsed 4 h with 1 μ Ci ^{3} H-Thymidine.

Similar results were also obtained using the CT6 IL-2 dependent cell line.

stimulated CTL precursors. Our results also indicate that HI inactivated virus fails to trigger helper cells in vitro.

Discussion

The purpose of this investigation was to determine why inactivated forms of HSV-1 fail to prime mice in vivo for CTL responses. Our previous studies had indicated that splenocytes from mice primed in vivo with infectious HSV-1 could be restimulated in vitro with infectious, but not heat-inactivated virus, to generate strong anti-HSV CTL responses (139, 146, 147). However, near maximal levels of CTL activity were induced if cultures were supplemented with helper factors (139). Thus, the inability of inactivated virus to induce CTL was associated with an inadequate stimulation of helper cells. In the present report, we demonstrate that insufficient priming of helper T cells in vivo is the reason why inactivated virus fails to prime for a CTL response. To demonstrate this point, cells with putative helper activity were irradiated to prevent their differentiation into CTL. These cells were cocultured with antigen-stimulated responder cells that failed to generate CTL in the absence of help. We demonstrated that, whereas splenocytes from mice given infectious virus provided help, cells from animals which received inactivated virus could not. When splenocytes from naive mice were used, the response was minimal but was considered as a measure of CTL since the cytotoxicity was virus specific and H-2 restricted. Furthermore, the effector cells generated were eliminated by treatment with anti-Lyt 2.1 plus complement, a

property of CTL but not NK cells (126). The inability of splenocytes from mice given inactivated virus to stimulate a CTL response could be explained other than by a failure to prime helper cells. Alternative explanations including the induction of active suppression, or that the quantity of inactivated virus given was insufficient to be antigenic. This latter idea was considered unlikely since the doses of viurses used to stimulate antibody responses (113), which are thymus dependent responses in the mouse (23).

To analyze the possibility of suppression, irradiated splenocytes from animals given live or inactivated virus were added in graded numbers to antigen-stimulated immune spleen cells and the effect on the magnitude of the in vitro induced CTL response measured. By this approach, evidence of suppression was not forthcoming. However, it is well known that certain suppressor cells are radiation sensitive (151), and so could be eliminated in our experimental protocol. Consequently, unirradiated splenocytes were stimulated in vitro with antigen and the cell free supernatant fluids were analyzed for suppressive activity against secondary CTL induction in vitro. Positive evidence for suppression was lacking, but the case against suppression is particularly hard to make with negative experiments. Moreover, our test system was not ideal since it measured effects against secondary CTL induction, not primary induction, where requirements may be different. We are currently attempting to measure suppression against primary CTL induction in the helper cell dependent system.

There are reasons to expect that the requirements for primary and secondary anti-HSV CTL induction in vitro have similarities. This notion

is based on our observation that in modifying the helper cell dependent system to amplify the CTL response, splenocytes from animals given inactivated virus still failed to provide help. The modified assay was, in effect, a secondary induction system as responders were immune splenocytes (from animals given infectious virus), but depleted of helper cells by the removal of $\text{Lyt } 1^+$ cells by negative selection. Secondary CTL induction did not occur in such cultures unless an extraneous source of help was added. This could be provided in two ways. First, by the addition of soluble helper factors such as interleukin-2 containing ConA sup, or else by the addition of helper cells. Once again, whereas help was provided by splenocytes from mice given infectious virus, splenocytes from mice which received inactivated virus could not. Furthermore, we also demonstrated that only the splenocyte population from mice given infectious virus could generate detectable levels of IL-2 upon stimulation in vitro with antigen. This observation further indicates that inactivated virus in vivo fails to prime for help. However, inactivated virus is capable of driving CTL precursors in vitro. Thus, immune $\text{Lyt } 1^+$ cell-depleted splenocytes respond to either infectious or inactivated virus as long as an extraneous source of helper factor is added.

The failure to trigger help is not the only explanation for the apparent lack of in vivo immunogenicity of inactivated HSV for CTL induction. Elsewhere, we have shown that inactivated viral antigens fail to expand the CTL precursor frequency to the same extent as does infectious virus (138). However, splenocytes from mice given inactivated

virus had higher frequencies of CTL precursors than did splenocytes from HSV naive mice. In an attempt to amplify responses, we employed as responder cells splenocytes from animals given HI virus, and used these to monitor for helper effects of various irradiated splenocyte populations. The pattern of results were the same as with naive and $\text{Lyt } 2^+$ responders. Furthermore, the magnitude of responsiveness barely exceeded those obtained from naive cells, an observation we take to indicate that CTL precursors in the HI virus stimulated population were still minimal.

One observation deserving of comment but without a satisfactory explanation was that, whereas naive splenocytes were induced to respond with anti-HSV CTL by the addition of helper cells, adding helper factors alone to antigen-stimulated cells was not inductive. Primed antigen stimulated CTL respond maximally to helper factors as published previously (139, 146) and replicated in this communication. We have no explanation for the observed differences between helper cells and factors for primary CTL induction, but possibilities include that the primary induction requires some form of physical interaction between helper cells and CTL precursors or that an additional factor, not present in our ConA-stimulated supernatant fluid, is required for primary induction.

The evidence presented herein together with that published elsewhere (139, 146), indicate that the CTL precursor and helper cell precursor in vitro and in vivo have differential requirements for induction by HSV viral antigens. We contend that the requirements for helper cell recognition are more stringent than are those for CTL precursor

recognition. Certainly it is known in most systems investigated (37, 198), including HSV (146), that recognition by helper and cytotoxic T cells is different with respect to Ia antigen corecognition. Possibly inactivated virus can associate effectively with the D or K MHC elements, with which the CTL precursors are preoccupied, whereas they cannot form suitable associations with the Ia restricting elements. Another possibility is that free virus is capable of stimulating CTL precursors, but that infectivity to provide adequate membrane antigen expression is required to stimulate helper cells. These problems are currently under investigation in this laboratory. Whatever turns out to be the mechanism, our results do have application to practical studies on HSV vaccines. Purified HSV glycoproteins, which have failed to induce detectable levels of cellular immunity in our hands (28), may possibly be made efficacious by the concomitant administration of nonspecific helper cell factors.

VI. CONCLUSIONS

1. ConA-stimulated spleen cell supernatant fluids (ConA supe) can restore a secondary in vitro CTL response to HSV-1 in the absence of adherent accessory cells. These culture fluids can be supplied as late as 48 hours after antigen in a 5-day culture and still maximally restore CTL responsiveness.

2. Syngeneic resident peritoneal cells can also restore the CTL responsiveness of adherent cell-depleted cultures, but can be added not later than 24 hours after antigen in a 5-day culture.

3. ConA supernatant fluid can restore the secondary in vitro CTL responsiveness to heat-inactivated virus.

4. The addition of parental strain resident peritoneal cells which have been preincubated with HSV-1 to secondary in vitro cultures of adherent cell-depleted F_1 hybrid splenocytes, results in the preferential stimulation of F_1 responders which are MHC identical.

5. F_1 hybrid splenocyte cultures supplemented with ConA supernatant fluid and stimulated with virus exhibit CTL reactive against both parental haplotypes.

6. Elimination of Ia-positive cells from either whole splenocytes, or from adherent cell fractions subsequently added back to adherent cell-depleted cultures, results in the ablation of CTL responsiveness.

7. Prevention of secretory activity by Ia-positive adherent cells sharply impairs their ability to act as accessory cells, a condition that can be overcome by the addition of IL-1 to cultures.

8. All Ia-negative cells tested failed to subserve accessory cell function, even in the presence of extraneous IL-1.
9. Ia antigen expression and IL-1 secretion are not in themselves sufficient to provide accessory cell function, since infected Ia-positive B cells fail to provide accessory cell function in the presence of IL-1.
10. Infectious HSV-1 is capable of priming helper T lymphocytes in vivo, whereas inactivated forms of HSV-1 are not.
11. By the approaches employed, UV and heat-inactivated HSV-1 preparations failed to elicit suppressor activity.
12. IL-2 proliferative activity was present only in concentrated supernatant fluids from splenocytes which had been primed in vivo and and restimulated in vitro with infectious HSV-1.

VII. UNIFYING DISCUSSION AND SPECULATIONS

At the time these studies began, much of the information regarding the cellular interactions required for the activation of cytotoxic T lymphocytes (CTL) had to do with the CTL response to alloantigens (105, 125, 186, 187). This system is frequently employed for the study of CTL responses because alloantigens readily stimulate high levels of CTL activity. However, the in vivo importance of CTL is likely to be the elimination of aberrant tumorigenic cells and self cells infected with virus.

Our laboratory had made the observation that a nylon wool adherent cell played an essential role in the generation of CTL against HSV-1. It had also been determined that the requirement for this cell could be bypassed with the addition to culture of ConA-stimulated spleen cell supernatant fluids. These fluids contain a substance(s) referred to initially as T cell growth factor (TCGF). TCGF has been reported to assist in the generation of CTL by nonspecifically driving the proliferation and differentiation of antigen-activated CTL precursors (32, 116, 117, 131, 183). Reconstitution of CTL responsiveness to HSV-1 in adherent cell-depleted cultures could be accomplished with accessory cells only if they were added within the first 24 hours of a 5 day culture. By comparison, TCGF could reconstitute CTL responsiveness as late as 48 hours into culture. Since TCGF was later-acting than accessory cells in the anti-HSV CTL response, it was reasoned that the

critical role of the adherent cell is participation in the antigen-dependent, activation process for T helper lymphocytes.

A requirement for antigen presentation to the CTL precursor was not rigorously established. Splenocytes from (BALB/c $\times$ C3H/HeJ) F_1 mice were depleted of adherent cells, and cocultured with parental strain resident peritoneal cells (PC) which had been preincubated with HSV-1 and washed. Parental PC preferentially restored the CTL responsiveness of the MHC identical subset of F_1 splenocytes. These results suggested the requirement for H-2 restricted presentation of antigen to the CTL precursor. Since TCGF can restore the CTL response to HSV-1 in the absence of adherent cells, it is clear that if an antigen presentation requirement for the CTL precursor exists, it can be performed by cell types other than the adherent cell required for helper cell activation. Further investigations should attempt to establish a role for antigen presentation to the CTL precursors. To establish this, cells bearing the K or D region MHC antigens of a given haplotype would be infected with HSV-1 for a sufficient period to insure membrane expression of viral antigens. The cells then would be fixed with glutaraldehyde to prevent the release of new virions from infected cells. The infected, fixed cells can be added to adherent cell-depleted F_1 splenocytes having the appropriate parentage, and supplemented with TCGF. If the virus-infected cells preferentially restore the CTL response to antigen in the context of their own haplotype, the results will suggest that the CTL precursor recognizes antigen in the context of H-2 antigen during induction, and thus requires physical presentation.

In the present study, it was shown that the adherent accessory cell must express Ia antigens on its surface, as well as secrete a soluble mediator, interleukin-1 (IL-1). Ia-negative cell lines, including the macrophage cell line P388D₁, failed to serve accessory cell function in adherent cell-depleted cultures. Only cell populations which were known to contain varying percentages of Ia-positive macrophages and dendritic cells were able to restore CTL responsiveness in depleted splenocytes. These data could have reflected other differences between the Ia-negative cells tested and the Ia-positive macrophages, particularly in light of the observation that Ia-positive B cells, which are infectable by HSV-1 and express the viral antigens in their membranes, could not serve as accessory cells.

Other investigations have demonstrated that the Ia antigen expression of macrophages is a regulatable phenomenon (26, 167, 168). Resident PC, which contain on average 10% Ia-positive cells, reverted to a population containing fewer than 1% Ia-positive cells following two day days of in vitro culture. I found that Ia-positivity could be restored to such cells if they were cultured a further two days with ConA-stimulated spleen cell supernatant fluids. Thus, it became feasible to compare the Ia-negative and Ia-positive forms of the same cell for their capacity to serve as accessory cells in depleted cultures. Ia-positive resident PC could restore CTL activity to adherent cell-depleted cultures, but Ia-negative PC failed to do so.

IL-1, known to be a product of macrophages (38), has been implicated in the generation of CTL responses to alloantigen (38, 77). In one

report, IL-1 could apparently replace the adherent cell requirement (77), but this appears not to be the case in the anti-HSV CTL response studied here. When antigen-infected P388D₁ cells, which had been stimulated to express Ia antigen, were added to cultures with IL-1, CTL activity was generated in vitro from adherent cell-depleted splenocytes. Neither Ia-positive P388D₁ cells nor IL-1 alone was stimulatory. Ia-positive, antigen-infected PC, which were glutaraldehyde-fixed to prevent the secretion of soluble products, could assist in the generation of anti-herpes CTL in depleted cultures which had been supplemented with IL-1. These results suggest that both Ia antigen expression and IL-1 secretion are essential qualities of the accessory cell population. However, Ia-positivity and IL-1 secretion are not in themselves adequate to provide accessory cell function since HSV-1-infected Ia-positive B cells could not restore CTL responsiveness even in the presence of extraneous IL-1.

Earlier work done by others in this laboratory had shown that heat-inactivated preparations of HSV-1 could stimulate in vitro secondary CTL responses in adherent cell-depleted cultures in ConA-stimulated spleen cell supernatant fluids were added. In light of the accessory cell and TCGF experiments, it was believed that the failure of inactivated HSV-1 to activate CTL might reflect an inability of such preparations to adequately stimulate T cell help. To address this problem, an in vitro system was developed in which helper deficient CTL responder populations and putative helper cell populations were cocultured. Helper cell populations were prepared by priming mice

in vivo with infectious or inactivated HSV-1, and irradiating the splenocytes at 1500 rads to selectively abrogate any CTL activity. Helper cell activity was evident only in irradiated splenocytes from mice which were injected with infectious HSV-1. However, irradiated splenocytes from mice which had been primed in vivo with inactivated HSV-1 could not assist in the induction of CTL responses from helper-deficient responder cells. Interleukin-2 (IL-2), a component of so-called TCGF, was undetectable in supernatant fluids of splenocytes primed with inactivated virus and restimulated in vitro with infectious HSV, also indicating that inactivated HSV-1 fails to prime helper cells in vivo. In contrast, IL-2 was detected in supernatant fluids from splenocytes primed in vivo and restimulated in vitro with infectious HSV-1.

The question of whether or not inactivated preparations of HSV-1 can stimulate the active suppression of anti-HSV CTL has not been definitely resolved. No suppression of CTL activity could be detected in secondary in vitro splenocyte cultures when inactivated HSV-primed splenocytes were added. Similarly, there was no detectable suppression of the secondary in vitro anti-HSV response by supernatant fluids from inactivated virus-stimulated splenocytes. However, suppressor cells may have been destroyed in the cell-mixing experiments by x-irradiation prior to culture of the inactivated virus-primed cells and/or suppressor factors may have been denatured. In addition, suppression may have been negated by factors in supernatant fluids. Cell-mixing experiments should be done in which the ability of inactivated HSV-primed splenocytes to

suppress primary in vitro CTL responses can be measured. In any case, my results indicate that the failure of inactivated HSV-1 to stimulate CTL responses is due to inadequate activation of T cell help, an effect which can under some conditions be overcome by the addition of a cell-free source of helper factors.

A sequence of cellular interactions for the CTL response to HSV-1 can be postulated from these data, and has been detailed in a portion of Figure I (page 22). Two distinct antigen-activated sequences appear to occur, one of which is dependent upon the successful activation of the other. The first sequence, the antigen activation of T helper cells, is an independent sequence and begins with the accumulation of HSV-1 on the surface of an Ia-positive accessory cell. This cell in some fashion physically presents and/or processes antigen, which the helper T lymphocyte precursor then recognizes in the context of Ia antigens. The accessory cell also synthesizes and secretes IL-1. The combined signals of antigen and IL-1 are postulated to drive the helper T lymphocyte into proliferation and direct it to manufacture a number of soluble mediators, including IL-2 and T cell differentiation factor (TCDF). Based on our data regarding the kinetics of addition of PC and TCGF to cultures, it may require as much as 48 hours for this sequence to achieve peak levels of activity.

The second sequence, which is CTL differentiation and expansion, is dependent on the successful activation of the helper cell sequence. HSV-1 is probably presented in the context of K or D antigens on accessory cells to the CTL precursor. The stimulation of the CTL precursor by

antigen appears to occur more readily than does the stimulation of the helper cell precursor, since inactivated virus preparations effectively activate the former, but not the latter. It is not understood whether or not inactivated HSV-1 envelope proteins get into the membrane of presenting cells and associate with K and D antigens. De novo protein synthesis by HI-HSV-1 is unlikely, and thus if the CTL precursor requires H-2 restricted antigen presentation, the nature of the association of HI-HSV with the presenting cell is unclear. Antigen stimulates the CTL precursor to express the appropriate receptors for IL-2 and TCDF (32, 183). As they become available, IL-2 and TCDF bind to the antigen-stimulated CTL and program the cells to multiply and undergo final differentiation into cytotoxic T lymphocytes.

Overall, the information from the literature and the results presented in this dissertation indicate that a vaccine which stimulates CTL activity must be capable of activating both the helper T cell and CTL precursors. Alternatively, a vaccine could contain viral proteins supplemented with helper cell factors. The development of such a vaccine would necessitate an increasingly biochemical framework, outlining the interactions of clearly-defined effector and receptor molecules, and the physicochemical consequences of the interactions. An improved understanding of the cellular interactions and activation requirements of the CTL response could possibly lead to the development of more effective anti-viral vaccines.

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