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AN ANALYSIS OF THE MURINE CD4$^+$,CLASS II MHC-RESTRICTED CYTOTOXIC T LYMPHOCYTE RESPONSE TO HERPES SIMPLEX VIRUS TYPE 1

A Thesis

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Gerasimos Kolaitis

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To my family

for their unselfish and continued love and support.

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ABSTRACT

The role of $CD4^+$ T lymphocytes in the effector phase of cytotoxic T lymphocyte (CTL) responses to herpes simplex virus type 1 (HSV-1) was characterized. Their contribution in the CTL response of Balb/c mice was confirmed through antibody (Ab) inhibition experiments. Specific requirements for antigen (Ag) processing pathways involved in the sensitization of $CD4^+$ and $CD8^+$ T cells were examined.

Two effector CTL populations were distinguished on the basis of phenotypic profiles and MHC restrictions. The study suggests that the CTL response to HSV-1 is the consequence of the cumulative effects of $CD4^+$ and $CD8^+$ T cells. $CD4^+$ CTL are restricted by class II MHC determinants, inasmuch as anti-IA inhibits their function. On the contrary, anti-IA does not inhibit the response against targets expressing only class I MHC determinants.Target lysis by either effector population is not mediated through soluble factors and requires effector-target conjugate formation.

Neither $CD4^+$ nor $CD8^+$ CTL lyse targets pulsed with vaccinia recombinants of gC or gD. Furthermore, UV-inactivated HSV-1 fails to elicit a CTL response, inasmuch as targets pulsed with UV-treated virus are not killed. Balb/c derived B lymphomas are partially inhibited by either chloroquine or emetine in presenting Ag to whole CTL populations. Anti-CD4 acts in concert with emetine to abolish the response entirely. In contrast, CTL recognition of class I bearing fibroblasts is completely ablated in the presence of emetine treated targets, whereas it remains unhindered against targets pretreated with chloroquine.

We propose that the Balb/c CTL response to HSV-1 is effected by $CD4^+$, class II and CD8+ class I MHC-restricted T cells with requirements for endosomally processed or de novo synthesized Ag respectively.

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I. Literature Review

Introduction

Differentiation of thymocytes into mature T lymphocytes is characterized by phenotypic changes and the concomitant establishment of their functional limitations. The two major T cell subsets thus evolved, are generally sequestered into two broad effector populations, expressing either the CD4 (otherwise known as L3T4, Leu3, or T4), or the CD8 surface glycoproteins. These two populations, $CD4^+$ and $CD8^+$ T cells, were initially regarded as functionally distinct on the basis of their restriction requirements and biological capacities. Thus, $CD4^+$ T lymphocytes were thought to recognize foreign antigen (Ag) predominantly in the context of the major histocompatibility (MHC) class II proteins and were designated as the helper/inducer T cell population. On the other hand, the $CD8^+$ population was assigned cytolytic and/or suppressive functions, and was believed to recognize Ag strictly in conjunction with class I MHC molecules (119, 138, 99, 80, 105).

Since the observations by Moretta et. al. (83), extensive research has ensued not only challenging the rigidity of such a model, but moreover, providing substantial evidence attesting to the heterogeneity of T cells. In immune responses as variable as those against alloantigens, viral, protein, and bacterial Ags, the picture that has emerged is one of diversity. $CD4^+$ T cells have thus extended beyond a capacity to provide help for immunoglobulin (Ig) production, and/or effect delayed type hypersensitivity (DTH) reactions, and have henceforth assumed a role initially prescribed to the $CD8^+$ population, namely cytotoxicity (22, 88, 123, 135). Similarly, although it is still a matter of considerable controversy, the requirement of $CD4^+$ T

cells for clonal amplification of $CD8^+$ cytotoxic T lymphocytes may be bypassed (28). Additionally, there is evidence to suggest that $CD8^+$ cells can produce sufficient interleukin-2 (IL-2), when stimulated with phytohaemagglutinin (PHA) and phorbol myristate acetate (79), to support their proliferation, and generally assume helper T cell functions (121, 125). Furthermore, the presumed affinity of CD4 or CD8 for MHC class II and class I respectively may not be absolute. Hence, $CD4^+$ T cells have been found to react with class I bearing targets, whereas the $CD8^+$ subset may include a minor population of cells capable of recognizing targets compatible only in MHC class II Ags (114, 113, 40, 120, 70, 112).

It is therefore possible that exclusivity regarding function and MHC restriction requirements is not dictated by phenotypic differences, and that functional characteristics are not restricted to a particular T cell subset. Rather a multipotential may indeed exist within individual cells, its realization dependent on conditions of culture and/or their state of differentiation (22, 81, 90).

Molecular and Functional Characteristics of the CD4 Receptor

Biochemical studies, aided by the isolation and sequencing of cDNA, indicate that the CD4 molecule is a transmembrane protein with significant homology to the immunoglobulin (Ig) gene family and the MHC class II determinants. Maddon et. al. have demonstrated that CD4 is an integral membrane glycoprotein with an overall structure consisting of an amino-terminus variable-like domain, a joining-like region, a membrane spanning region, and a highly charged cytoplasmic domain. Immunoprecipitates of both CD4 transfected cells and $CD4^+$ T lymphocytes contain a band that migrates with a relative molecular weight of approximately 55 kD.

Furthermore, within the region of the first 94 amino acids there is a conservation in 8 of 14 invariant residues between CD4 and the variable region of the Ig light chain. This region contains two cysteine residues 66 amino acids apart, theoretically capable of forming intrastrand disulphide bonds, a notion supported by the protein's mobilities under reducing and nonreducing conditions. In addition to individual amino acid homologies with Ig variable and constant domains, CD4 exhibits a characteristic pattern of folding, in which a series of anti-parallel β-strands fold into two β-sheets held together by disulphide linkage and other hydrophobic interactions (71).

The implications of the homology observed have since been expanded to include functional attributes of the CD4 receptor. It is a common phenomenon for Ig and MHC molecules to exist as dimers, as for example in the interactions between the light and heavy chains on individual antibody (Ab) molecules, or the α and β subunits of MHC class II Ags. Although there is no evidence to suggest that CD4 appears as an aggregate of multiple subunits, its association with other membrane components on the same or different cells, including MHC Ags, has been the subject of numerous studies. Earlier research suggested that CD4 is intimately involved in the recognition of Ag by associating with the class II products of the MHC locus. This association is presumably effected by binding to a nonpolymorphic region of MHC, thus increasing the overall avidity of the T cell to its target or Ag presenting cell (APC) bearing the appropriate MHC protein (124, 80, 41, 78, 72, 26). A wide array of monoclonal antibodies (moAb) against CD4 have been shown to inhibit the proliferative response of $CD4^+$ T cells in mixed lymphocyte reactions (MLR). Because the inhibitory effect can be augmented by either increasing the Ab concentration or decreasing the number of stimulator cells, it was initially postulated that anti-CD4 competed with Ags, presented by the stimulator cells, for T cell membrane sites involved in antigenic

recognition.CD4 was thus implicated in the recognition of Ag and MHC (26).

Using HLA-SB-specific $CD4^+$ cytotoxic T lymphocytes (CTL), Biddison et al. introduced a model whereby CD4 could bind to a nonpolymorphic region of MHC class II Ags, (10), which appears to strengthen the interactions of $CD4^+$ T cells with their respective targets or stimulators (120). Examination of $CD4^+$ clones has convincingly shown that recognition of Ag-MHC class II complexes is variably affected by a number of anti-CD4 moAb preparations. The inhibition appears to correlate with the overall sensitivity of a particular T cell clone to Ag (109). Hence, clones that react well with low doses of Ag and are moreover not easily inhibited by Ab raised against the I region of the MHC locus, in other words, cells that are relatively sensitive to Ag are simultaneously difficult to inhibit with anti-CD4 Ab. In parallel, at high doses of Ag, T cell clones that are otherwise of intermediate or low sensitivity to antigenic stimulation, may indeed bypass the requirement for CD4 (41). Moreover, as demonstrated by Marrack et al (72), clones of inherently low responsiveness that have lost the CD4 protein retain only partial reactivity and considerably below that of their $CD4^+$ counterparts. On the other hand, $CD4^-$ mutants of originally highly responsive clones retain their responsiveness to antigenic stimulation .

Subsequent research on the relative role of CD4 in T cell triggering has challenged the functional restrictions inherent in the previous model. The hypothesis that this protein acts solely as an ancillary structure, increasing the avidity of $CD4^+$ T cells for Ag-MHC complexes, are in apparent contradiction with a number of studies indicating a more active participation of CD4 in post binding events. Although these studies do not refute the proposed model, they do explore the possibility of alternative mechanisms whereby CD4 might be more directly involved in T cell activation.

It was initially observed that moAb against CD4 is capable of inhibiting concanavalin A (Con.A) induced IL-2 production under conditions free of MHC class II requirements. Since cells expressing MHC determinants were absent from the assays, it would not be possible for Ab to block the interaction of CD4 with MHC. Wassmer et al. (136) suggested that the inhibition was the result of a negative signal transmitted upon Ab-CD4 complex formation . In the presence of MHC determinants, CD4 may conceivably interact with MHC and transduce a similar signal that could regulate a T cell's response, depending on the strength of the antigenic stimulus received. Thus, P. Marrack's findings (72) may alternatively be interpreted if one considers that T cells with low affinity Ag receptors might be susceptible to the down regulating signals of Ab-CD4 interactions . Conversely, high affinity interactions may overcome such negative signals, provided that the difference between the antigenic stimulus and the inhibitory signal transduced by CD4 yields an overall positive tertiary message. Consequently, as predicted, increasing concentrations of a mitogen, such as Con.A, can indeed overcome the inhibitory effects of anti-CD4 Ab (136).

Further evidence in support of the " negative signal " hypothesis came from the reports of Fleischer et al.(30), and Beckoff et al.(6), wherein $CD4^+$ CTL were triggered in the presence of antibodies against the CD3-T cell receptor complex (CD3-TcR), or mitogens such as Con.A and PHA, either in the absence of MHC bearing targets or in the presence of Ia^- fibroblasts. It was observed that binding of an antibody to CD4 resulted in the inhibition of cytotoxicity and proliferation. The inhibition was dependent on the concentration of anti-CD3. Therefore, by increasing the concentration of anti-CD3 the modulatory effects of anti-CD4 could be bypassed. Thus, the effect of ligand binding to CD4 is inversely related to the magnitude of the signal received by the CD3-TcR complex (30, 6). Results of a similar nature were

obtained by Bank and Chess (5). However, in addition to the inhibitory effects of anti-CD4 Ab on the proliferative response of $CD4^+$ T cells, it was shown that the overall effect was time dependent with maximal inhibition occurring within the first 24 hours of CD4 perturbation. In physiological terms the " negative signal " model could provide a theoretical means of justifying the reason low affinity interactions between T lymphocytes and their natural ligand, namely self MHC, in the host does not usually result in the induction of effector functions, thus observing tolerance. By extension, high affinity interactions, such as those between CTL and virally infected cells, may overcome the CD4 transduced inhibitory signals, thereby assuring a response against appropriate targets (30). In other words, in the presence of MHC without concomitant antigenic stimulation there would be no activation of T cells, ensuring that interactions of $CD4^+$ T cells with accessory cells devoid of Ag does not result in unwanted immune responses (5).

In contrast to the " negative signal " hypothesis it has been demonstrated that moAb B66, which recognizes CD4, transduces a positive signal to T cells at both the bulk and clonal level. Such an interaction results in mobilization of intracellular Ca^{++} stores, expression and release of IL-2, and eventual proliferation of the cells (18).

How such signals, be they positive or negative, are actually transmitted is not known. Evidence suggests that signal transduction through CD4 may involve the activation of $p56^{lck}$, a protein with tyrosine kinase activity which has been shown to phosphorylate the ζ subunit of the CD3-TcR complex (134). The ensuing phosphorylation may provide the initial signal for comodulation of CD4 with the CD3-TcR complex, culminating in IL-2 production, IL-2 receptor expression, and proliferation of the cells (94, 77, 101). These observations and further research suggests that CD4 is physically associated with the T cell receptor (TcR) (13, 134).

Certain anti-TcR monoclonals that activate T cells may do so by inducing a conformational change on the TcR thereby encouraging its association with CD4. According to this postulate, any combinations between anti-CD4 and anti-TcR preparations resulting in conformational changes that facilitate CD4-TcR complex formation may induce T cell responsiveness, whereas, conformational changes that block this association may inhibit T cell activation (101). To extend the latter hypothesis, it is conceivable that what are thought to be signals transduced upon perturbation of CD4 may be the direct result of changes in the three dimensional structure of CD4 and the TcR following binding to their respective ligands. Thus, responsiveness or nonresponsiveness may be due to enhanced CD4-TcR complex formation and not necessarily the result of any particular signals. If complex formation is the prerequisite for T cell activation, any interference that prevents it from occurring may also inhibit activation of the T cell. Similarly, promoting complex formation would result in enhanced T cell responsiveness.

Whether CD4 is capable of transducing any signals into a cell, the results to date uniformly suggest that it is capable of regulating the state of activation of a T cell independently of interactions with accessory cells. In a recent review, Janeway (51) argues in favor of " co-receptor " rather than mere bystander " accessory " functions for the CD4 molecule. However, it is plausible that CD4 subserves two distinct functions, namely interaction with MHC class II determinants, and transduction of subsequent signals either directly or via another protein. In the former instance, CD4 is perceived to be an accessory molecule, whereas transduction of any signals predicates true receptor-like properties.

Processing Requirements for Ag Presentation

The T cell receptor is composed of a heterodimer capable of co-recognizing combinations of Ag and MHC class I or II on the surface of APC or targets (1, 43, 89, 107). The two chains of the TcR, α and β, form a functional polymer with the invariable glycoprotein CD3 (115) present on all mature T cells regardless of their CD4 or CD8 phenotype. The penultimate requirement for T cell activation is Ag presentation, after internal degradation and processing, as a complex with MHC class I or II determinants. In fact, a number of peptides that are antigenic for MHC class II restricted T cells are shown to directly bind to purified Ia molecules (4).

Presentation of Ag to MHC class II-restricted T lymphocytes is sensitive to lysosomotropic agents that interfere with the pH of endosomes, thereby implicating an acidified intracellular compartment in the process (84). Such observations indicate that the antigenic stimuli recognized by T cells may not be intact Ag but rather a processed form of it (7, 110, 45).

Initial observations suggested that there is a marked difference in the presentation and processing requirements of the two T cell subsets. Morrison et al. (84) have demonstrated that whereas $CD4^+CD8^-$ Ia-restricted, Ag-specific cytotoxic T lymphocytes could lyse MHC compatible targets treated with a soluble form of influenza haemagglutinin (HA), they do not require ongoing protein synthesis in their respective targets. However, the effectors remain virtually ineffective against targets treated concomitantly with HA and chloroquine, or targets infected with HA-recombinant vaccinia constructs. On the other hand, class I-restricted $CD4^-CD8^+$ CTL recognized only targets that were productively infected with influenza or a vaccinia recombinant. Protein synthesis was a prerequisite, whereas chloroquine

treatment of infected targets did not interfere with their recognition by T cells. This led to the conclusion that class II-restricted T cells recognize protein fragments that have undergone endosomal processing, whereas their class I-restricted counterparts recognize Ags that escape endosomal degradation, or are synthesized de novo.during viral replication In order to account for these differential requirements in responses to specific Ag, the model of exogenous and endogenous processing was proposed (84). Thus, it was postulated that any proteins entering the lysosomal compartment could potentially interact with class II MHC determinants, whereas the association of Ag with class I molecules necessitated de novo protein synthesis.

Further research in a number of laboratories has added support to the model as proposed by Morrison et al. (84). For instance, it has been demonstrated that T cells, or appropriate APC, treated with HA peptides can present Ag (45). Similarly, Lanzavecchia et al. (63) have shown that soluble gp120 and OKT9 moAb, when used as Ags, can sensitize T cells to subsequently present degraded forms of these Ags to responder T cell clones. Moreover, the presentation was sensitive to treatments with leupeptin, attesting to the requirement for endosomal proteases in the processing step.

Contrary to the above model, there are reports whereby proteins, or fragments of proteins exogenously introduced into a cell can be presented to class I-restricted T cells, provided that the endosomal compartment is bypassed. Thus, cyanogen bromide or trypsin-derived fragments of ovalbumin (OVA) can stimulate class I-restricted CTL, presumably by binding directly to the MHC determinants (17). Furthermore, OVA introduced into the cytoplasm through osmotic lysis of pinocytic vesicles gave similar results (82). The discrepancies between class I and II presentation requirements, inherent in the "exogenous versus endogenous hypothesis" were further questioned by results with soluble hapten-protein conjugates (106), the nonstructural T protein of

SV40 virus (39,126) and immediate early gene products of herpesvirus replication(75, 57), a chimeric HA molecule (137), and the polymerase gene products of influenza (9). Similarly, truncated influenza nucleoprotein (NP), introduced into cells via transfection (2), as well as synthetic peptides of NP, were sufficient in inducing cytotoxicity of class I-restricted CTL.These results allowed Townsend et al. (130, 131) to speculate that in agreement with class II MHC restriction, class I-restricted CTL may indeed recognize fragmented or degraded forms of viral Ags. It is also of interest to note that fusion active Sendai virus is able to sensitize targets for CTL in a class I mode without ongoing de novo protein synthesis, whereas Sendai virus lacking fusion activity fails to do so. In the latter instance endocytosis of the fusion inactive virions might result in the lysosomal degradation of viral Ags otherwise required for class I-restricted CTL recognition (36).

Be it exogenously or endogenously introduced, it appears necessary that Ag be processed prior to presentation . The differences observed between class I and class II processing requirements may thus be due to the intracellular compartment involved in the degradation of proteins. In a model proposed by Germain, (33) and more recently expanded by Long (69) proteins entering the endosomal compartment, either via pinocytosis or receptor-mediated endocytosis, undergo limited proteolytic degradation, and the fragments generated may associate with MHC class II determinants, either within the endosome, a compartment that has been shown to be rich in Ia (42), or on the surface of a cell. On the other hand, proteins endogenously synthesized may undergo degradation within a compartment distinct from the endosomes, perhaps the region of the transitional Golgi stacks. Denatured or fragmented molecules may thus escape further lysosomal processing and reach the surface of a cell, either alone or in association with newly synthesized class I determinants (33). Whereas the endosomal

compartment is sensitive to lysosomotropic agents which interfere with the function of acid proteases (141, 19), the cytosolic region, where protein derivatives that associate with class I molecules are formed, is not affected. Interestingly, although it has been shown that externally added synthetic peptides can be presented at the surface of a cell, the site of Ag association with MHC remains at best speculative (132).

A Functional Comparison of $CD4^+$ and $CD8^+$ T lymphocytes

Since the discovery of differentiation antigens, attempts have been made to characterize T cells on the basis of their surface phenotype, and prescribe distinct functional properties to the subsets thus created. It was found in both the human and murine systems that the majority of T lymphocytes capable of providing help for Ig production bore the CD4 marker, whereas cytolytic and suppressor cells were of the CD8 phenotype (50, 93). However, it was soon realized that these differences were more imagined than real, and that phenotype does not necessarily predicate function. Hence, cells with helper, cytotoxic, or suppressor abilities were found that seemed to violate the originally set distinctions (55, 123, 126). Moreover, these observations are not isolated instances, and may in fact constitute the rule rather than the exception. For instance, $CD4^+$ Ia-restricted T cells comprise the predominant CTL phenotype in human HSV-1 and measles virus infections (139, 49, 108).

It is conceivable that at least part of the reason similar observations were not reported earlier, was culture conditions not conducive to the generation of such CTL. Thus, as demonstrated by Krensky et al. (59, 60), allospecific cytotoxic T cells established by continuous culture with stimulators expressing only HLA-DR6 MHC determinants, are $CD4^+$ and DR6-specific. However, in the presence of stimulators

positive in both class I and class II MHC antigens, the predominant phenotype of CTL generated over long term cultures is $CD8^+$ and HLA-A or B- specific (59, 60).

The functional distinctions between $CD4^+$ and $CD8^+$ CTL clones were further obscured with the discovery of MHC restriction overlap. Thus, both $CD8^+$ class II, and $CD4^+$ class I-restricted CTL have been reported in the literature of the last few years (31, 32, 58, 119,). Although the phenomenon of MHC restriction overlap remains a rather rare occurrence, it nonetheless weakens the assumed relationship of CD4 and CD8 molecules to T cell function and specificity.

The similarities between $CD4^+$ and $CD8^+$ CTL notwithstanding, differences do exist in the presumed mode of action of the overall lytic response. There are a number of instances whereby lysis effected by $CD4^+$ clones, unlike their $CD8^+$ counterparts, occurs partly via soluble mediators, perhaps lymphotoxin(s), thus bypassing the requirement for direct conjugate formation between effectors and targets (127, 128, 129).

Whether diversity is an acquired phenomenon, following extensive in vitro expansion, or rather represents a stable repertoire, is still a matter of considerable debate. Long term culture of influenza-specific T cells have resulted in the generation of otherwise undetectable levels of $CD4^+$ CTL activity (29, 44). Cytotoxicity was acquired after four weeks of cloning, and the degree of lysis observed increased over extended culture. Similar results were obtained with alloreactive T cells specific for HLA-SB antigens (10). Although these studies do not contest the possibility of cytolytic $CD4^+$ lymphocytes being artifacts of in vitro conditions, rather than true CTL, they clearly demonstrate that certain functions may be acquired without compromising antigenic specificity. It is moreover plausible that diversification may be the result of distinct stages in the differentiation of a single cell, or that the

characteristics manifested in time might correspond to the effects of a number of diverse cells with a common precursor. Furthermore, the nature of the antigenic stimulus may dictate the magnitude of such responses. It appears, for example, that in humans seropositive for HSV-1, and to a lesser extent cytomegalovirus (CMV), the majority of freshly isolated CTL express CD4 and are restricted by HLA-DR determinants (68, 104).

Alternatively, the answer may lie entirely on the frequency of the $CD4^+$ CTL response. Thus, although influenza and Epstein-Barr virus-specific $CD4^+$ class II MHC-restricted CTL are rare or undetectable in primary cultures, upon in vitro stimulation of peripheral blood leukocytes from seropositive individuals, and subsequent depletion of the $CD8^+$ population, a response is clearly observed. Based on these results, Bourgault et al. (14) have postulated that the scarcity of the $CD4^+$ CTL response is a matter of absolute numbers of cells present in a particular assay. Therefore, expansion of intrisically low numbers of $CD4^+$ T cells, numbers that do not exceed 10% of the total CTL population, may preferentially bias their in vitro detection.

A Speculative Role of $CD4^+$ CTL in Immunoregulation

Discrimination of self from non-self is an integral part of immune responses, without which the results could be deleterious, manifest in many an autoimmune disease. Truly there is, perhaps, a disadvantage in complying with such restrictions, but random induction of cell mediated or humoral immunity certainly does not provide a logical alternative. The scenario, therefore, becomes one of control, be it prior to initiation of a particular response, or its eventual quiescence. Although much of

research has focused on the subject of immunoregulation, predictions and models are far from being complete, or at times physiologically sound.

The two major T cell subsets were initially thought to encompass a class of cells, perhaps of distinct phenotypes, whose functions would include modulation of the immune response, by providing negative signals culminating in downregulation. However, their isolation and characterization has proven a much more herculean task than anticipated. To date, suppressor cells have eluded attempts to be clonally isolated, and moreover, their restriction elements, believed to reside in the J region of the class II MHC locus, in addition to their receptors remain still rather inferential (114, 117).

In recent years models have been proposed assigning suppressive functions to the already well characterized $CD4^+$ and/or $CD8^+$ T populations (52, 62, 63, 86, 139). Since the observations that T cells of the CD4 phenotype can exhibit cytolytic activities, it is believed that under appropriate stimulation B cells, and APC in general, may indeed serve as targets. Thereby, a means of immunoregulation may be accounted for, the negative pressures for the regulation of Ab responses and suppression of APC-induced T cell activation being exerted by $CD4^+$ T lymphocytes.It is well documented that B cells are capable of presenting Ag in a manner not dissimilar to that of other APC (127, 19, 20, 61, 42). Therefore, it is conceivable that Ag sensitized B cells can be recognized as targets resulting in their demise. B lymphomas are routinely used as targets for $CD8^+$ class I-restricted effectors. Additionally, there is increasing evidence to suggest that they may be recognized and lysed in a class II-restricted fashion (55, 111, 104). One of the most recent reports to that effect by Shinohara et al. (113), clearly demonstrates that $CD8^+$ Ia-restricted CTL specific for keyhole limpet haemocyanin (KLH), in addition to OVA-specific class II-restricted cells, can effectively eliminate B cells sensitized with the appropriate Ag.

The crucial limiting factors, in such responses, are the ability of a cell to internalize Ag into the endosomal compartment for processing, and the level of Ia expression. The former requirement is theoretically satisfied by any cell actively engaged in endocytosis, either via pinocytic, or endocytic vesicles. Cells that fall within this category include macrophages and dendritic cells. Alternatively, cells bearing receptors for Ag, such as the Ig receptors on the surface of B cells, could also meet the requirement, endocytosis mediated via the actual receptor. A similar situation occurs with T cells in that they can process and present degraded forms of Ag, provided the sensitizing antigenic proteins have an affinity for T cell surface structures for internalization and subsequent recognition to occur (63).

The second prerequisite, the expression of Ia, is satisfied by most classically considered APC. Furthermore, cells otherwise Ia negative, such as fibroblasts and, at least in humans, T cells, can be induced to express class II MHC molecules, either in response to, or as a consequence of disease (27, 140). Additionally, interferon-γ (IFN-γ) can potentiate Ia expression in fibroblasts and endothelial cells (21, 76) as well as in macrophages and B cells (97, 118).

If, as has been described in vitro, the potential of $CD4^+$ T cells to effect cytolysis subsists in vivo, the question becomes one of degree. At least conceptually, there are no reasons this T cell subset cannot function in a suppressive mode by directly eliminating Ia^+ cells that have already been Ag primed.

The outcome of the response in eliminating B cells is quite obvious, namely downregulation of Ig production. However, with either macrophages or T cells as targets the issue becomes more complicated. Unlike B cells, macrophages and dendritic cells, both of which are rich in Ia and capable of Ag presentation to class II-restricted T cells, are not clonally expanded or distributed. Their role as non-specific

effectors of immunity, and their involvement in the initiating phase of an immune response may be compromised. Moreover, pressure may be exerted on any particular system to compensate for their premature removal from the circulation (62). On the other hand, T cells subserve a variety of functions critical to the overall immune response. As a consequence, their dowregulation might jeopardize many aspects of immunity, the end result of which might be a general state of immunosuppression. Indeed, their capacity to present Ag might be more of a handicap than a benefit. For instance, research indicates that processing and presentation of soluble gp120, in HIV seropositive individuals, by $CD4^+$ T cells may predispose such cells for lysis. Because gp120 is readily shed into the circulation (63), the possibility exists that it may bind to uninfected $CD4^+$ T cells and target them for destruction by $CD4^+$ gp120-specific CTL, thereby at least partly accounting for the overall decrease in the numbers of $CD4^+$ T cells observed in AIDS patients.

Whether such a scheme of immunoregulation does occur in vivo remains to be seen. However, it is certainly no more radical a hypothesis than advocacy of a separate T cell subset with suppressive abilities. In fact, considering the overall frugality in the number of cell types involved in immunity, this proposal may indeed present a reasonable model for understanding immunoregulation, or at least one aspect of it.

Rationale for the Present Study

Numerous studies have demonstrated the phenotypic and functional heterogeneity of T lymphocytes. Unlike the initially proposed rigidity of cell mediated responses, it has become increasingly evident that diversity may indeed account for a great number of functional properties, at least inasmuch as they occur in vitro. $CD4^+$

CTL, at either a bulk or clonal level, have been convincingly described in many situations(88, 22, 123, 135, 128, 108). Likewise, the ability of the $CD8^+$ population to secrete lymphokines is well documented (3, 37, 77, 81). Furthermore, restrictions posed by MHC determinants may not necessarily reflect absolute conditions, as was once thought. $CD4^+$ class I-restricted and, to a somewhat lesser extent $CD8^+$ class II-restricted T lymphocytes are frequently demonstrated in the literature (70, 112, 113, 114).

Although the subject of cell mediated responses is becoming ever more complicated, it seems appropriate that common grounds are established and any similarities between systems studied not overlooked. Considering the predominance of the $CD4^+$, Ia-restricted, HSV-1-specific CTL response in humans (24) it was of interest to describe a correlate in the murine system.

The murine model for HSV-1 has provided substantial information regarding the role of CTL in recovery from infection (64, 67, 92)The overwhelming majority of murine HSV-1-specific responses reported in the literature appear to be class I-restricted and dependent on the $CD8^+$ population. Both structural glycoproteins (38, 66, 98), and nonstructural immediate early proteins (73, 75) have been implicated as target Ags for $CD8^+$ CTL. However, not much is known about the state of $CD4^+$ CTL, inasmuch as they represent a true functional T cell subset at the bulk level where pressures exerted by clonal conditions may not apply

The present study was designed to investigate the heterogeneous nature of CTL in mice with an emphasis on the characterization of a $CD4^+$ CTL response as it relates to the bulk primary cell mediated response. A comparison is drawn between class I and class II-restricted CTL regarding the degree of cytotoxicity observed and the requirements for infectious or UV-treated HSV-1. In addition, the importance of

protein synthesis and the necessity for an intact endosomal compartment in the sensitization of targets is examined.

Ultimately, the significance of this work lies in its potential for the development of vaccines and the possible role of $CD4^+$ CTL as immunoregulators of a more direct manner, by eliminating Ia^+, HSV-1 sensitized APC, and B lymphocytes from circulation. The overall implications of such dynamics are discussed in a later section.

II. Specific Aims

1. Resolution of the IA and IE-restricted herpes simplex virus-specific proliferative response of primary lymphocyte cultures.

2. Analysis of the requirements for infectious or UV-inactivated herpes simplex virus on MHC class I and II-restricted primary CTL responses.

3. Examination of the Ag-specificity of the primary CTL response.

4. Characterization of the contribution of the $CD4^+$, IA^d-restricted T lymphocyte population in the overall CTL response to herpes simplex virus type 1.

5. Determination of the necessity for direct conjugate formation in the primary herpes simplex virus type 1-specific CTL response.

6. Definition of the requirements for protein synthesis and an intact endosomal compartment for the recognition of targets in MHC class I and class II-restricted CTL responses.

III. The Role of Ia in the Recognition of Herpes Simplex Virus Type 1 Infected Stimulator Cells by Primary Lymphocyte Cultures

List of Abbreviations

CTL: cytotoxic T lymphocytes.

CMV: cytomegalovirus.

HSV-1: herpes simplex virus type 1.

MOI: multiplicity of infection.

Ag: antigen.

[^{3}H]-TdR: tritiated thymidine.

IFA: indirect fluorescence assay.

moAb: monoclonal antibody.

TCGM: T cell growth medium.

HI FBS: heat inactivated fetal bovine serum.

Introduction

The response to many viral infections is characterized by the concerted effort of the immune system in mobilizing as many of its components as possible, and particularly those associated with the cell mediated response. Cell mediated immunity has been under considerable scientific scrutiny for many years and its significance in viral clearance is without dispute. Classically described, cell mediated immunity is divided into two constituent parts, each comprised of individual T cell subsets

generally believed to differ in effector function as well as surface profiles (16) and requirements for Ag recognition (142).

One of the foremost prerequisites for the efficacy of primary cell mediated responses in combating infection is amplification and expansion of precursor cells, without which beneficial results could not be attained. For instance, it is not biologically sound to assume that a frequency of less than 1/200,000 HSV- specific CTL precursors, a number which translates into 10 cells per lymph node, present in non-immunized mice (100) could successfully provide a means of defense. Obviously, if that were possible primary productive infections could never occur, and the situation would mimic memory responses. It is only upon immunization that the ratio of Ag specific T cells to the total number of cells present can be adequately reduced to detect in vitro. Thus, in HSV-1 seropositive individuals the frequency of CTL precursors is reported to approximate 1/4000 - 1/8000 (104), whereas in HSV-1 primed mice the frequency of virus specific CTL exceeds 1/5000 (100). Hence, one of the principal means available of assessing cellular immune responses in both humans and animal models alike is the lymphoproliferation assay.

The results reported in this section indicate that in the Balb/c strain of mice the Ia-restricted T cell population accounts for a significant portion of the overall lymphoproliferative response to HSV-1. The effect of anti-IA^d moAb is examined concomitantly with the requirement for HSV-1 sensitization of stimulator cells

Materials and Methods

Animals.

Female Balb/c (H-2^d) mice were obtained from Jackson Laboratories and used at 4 to 8weeks of age.

Viruses.

The KOS strain of HSV-1 was propagated on Vero cell monolayers and stored as infectious culture supernatant fluid. Vaccinia^{tk-} (Vac^{tk-}) was constructed and propagated by E. A. Allen.

Cell Lines and Antisera.

The L cell transfectants RT 2.3.H (IA^d; abbreviated RT 2.3) and RT 10.3H2 (IE^d; abbreviated RT 10) were prepared and provided by R. N. Germain (34, 35). The cells were maintained in continuous culture using the selective medium HAT (Sigma Chemical Co. Ltd.) in DMEM (Gibco-BRL), supplemented with 1% pen/strep, 10% HI FBS (Gibco-BRL) and 1% each of L-glutamine and non-essential amino acids. The day before any functional assays the cells were washed twice, and HT (Sigma Chemical Co. Ltd.) was substituted for HAT.

The B lymphoma line A20-1.11 (abbreviated A20)was a kind gift of D. W. Horohov and was maintained in RPMI-1640 supplemented with 1% pen/strep and L-glutamine, 10% each of NCTC-109 medium and 10% HI FBS, and 5 x 10^{-5} mM 2-mercaptoethanol. LT-A-30 (abbreviated LT-A).cell lines are a kind gift of Ian Hayes and maintained in DMEM supplemented with 1% pen/strep and L-glutamine, and 10% HI FBS. Anti-IA^d (IgG_{2a}) was purchased from Beckton Dickinson.

Mouse Immunizations and Preparations of Lymph Node Cultures.

Four to 8 week old Balb/c mice received multiple injections of 5 x 10^6 $TCID_{50}$ of HSV-1 per site by various routes of inoculation. HSV-1 immune mice received a single injection in each of the hind leg footpads and the base of each ear in 0.04 mls of volume. At five days post-immunization the popliteal and retropharyngeal lymph nodes were excised and single cell suspensions of 4 x 10^6 cells/ml were cultured in six-well cluster plates (Corning Glass Works) in a total volume of 5 mls of TCGM. The medium consisted of RPMI 1640 (Gibco-BRL) and NCTC-109 (Gibco-BRL) in a 1/1 ratio, supplemented with 1% pen/strep, 5 x 10^{-5} mM of 2-mercaptoethanol (2-ME) and 10% HI FBS. The cell suspensions were incubated in a humidified 37° atmosphere, containing 5% CO_2, for 5 days in the absence of any restimulation, prior to use in proliferation assays.

Measurement of Lymphocyte Proliferation.

The incorporation of [^{3}H]-TdR into cellular DNA was used as a measure of proliferation. Typically, at 5 days post-initiation of lymphocyte cultures A 20 B lymphomas (H-2^d), RT 2.3 (H-2^k, IA^d), and RT 10 (H-2^k,IE^d) transfected cells in addition to LT-A fibroblasts (H-2^k) were incubated with or without HSV-1 and Vac^{tk-} at MOI of 5 in RPMI 1640, supplemented with antibiotics, L-glutamine 2-ME, and 10% HI FBS prior to being used as the stimulator population. After seven hours of incubation the cells were X-irradiated (3000 rads), washed twice and resuspended in TCGM containing 5% FBS. Subsequently, 100 µl of the cell suspensions were transferred onto 96 well plates and serial dilutions made. Where indicated, anti-IA^d at 6.25 µg/well was incorporated into the culture at this point. At all times, the volume of the cells alone or with Ab equaled 100 µl.

The responder population was pooled, washed once and resuspended at 4 x 10^5 cells/ml. Seventy five µl of the cell suspension and 25 µl of [^{3}H]-TdR at 40 µCi/ml was then added to the stimulator population and incubated at 37° and 5% CO_2. The final culture consisted of 3 x 10^5 responders and various numbers of stimulators (as per experiment) in a total volume of 200 µl/well of TCGM containing 5% HI FBS, and 1 µCi of [^{3}H]-TdR. Culture was arrested at twelve hours post [^{3}H]-TdR treatment and the cells harvested onto glass fiber filters (Skatron Inc.) with a semi-automated cell harvester (Cambridge Technology, Inc.). The filter papers were immersed in 1 ml of Ecolume (ICN Biomedicals, Inc.) and counted on a Beckman LS 7000 liquid scintillation spectrophotometer. Results are expressed as the mean of 6 replicate wells.

Anti-Ia Dose Response.

The dose response to anti-IA^d moAb was measured as the degree of inhibition of cellular proliferation. Responder lymph node cells from 5 day old cultures were incubated at 3 x 10^5 cells per well of 96 well plates in the presence of 5 x 10^5 RT 2.3 or A20 cells and various concentrations of anti-IA^d. The cells were treated with or without HSV-1 (MOI:5) for seven hours and thereafter X-irradiated (3000 rads) prior to being used as the stimulator population in proliferation assays. All wells received 1 µCi [^{3}H]-TdR and cultured for twelve hours in a total volume of 200 µs of TCGM containing 5% HI FBS prior to harvesting. Results are expressed as the mean value of the percent inhibition of [^{3}H]-TdR uptake of four replicate wells.

Results

Resolution of the IA an IE-Restricted Lymphoproliferative Response; Requirement for HSV-1 Infected Stimulator Cells.

The relative contributions of IA and IE-restricted T cells in the overall proliferative response was assessed by stimulating 3 x 10^5 cells derived from 5 day old primary lymph node cultures with 2 x 10^5 each of RT 2.3 and RT 10.L cell transfectants. The stimulators were treated with Vac^{tk-} and HSV-1 (MOI:5) or given no Ag stimulation, followed by X-irradiation and finally assayed for [^{3}H]-TdR-uptake. LT-A, HSV-1 infected fibroblasts were used as the allogeneic control.

As is shown in Figure 1, primary cultures of lymph node cells from acutely infected mice respond to both the IA^+ and IE^+ stimulator cells that had previously been treated with infectious HSV-1. Conversely, uninfected or Vac^{tk-} infected RT 2.3 and RT 10 cells fail to induce proliferation of lymphocytes to any degree above that provided by the LT-A allogeneic controls thus, indicating that the response is HSV-1-specific.

Confirmation of the IA-Restricted T cell Proliferative Response.

To confirm the MHC restriction requirements of the response, 5 x 10^5 RT 2.3 and A 20 cells, treated with or without HSV-1, were cocultured in the presence or absence of various concentrations of anti-IA^d moAb and 3 x 10^5 lymph node preparations. HSV-1 infected LT-A fibroblasts were included in the assay as allogeneic controls.

The data presented in Figure 2 provide evidence in support of an HSV-1 specific and IA-restricted response. IA^+ RT 2.3 cells previously sensitized with virus

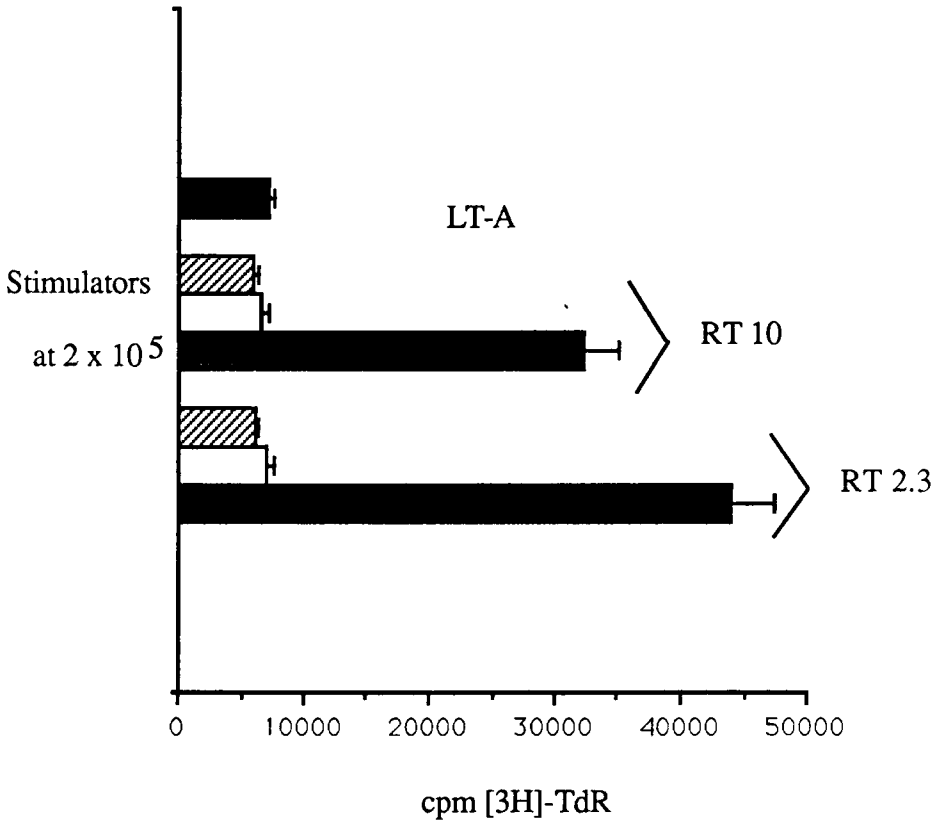

Figure 1 Resolution of the IA- and IE-restricted Lymphoproliferative Response

Responder H-2^d lymph node preparations at 3 x 10^5 were incubated with RT 2.3 (H-2^k; IA^d) and RT 10 (H-2^k; IE^d) stimulator cells Stimulators were infected with HSV-1 (solid bars), Vac^{tk-} (open bars) or left uninfected (hatched bars). LT-A (H-2^k) allogeneic controls were treated with HSV-1 (solid bars).

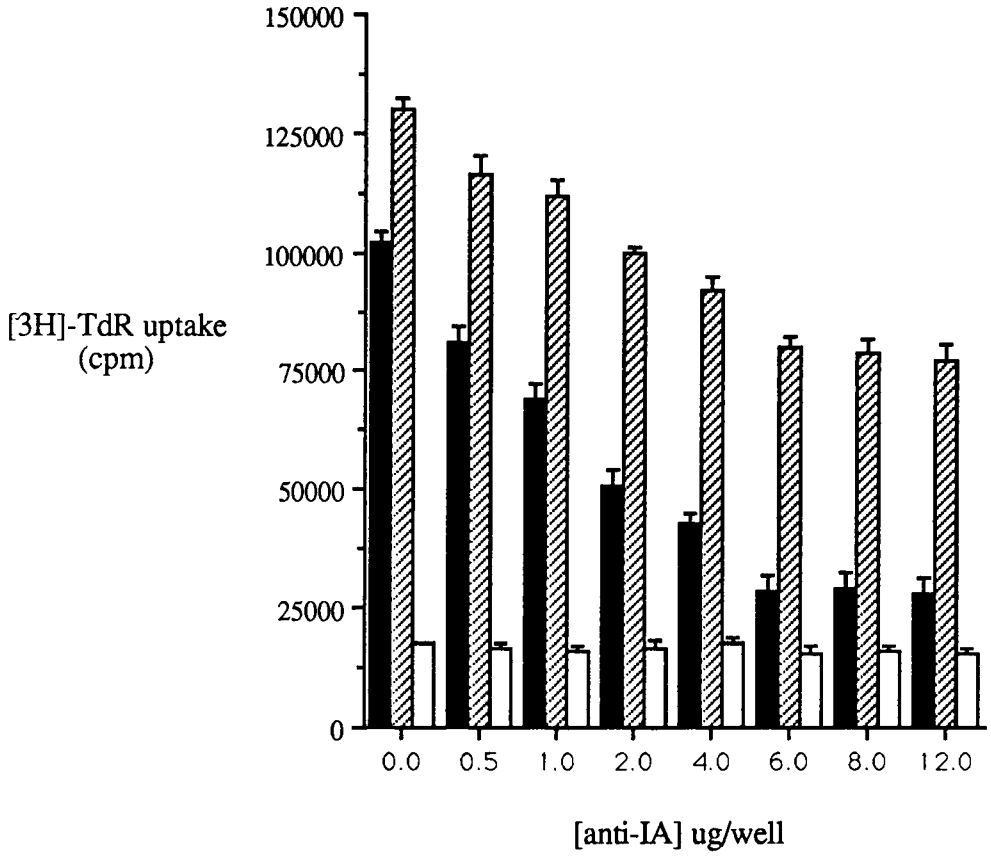

Figure 2 Anti-IA^d Dose Response Kinetics

Responder Lymph node preparations were incubated at 3 x 10^5 cells with 5 x 10^5 each of RT 2.3 (solid bars), A 20 (hatched bars), and LT-A (open bars) stimulators. All stimulator cells were infected with HSV-1

are capable of priming lymph node cultures from acutely infected mice to proliferate. The response is IA-restricted inasmuch as anti-IA^d moAb can inhibit [^{3}H]-TdR uptake. Moreover, the Ab effects are dose dependent with maximal inhibition occurring at about 6 μg of Ab per 5 x 10^5 stimulators. As little as 0.5 μg of soluble anti-IA reduces the response by almost 20% of the untreated controls. Although the inhibition observed does not exceed 86% of the maximal response, it is nonetheless, specific, as neither the HSV-1 untreated controls, nor the anti-IA^d treated, HSV-1 infected LT-A cells are rendered ineffective in providing an antigenic stimulus. In fact, uninfected RT 2.3 cells only marginally elicit a response, not exceeding that induced by LT-A, HSV-1-primed fibroblasts.

Dependence of T Cell Proliferation on Ag and IA Concentrations.

Further evidence for MHC restriction and antigenic specificity is presented in Table I. As an indirect measure of Ag dose requirements, RT 2.3 and A 20 cells pre-exposed to HSV-1 and Vac^{tk-}, or left untreated, were incubated at various numbers with 3 x 10^5 responders. Anti-IA^d at 6.25 μg/well was included in the HSV-1 treated cultures.

Whereas anti-IA^d reduces the stimulatory effects of HSV-1 pretreated RT 2.3 cells to about the level of the allogeneic response, similar treatment of the A 20 B lymphomas only partially inhibits the proliferative response.

Discussion.

The results presented herein provide preliminary evidence of IA- and IE-restricted T cell responses in Balb/c mice. L cells transfected with either IA or IE and

Table I

Class II MHC-Restricted Lymphoproliferation

		[^{3}H]-TdR uptake (% Inhibition)[a]			
			HSV-1 +/- anti-IA[d]		
Stimltrs	No (x 10^5)	uninfected	-	+	Vac
RT 2.3	1	2026	19736	2712 (86)	2585
	2.5	5313	41612	8370 (80)	5911
	5	9795	79494	22380 (72)	10005
	10	15626	131347	43578 (67)	20130
A20	1	2137	23616	12618 (47)	3864
	2.5	7691	47761	28184 (41)	8009
	5	9921	84917	56524 (33)	11240
	10	16750	151597	105427 (30)	22613
LT-A	1	ND	3690	ND	ND
	2.5	ND	8773	ND	ND
	5	ND	16017	ND	ND
	10	ND	23278	ND	ND

Stimulators were infected with virus or left untreated for 6 hours prior to coculture with 3 x 10^5 responders. The assay was run for 12 hours.

[a] Values represent the mean of 6 replicates

subsequently primed with HSV-1 are potent stimulators of lymphoproliferative responses. Due to the otherwise disparate H-2 phenotype between the stimulators and the responder preparations, it is possible that the response is elicited by Ags other than IA or IE determinants. However, because HSV-1 sensitization of the stimulators appears to be a prerequisite for [^{3}H[-TdR uptake, the possibility seems rather unlikely. There is no evidence to suggest that any determinants other than MHC molecules are capable of presenting Ag (4).Furthermore, alloantigen specific T cells are accounted for by the response to LT-A (H-2^k) stimulators.

The apparent difference in the relative strength of the response to either IA$^+$ or IE$^+$ HSV-1 stimulators in favor of the former, may reflect relative levels of Ia expressed by the respective populations. Not shown are the results obtained from indirect fluorescence assays (IFA) in an attempt to characterize surface expression of IA^d. Greater than 90% of the cells exhibited significant punctate fluorescence when examined under UV-microscopy. Similar determinations with the IE$^+$ cells were not performed. The level of MHC class II surface expression notwithstanding, it remains possible that in Balb/c mice the majority of Ia-restricted, HSV-1-specific T lymphocytes, as assayed in proliferation experiments, may exhibit a predilection for IA^d determinants over those of IE^d.

Confirmation of the IA-restricted proliferative response was made possible through Ab inhibition experiments. Thus, anti-IA^d treatment of RT 2.3 cells reduces the response by as much as 86% in a dose dependent manner. Significantly, as little as 0.5 μg of Ab reduces [^{3}H]-TdR uptake by as much as 20%, with maximal inhibition occurring at approximately 6 μg of the Ab. In mathematical terms the dose response to anti-Ia is not surprising. Thus, 0.5 μg of anti-IA^d is the equivalent of approximately 1.5×10^{12} molecules of Ab per 5×10^5 stimulator cells. Considering the bivalency of

the Ab used, the number of antigenic sites available for binding at any particular instance may theoretically be no fewer than half of the total number of Ab molecules present, that is 7.5 x 10^{11} sites per culture well. The reason for this manipulation is the assumption that only one of the Ag sites per Ab molecule may spatially approximate its ligand at any one time.

Evidence suggests that the number of MHC proteins expressed on the surface of a cell generally ranges between 5 x 10^4 and 9 x 10^4 (25, 54). Therefore, at 5 x 10^5 stimulators per well the number of IA molecules may not surpass a total of 5 x 10^{10}. Hence, 0.5 μg of anti-IA should be sufficient to secure all available IA sites within the culture. However, other factors such as non-specific binding, the time of incubation, and the number of IA molecules recycled or synthesized anew, may interfere with the optimal inhibitory response.

Whether Ab induced inhibition is due to capping and internalization of Ia-Ab complexes,or the direct result of steric hindrance and/or competition for TcR epitopes, cannot be deduced from the present data. However, since HSV-1 pretreatment of the stimulator population is required for induction of [^{3}H]-TdR uptake, the mode of action of anti-IA^d moAb becomes immaterial to the argument.

Although not directly demonstrated, it appears that a portion of the proliferation elicited by A 20 cells may be attributed to class I-restricted T cells. Thus, whereas anti-IA perturbation of RT 2.3 cells results in a marked decrease in [^{3}H]-TdR-uptake, similar treatment of A 20 cells only marginally interferes with the proliferative response. Alternatively, the difference in the degree of inhibition may be attributed to the fact that whereas the responder and the RT 2.3 stimulator populations are histocompatible only in the IA locus of MHC, the A 20 B lymphomas and the lymphocyte cultures share similar profiles across the entire region of MHC. Therefore,

the residual response induced by A20 cells incubated with anti-IA^d may reflect the presence of the IE-resticted, HSV-1-specific responding population of cells. Indeed evidence for the existence of T cells restricted to IE is presented in Figure 1 Alternatively, the amount of IA expressed on the RT 2.3 and A20 cells available for cross linking with the TcR may differ significantly, in favor of the latter cell type.

Collectively, the results presented in this section provide unequivocal evidence in support of an Ia-restricted lymphoproliferative response, as has previously been shown (138, 139). The response is HSV-1 dependent, as neither uninfected nor Vac^{tk-} treated stimulator cells can induce freshly isolated lymph node preparations to proliferate. Furthermore, the response is at least partly restricted to IA, inasmuch as anti-IA^d moAb can inhibit [^{3}H]-TdR uptake by the cultures.

IV. Antigen Specificity for CTL Responses to Herpes Simplex Virus Type 1. Requirement for Infectious Virus

List of Abbreviations

CTL: cytotoxic T Lymphocytes

CMV: cytomegalovirus

HSV-1: herpes simplex virus type 1

OVA: ovalbumin

MOI: multiplicity of infection

Ag: antigen

[^{3}H]-TdR: tritiated thymidine

moAb: monoclonal antibody

CMI: cell mediated immunity

TCGM: T cell growth medium

Introduction

Many studies bear testimony to the role of T lymphocytes in the clearance of virally infected cells (100, 105). The response was initially described as one supported entirely by the CD8$^+$ T lymphocyte population, considered cytotoxic and/or suppressive in nature. The model was based on the hypothesis that surface marker profiles were strict correlates of the relative functions of T cells (16, 96). More recently however, it has become apparent that the simplicity implied in the model is not sacrosanct. Instead, it appears that the CD or Ly phenotype may actually reflect the

MHC restrictions of a particular cell (122, 139). Hence, functionally heterogeneous cells can be found within each subset population of T lymphocytes. Thus, class II-restricted CTL have been described in numerous systems, such as OVA (127), CMV (68), and HSV-1 (104, 138, 139). With few exceptions, the majority of the studies describing $CD4^+$, class II-restricted CTL are performed primarily with cloned cells (86, 119, 139), hence the argument that these CTL may represent an aberrant phenotype generated through in vitro pressures (29). However, as was demonstrated by Schmid, and Jacobson in human HSV-1 (104), and measles virus infections (49) respectively, $CD4^+$, class II-restricted T cells may indeed comprise a predominant CTL population.

The results reported in this section indicate that at least in the Balb/c strain of mice the $CD4^+$ T cell subset may account for a significant proportion of the overall CTL population.Furthermore, the immunogenic potential of UV-inactivated HSV-1 as well as the role of gC and gD in the CTL response were examined.

Materials and Methods

Animals.

Female Balb/c ($H\text{-}2^d$) mice were obtained from Jackson Laboratories and used at 4 to 8 weeks of age.

Viruses.

The KOS strain of HSV-1 was propagated on Vero cell monolayers and stored as infectious culture supernatant fluid. UV-inactivated virus (UV-HSV) was prepared by exposure of 0.5 ml of viral stock to a germicidal lamp (Sylvania Electric Products.)

at a distance of 3 cm for 2 minutes. The infectivity of the virus was thus reduced to less than 10% of the original stock. Vaccinia^{tk-} (Vac^{tk-}) and Vaccinia recombinants of HSV-1 glycoproteins gC (VgC) and gD (VgD) were constructed and propagated by E. A. Allen. The VgC construct was stored as purified virion preparations.

Cell Lines.

The L cell transfectants RT 2.3 (IA^d) and RT 10.(IE^d) were prepared and provided by R. N. Germain. The cells were maintained in continuous culture using the selective medium HAT (Sigma Chemical Co. Ltd.) in DMEM (Gibco-BRL), supplemented with 1% pen/strep, 10% HI FBS (Gibco-BRL) and 1% each of L-glutamine and non-essential amino acids. The day before any functional assays the cells were washed twice and HT (Sigma Chemical Co. Ltd.) was substituted for HAT.

The B lymphoma line A 20 was a kind gift of D. W. Horohov and originally characterized by Kim et al. (56). The cells were maintained in RPMI-1640 supplemented with 1% pen/strep and L-glutamine, 10% each of NCTC-109 medium and 10% HI FBS, and 5 x 10^{-5} mM 2-mercaptoethanol. EMT6 and LT-A.cell lines were maintained in DMEM supplemented with 1% pen/strep and L-glutamine, and 10% HI FBS.

Mouse Immunizations and Preparations of Lymph Node Cultures.

Four to 8 week old Balb/c mice received multiple injections of 5 x 10^6 $TCID_{50}$ of HSV-1 per site by various routes of inoculation. HSV-1 immune mice received a single injection in each of the hind leg footpads and the base of each ear in 0.04 ml of volume. At 5 days post-immunization the popliteal and retropharyngeal lymph nodes were excised and single cell suspensions of 4 x 10^6 cells/ml were cultured in six-well

cluster plates (Corning Glass Works) in a total volume of 5 ml of TCGM. The medium consisted of RPMI 1640 (Gibco-BRL) and NCTC-109 (Gibco-BRL) in a 1:1 ratio, supplemented with 1% pen/strep, 5 x 10^{-5} mM 2-mercaptoethanol (2-ME) and 10% HI FBS. The cell suspensions were incubated in a humidified 37° atmosphere, containing 5% CO_2, for 3 days in the absence of any restimulation. The effectors were subsequently used in CTL assays.

Determination of CTL Activity.

Targets were labelled with 250 μCi of [51Cr] per 2 x 10^6 cells and treated with or without HSV-1, UV-inactivated virus, VgC, or VgD at MOI of 5 or 10 in serum free medium for 4 hours at 37°. Controls consisted of uninfected or Vac^{tk-} infected cells. Following infection the various target groups were washed three times and resuspended in TCGM.

In parallel, lymph node suspensions of 3 day cultures were pooled in TCGM and used as the effector population at various numbers (as per assay) in 96 well V-bottom plates to which 1 x 10^4 targets were subsequently added. The plates were then centrifuged (200 g for 5 minutes) and incubated for an additional 4 hours. Radioactivity released into the medium was measured and specific cytotoxic activity determined using the formula: % specific release = (experimental - spontaneous) / (total - spontaneous) x 100%.Each determination was performed in replicates of 4 with spontaneous release values of below 10% and 20%. The results are expressed as the mean values of replicate wells.

Cold Target Competition Assay.

HSV-1 infected, [^{51}Cr]-labeled A 20 and EMT6 cells were mixed with unlabeled HSV-1 treated or untreated targets at a ratio of 1:5. CTL activity was determined as described above against 1 x 10^6 effectors

Results

Demonstration of HSV-1 Specificity and MHC Restriction of the CTL Response.

Perhaps the most direct manner of assessing the degree of clearance of HSV-1 infected cells is the [^{51}Cr]-release assay,inasmuch as the radioactivity released reflects the relative number of targets lysed. Prefatory experiments were designed to confirm the Ag specificity of CTL examined against a panel of targets sharing compatibility in either class II (RT 2.3, RT 10) or class I (EMT6) MHC determinants. Target populations expressing the products of both MHC loci (A 20) were included in an effort to evaluate the maximal response, MHC restrictions notwithstanding.

Typically targets were treated with or without HSV-1 (MOI:5) in the presence of $Na_2{}^{51}CrO4$. Four hours subsequent to incubation the cells were combined with various numbers of effectors. E/T ratios ranged from 12.5 to 100 effectors per target. The mixture of effectors and targets was then incubated for an additional 4 hours and the amount of [^{51}Cr] released measured. LT-A cells were used as allogeneic controls.

The results depicted in Table II are representative of 3 separate experiments. As shown, A 20 and EMT6 histocompatible targets are lysed in an HSV-1 dependent fashion Moreover, the extent of lysis is directly proportional to the number of effectors present. In contrast, neither IA^+ RT 2.3 nor IE^+ RT 10 transfected fibroblasts are recognized by the CTL population. Similarly, lysis of uninfected MHC

Table II

CTL Recognition of Target Cells Sensitized with Infectious HSV-1

Expt No	Targets	E/T	% [^{51}Cr]-release (std. dev)[a] uninfected	HSV-1 Infected
Expt 1				
	A 20	12.5	10.0 (2.6)[b]	54.1 (2.7)
		25.0	11.7 (2.1)	62.9 (1.5)
		50.0	16.5 (3.6)	68.4 (3.4)
		100.0	21.3 (3.6)	76.2 (3.2)
	EMT6	12.5	0.9 (1.0)	29.9 (3.8)
		25.0	1.9 (1.4)	43.4 (1.3)
		50.0	3.5 (1.9)	67 6 (3.1)
		100.0	9.1 (2.4)	85.0 (3.3)
	RT 2.2.3H	100.0	ND	3.6 (1.6)
	RT 10.3H2	100.0	ND	0.9 (0.7)
	LT-A	100.0	ND	5.2 (1.6)
Expt 2				
	A.20	5.0	1.4 (1.1)	33.5 (3.3)
		10.0	6.9 (2.7)	42.2 (2.4)
		20.0	12.4 (2.0)	56.0 (3.3)
		40.0	14.7 (1.7)	61.4 (3.2)
	EMT6	5.0	0.4 (0.8)	11.3 (1.8)
		10.0	0.5 (0.6)	19.2 (2.1)
		20.0	2.0 (1.4)	28.9 (1.4)
		40.0	5.4 (1.4)	44.3 (1.8)

Targets were infected with HSV-1 (MOI: 5) for 4 hours prior to coculture with the effector population. The assay was run for an additional 4 hours.

[a] Spontaneous release did not exceed 20%.

[b] Values represent percent [^{51}Cr] released and are expressed as the mean of 4 replicates.

compatible and HSV-1 treated allogeneic LT-A controls is not observed.

Comparison of Infectious and UV-Inactivated HSV-1 in Sensitizing Cells for CTL Recognition.

Lymph node preparations from HSV-1 immune mice were cocultured with a panel of targets pre-exposed to either infectious HSV-1 or UV-inactivated virus at MOI of 5 and 10, or no antigenic stimulation. HSV-1 treated LT-A cells were included as allogeneic controls. The E/T ratios tested were 50 and 100/1.

As demonstrated in Table III, UV-inactivation of HSV-1 dramatically reduces any stimulatory effects of the virus in sensitizing targets for CTL lysis. Whereas a dose of 5 $TCID_{50}$ of infectious HSV-1 renders cells susceptible to lysis by CTL, as much as 10 virions of UV-inactivated virus fail to do so. Neither MHC incompatible, nor IA compatible RT 2.3 fibroblasts constitute appropriate targets under the conditions tested. Similarly, as in Table II, uninfected A 20 and EMT6 cells do not elicit a CTL response whatsoever.

Relevancy of gC and gD HSV-1 Glycoproteins as Immunogens in the CTL Response.

To examine the possible roles of gC and gD in the overall CTL response to HSV-1, vaccinia recombinants encoding for the proteins were used to infect A 20 B lymphomas and EMT6 fibroblasts. Infection was allowed to proceed for 6 hours. Targets thus treated were subsequently incubated with primary lymph node cultures from HSV-1 immunized Balb/c mice. HSV-1 treated or untreated, and Vac^{tk-} infected cells served as positive and negative controls respectively, while LT-A cells constituted the allogeneic population.

Table III

CTL Recognition of Targets Sensitized with Infectious and UV-Inactivated HSV-1

		% [^{51}CR]-release (std. dev.)[a]				
			MOI			
			5.0		10.0	
Targets	E/T	uninfected	HSV-1	UV-HSV-1[b]	HSV-1	UV-HSV-1
A 20-	50.0	4.1 (2.5)	58.4 (4.0)	ND	ND	5.6 (2.6)
	100.0	13.9.(3.8)	76.3 (4.0)	ND	ND	15.9 (2.1)
EMT6	50	1.5 (0.5)	50.4 (3.6)	ND	ND	8.4 (0.6)
	100.0	6.9 (1.1)	83.3 (3.2)	ND	ND	17.3 (1.5)
RT 2.3	50	ND	1.8 (0.5)	0.1 (0.8)	1.4 (0.4)	0.5 (0.7)
	100.0	ND	5.3 (1.0)	4.1 (1.5)	4.4 (0.8)	3.6 (0.5)
LT-A	50	ND	5.8 (1.2)	ND	4.9 (0.9)	ND
	100.0	ND	12.1 (2.4)	ND	16.1 (1.2)	ND

Targets were treated with or without infectious or UV-inactivated HSV-1 at MOI shown for 4 hours. Effectors and targets were incubated for an additional 4 hours before measuring [^{51}Cr] released. Values represent the mean of 4 replicates.

a Spontaneous release was below 20%.

b HSV-1 was irradiated for 2 minutes under a UV lamp at a distance of 3 cm. MOI represents infectious units prior to UV-inactivation

The data presented in Table IV clearly indicate that neither gC nor gD can effectively sensitize cells for CTL recognition. Whereas HSV-1 treatment significantly enhances the susceptibility of A 20 and EMT6 cells, targets treated with either of the vaccinia constructs are only minimally killed by the effector population. However, under no circumstances does lysis of gC or gD sensitized targets exceed that of the uninfected controls.

Relative Contributions of Class II- and Class I-Restricted CTL in the Overall CTL Response to HSV-1.

Due to differences in the class of MHC determinants expressed by either A 20 or EMT6 cells, a possible means of assessing the relative strengths of the class II- versus the class I-restricted CTL response is the cold target competition assay. The experiment was performed under the assumption that unlabeled targets expressing only class I MHC determinants (EMT6) should only partially inhibit the CTL response against [^{51}Cr]-labeled cells expressing MHC determinants coded for by the entire H-2 region.

A 20 and EMT6 cells were treated with or without HSV-1 in the presence or absence of radioactive [^{51}Cr] for 4 hours. Subsequent to antigenic stimulation, 1 x 10^4 labelled and 5 x 10^4 unlabeled targets were incubated in the presence of 1 x 10^6 effectors for an additional 4 hours prior to [^{51}Cr]-release measurements. The ensuing cultures consisted of 1 x 10^6 effectors and a ratio of unlabeled to [^{51}Cr]-labeled targets of 5/1. Inhibition of lysis was calculated as the percent reduction in the release of [^{51}Cr] below that induced by unlabeled, HSV-1 untreated A 20 and EMT6 target cells.

Table IV

CTL Recognition of HSV-1 Glycoproteins gC [a] and gD [b]

		% [^{51}Cr]-release (% std. dev.), E/T: 100/1				
			Antigen			
Expt No	Targets	uninfected	HSV-1	Vac.gC	Vac.gD	Vac
Expt 1						
	A 20	14.1 (2.5)	75.6 (1.3)	5.8 (2.4)	15.5 (1.9)	2.8 (1.5)
	EMT6	8.6 (1.0)	91.2 (2.8)	7.1 (1.1)	9.8 (2.0)	2.7 (2.7)
	LT-A	ND	3.5 (1.1)	ND	ND	ND
Expt 2						
	A 20	6.7 (1.4)	52.5 (2.5)	9.2 (1.4)	4.9 (2.0)	ND
	EMT6	7.8 (2.3)	89.0 (4.0)	8.0 (2.2)	5.5 (0.6)	ND
	LT-A	ND	5.4 (0.4)	ND	ND	ND

Targets treated with any of the vaccinia constructs were infected for 6 hours. HSV-1 treated targets were infected for 4 hours. [^{51}Cr]-release assays were performed over 4 hours

[a] Vac.gC was purified prior to use.

[b] Vac.gD was used as cell exracts. No purification was performed.

As shown in Table V, whereas HSV-1 infected cold A 20 cells inhibit lysis of HSV-1 sensitized , [^{51}Cr]-labeled A 20 targets by almost 30%, similarly treated EMT6 fibroblasts reduce the CTL response by less than 20%. On the other hand, inhibition of lysis of radioactively labelled and virally infected EMT6 targets by either of the HSV-1 sensitized, unlabeled cell types is of a similar magnitude.

Discussion

It is well established that protection and recovery from HSV-1 infection is a function of thymus derived cells (73, 91,104). Furthermore, the phenomenon of MHC restriction first reported by Doherty et. al. in 1976 (24) was expanded to apply to herpesviruses as well (44, 68, 85, 92).

The initial experiments reported herein were primarily designed under the assumption that identification of class I- or class II-restricted CTL might be reflected in the relative degree of lysis observed against targets expressing either class I, class II, or a combination of class I and II MHC determinants. Thus, the population of targets used in the assays performed consisted of cells with effector cell histocompatibility in either class I (EMT6) or class II (RT 2.3, RT 10) MHC. Additionally, with respect to the A 20 cell population, compatibility with CTL spans the entire H-2 region (56, R. Asofsky personal communication).

Cytotoxicity induced by the effector population in all instances was strictly dependent on HSV-1 as the stimulating Ag. Thus, in keeping with previous reports (66, 73, 75), targets that were either uninfected or treated with vaccinia failed to elicit a CTL response. Furthermore, lysis of allogeneic, HSV-1 infected LT-A fibroblasts was comparatively identical to that of uninfected controls.

Table V

Cold Target Competition Assay

		% [^{51}Cr]-Released. (% Inhibition) [^{51}Cr]-labelled Targets [a]	
Cold Targets	Ag	A 20+HSV-1	EMT6+HSV-1
A 20	none	58.4	ND
	HSV-1	40.9 (30.0)	42.8 (23.6)
EMT6	none	ND	55.3
	HSV-1	47.2 (18.0)	40.7 (25.4)

Cold targets treated with or without HSV-1 were mixed with [^{51}Cr]-labeled, HSV-1 treated syngeneic cells. The mixture was incubated with 1 x 10^6 effectors for an additional 4 hours.

[a] The ratio of cold to hot targets was 5/1.

Although the amount of [^{51}Cr] released by HSV-1 treated A 20 and EMT6 targets at E/T ratios in excess of 50/1 was of virtually identical proportions, results at E/T ratios of 40/1 or below clearly indicate a dichotomy. In fact, the smaller the ratio of effectors to targets the more pronounced the difference becomes. Thus, whereas the percentage of [^{51}Cr] released by A 20 cells at an E/T ratio of 20/1 exceeds 55% of the total attainable, the response against similarly treated EMT6 cells barely reaches the 30% mark. The consistency of the observation may indicate that the difference observed in the relative amounts of radioactivity measured is a reflection of the total numbers of MHC molecules per target population engaged in Ag presentation, and as a consequence, the aggregate number of cells responding. Hence, pronounced lysis in favor of A 20 lymphomas at low E/T ratios may represent the cumulative effects of class I- and class II-restricted CTL. By comparison, E/T ratios above 50/1 may assimilate sufficient numbers of class I-restricted effectors to obscure the discrepancy. Alternatively target susceptibility to lysis may differ significantly between the two cell types, thus biasing the system in favor of the A 20 cells.

Paradoxically, both IA$^+$ RT 2.3, and IE$^+$ RT 10 L cell derivatives failed to elicit a CTL response in all cultures tested. Whether this phenomenon signifies an inherent or acquired capacity of such cells to neutralize the lytic signals generated by CTL is not known. Arguably, susceptibility or resistance to CTL lysis is not an assumption unique to the present study. Similar results have been reported by Tite et al. (129) in a study of OVA specific, Ia-restricted CTL , and Hayward et al. (44) in research with human VZV infections. Alternatively, although IA and IE transfected cells are potent stimulators in lymphoproliferation assays in an Ia-dependent manner, it is plausible that epitopes for either IA or IE-restricted CTL are not generated during HSV-1 infections. If the latter hypothesis were true, then it must be presumed that

murine HSV-1 specific CTL, unlike their human equivalents (104, 138, 139), display no class II-restricted killing. Consequently, lysis of A 20 B lymphomas reported thus far must be interpreted as one supported entirely by the class I MHC-restricted CTL population, a probability not forthcoming. Perhaps a more acceptable explanation might be to assume an inherent enzymic deficiency skewed against generation of CTL epitopes and of no significance to proliferating T cells.

In an attempt to justify the apparent lack of CTL recognition of IA^+ and IE^+, HSV-1 infected targets, efforts were undertaken to bias the system in favor of a class II-restricted CTL response. Results obtained from research with influenza suggest that class II-restricted CTL recognition of targets is independent of viral infectivity (45, 84). Thus, UV-inactivated, non-infectious influenza viral particles can effectively sensitize targets for lysis in an Ia-restricted manner, and in so doing dispensing with the requirement for de novo protein synthesis of viral Ags. It was thereafter proposed that the difference between class I- and class II-restricted recognition of targets is predicated by the processing pathway involved in the generation of CTL epitopes (33).

Based on these assumptions, a comparison was drawn between the respective capacities of either UV-inactivated or infectious HSV-1 virions in sensitizing cells for CTL recognition. The requirement for infectious HSV-1 in the inductive phase of a CTL response has been demonstrated elsewhere (67, 105). The apparent failure of UV-inactivated virus to immunize mice was moreover assumed to reside in the limitations posed by the experimental designs used, inasmuch as class II-restricted CTL were not examined (73). As the present report indicates, UV-irradiation completely ablates HSV-1 immunogenicity, even at the level of target cell sensitization, regardless of the MHC restriction requirements of the effector population. Thus, contrary to influenza-specific CTL (15, 84), recognition of HSV-1

treated targets seems entirely dependent on infectious virus. Perhaps association of Ia and UV-inactivated HSV-1 is not conducive to CTL induction and recognition. Alternatively, UV-treated HSV-1 may preferentially activate suppressor cells in the culture system. However, because the effector population was not restimulated in vitro with any form of the Ag, be it infectious or non-infectious, immunoregulation would imply in vivo activation of suppressor cells. If such a scheme were plausible, the results obtained with infectious HSV-1 treated targets might be difficult to reconcile. It remains possible nonetheless, that in vivo generated suppressors may require a secondary stimulus, provided by the UV-HSV-1 treated targets before becoming fully functional. As seductive as this hypothesis may seem, it appears unlikely, inasmuch as UV-irradiation, contrary to heat inactivation of HSV-1, has not been shown to induce suppressor activity (47)

Although antigenic specificity of CTL against HSV-1 is without dispute, identification of target Ags involved in the response has been at best a difficult task. Nevertheless, both structural (38, 66, 98)as well as nonstructural (73, 75) HSV-1 proteins are currently regarded as target Ags for $CD8^+$ CTL. Additionally,gD was recently implicated in class II-restricted CTL responses (73). It was therefore of interest to describe a correlate in the Balb/c system. Unfortunately however, as the data indicate, neither gC- nor gD-specific, class I- or class II-restricted CTL were detected. Thus, contrary to a secondary CTL response in C3H/HeJ ($H\text{-}2^k$) mice (73) gD does not appear to constitute a target for primary CTL in Balb/c mice. As the gD preparations used in the two studies were of the same viral stock, it is unlikely that the results reported herein reflect technical limitations. Similarly, inasmuch as the mice were immunized with HSV-1, suppression of the response by VgD, as reported elsewhere (74), is seemingly improbable.

Failure of purified VgC to elicit Ia-restricted CTL may indeed reflect a limitation inherent in the preparation of targets. It is possible that endogenously synthesized gC is inappropriately processed for recognition by Ia-restricted CTL. Such is certainly the case with HA-specific, Ia-restricted CTL (84). Moreover, inability to detect a class I-restricted response against gC may suggest that the effectors are altogether absent.

Collectively, the results presented in the preceding section clearly underline the importance of infectious HSV-1 in sensitizing targets. Furthermore, the overall response, as implied by the data in tables II and V, appears to represent the cumulative effect of class I- and class II-restricted CTL. Finally, neither gC nor gD seems to elicit major CTL populations.

V. Demonstration of a Class II MHC-Restricted CTL Response in Balb/c Mice

List of abbreviations

CTL: cytotoxic T lymphocytes

TcR: T cell receptor

E/T: effector to target ratio

HSV-1: herpes simplex virus type 1

moAb: monoclonal antibody

Ag: antigen

HI FBS: heat inactivated fetal bovine serum

Introduction

In mice and man alike cytotoxic effector cells are derived from either of two T cell populations termed $CD4^+$ and $CD8^+$ on the basis of phenotypic characteristics (23, 95, 122). Furthermore, target Ags recognized by the individual subsets are primarily epitopes associated with distinct MHC determinants. Thus, $CD4^+$ CTL preferentially kill targets bearing class II MHC proteins, whereas CTL expressing CD8 restrict their effects to cells positive for class I MHC determinants (107, 121).

Whether CD4 or CD8 molecules act as recognition elements, or alternatively represent components of the lytic mechanism is a subject characterized by considerable uncertainty in the literature to date. Whereas it was initially proposed that these proteins serve solely as adhesion molecules (26, 80, 124), it is currently believed that

their dynamics, and in particular those of the CD4 molecule, include signal transduction (51, 53) suggesting their participation in TcR triggering. The role of CD4 in the differentiation and activation of T lymphocytes notwithstanding, its mere expression provides a powerful means of characterizing the T cell subsets involved in the responses studied.

Overwhelmingly, most of the research regarding the function of CD4 has relied on clonal situations. Despite the significance of T cell clones, the relevance of studies utilizing clonally derived cells to in vivo circumstances remains controversial.

The principal goal of the work presented in this section is to qualitatively distinguish the individual T cell populations involved in the CTL response against murine HSV-1 infected targets. Monoclonal Ab to CD4, CD8, and IA^d were used in inhibition assays, and the requirement for cognate effector-target interactions was examined.

Materials and methods

Animals.

Female Balb/c ($H\text{-}2^d$) mice were obtained from Jackson Laboratories and used at 4 to 8 weeks of age.

Viruses.

The KOS strain of HSV-1 was propagated on Vero cell monolayers and stored as infectious culture supernatant fluid. The virus was used at an MOI of 5 during a 4 hour infection period.

Cell Lines and Antisera.

The B lymphoma line A 20 was originally characterized by Kim et al. (56).and provided by D. W. Horohov. The cells were maintained in RPMI 1640 supplemented with 1% pen/strep and L-glutamine, 10% each of NCTC-109 medium and 10% HI FBS, and 5 x 10^{-5} mM 2-mercaptoethanol. EMT6 and LT-A.cell lines were maintained in DMEM supplemented with 1% pen/strep and L-glutamine, and 10% HI FBS.

The antisera 2.43 (IgG_{2b}) and GK 1.5 (IgG_{2b}) were used as sources of anti-Ly 2.2 and anti-CD4 respectively in the form of ascites from pristane primed and X-irradiated Balb/c mice. Anti-IA^d (IgG_{2a}) was purchased from Beckton Dickinson Immunocytometry Systems. Unless otherwise indicated, GK 1.5 and 2.43 moAb were used at 12.5 µg per well of culture. Anti-IA^d was used at 6.25 µg per 1 x 10^4 targets.

Mouse Immunizations and Preparations of Lymph Node Cultures.

Four to 8 week old Balb/c mice were injected with 5 x 10^6 $TCID_{50}$ of HSV-1 per site by various routes of inoculation. HSV-1 immune mice received a single injection in each of the hind leg footpads and the base of each ear in 0.04 ml volume. At 5 days post-immunization the popliteal and retropharyngeal lymph nodes were extracted and single cell suspensions of 4 x 10^6 cells/ml were cultured in six-well plates (Corning Glass Works) in a total volume of 5 ml of TCGM. The medium consisted of RPMI 1640 (Gibco-BRL) and NCTC-109 (Gibco-BRL) in a 1/1 ratio, supplemented with 1% pen/strep, 5 x 10^{-5} mM 2-mercaptoethanol (2-ME) and 10% HI FBS. The cell suspensions were incubated in a humidified 37° atmosphere, containing 5% CO_2, for 3 days in the absence of any restimulation The effectors were

subsequently used in CTL assays.

Determination of CTL Activity.

Targets were labelled with 250 μCi of [^{51}Cr] per 2 x 10^6 cells and treated with or without HSV-1 in serum free medium for 4 hours at 37°. Controls consisted of uninfected and LT-A HSV-1 treated allogeneic cells. Following infection the various target groups were washed three times and resuspended in TCGM.

Lymph node suspensions of 3 day cultures were pooled in TCGM and used as the effector population at various numbers (as per assay) in 96 well V-bottom plates to which 1 x 10^4 targets were added. The plates were then centrifuged (200g for 5 minutes) and incubated for an additional 4 hours. Radioactivity released into the medium was measured and specific cytotoxic activity determined using the formula:

% specific release = (experimental - spontaneous) / (total - spontaneous) x 100%

Each determination was performed in replicates of 4, with spontaneous release values between 10% and 20%. The results are expressed as the mean values of replicate wells.

Determination of Cognate Interactions; Innocent Bystander Assay.

LT-A allogeneic [^{51}Cr]-labeled cells at 1 x 10^4 prepared as described above, were mixed in a 1/1 ratio with unlabeled HSV-1 infected or uninfected MHC compatible A 20 lymphomas or EMT6 fibroblasts. The mixture was subsequently incubated with 1 x 10^6 effectors at 37°. Radioactivity released was measured as per CTL assay.

Results

Effect of Anti-IA on CTL Target Recognition.

The inhibitory effect of anti-IA on CTL target recognition was examined by preincubating A 20 and EMT6 HSV-1 treated cells with anti-IA^d moAb at 4° for 30 minutes prior to 4 hour [^{51}Cr]-release assays. Routinely the Ab was used at a concentration of 6.25 μg per 1 x 10^4 targets. Normal mouse serum controls.(n.s).at a 1/10 dilution were used as the reference for maximal cell mediated lysis. Percent inhibition was calculated as the percentage reduction in the radioactivity measured below that released by n.s. treated targets.

As shown in Table VI, anti-IA treatment results in partial inhibition of the CTL response against A 20 cells, whereas it only minimally affects CTL recognition of EMT6 targets. Inhibition of CTL lysis of A 20 cells ranged between values of below 29% and over 60% in the two experiments presented. The degree of inhibition against EMT6, HSV-1 infected cells at no time exceeded 11% and is of no statistical significance. Inhibition was consistently pronounced at E/T ratios below 50/1, perhaps reflecting relative numbers of class I- and class II-restricted CTL in the cultures.

Effect of Anti-CD8 on CTL Target Recognition.

To confirm the significance of $CD8^+$ T cells in the murine CTL response to HSV-1, anti-CD8 Ab was used in CTL inhibition assays. Briefly, effector populations from acutely infected Balb/c mice were preincubated with 12.5 μg of anti-CD8 ascites fluid for 30 minutes in 96 well plates to which 1 x 10^4 HSV-1 treated A 20 and EMT6 cells were transferred. E/T ratios of 6.25 to 50/1 were .

Table VI

Effect of anti-IA^d on CTL Recognition of HSV-1 Infected Targets

			% [^{51}Cr]-release (% Inhibition)[a]	
			Antisera	
Expt No.	Targets	E/T	n.s.	anti-IA^d [b]
Expt 1				
	A 20+HSV-1	12.5	47.3	26.9 (43.2)
		25.0	54.0	29.8 (44.9)
		50.0	61.9	43.9 (29.0)
		100.0	71.1	49.0 (31.0)
	EMT6+HSV-1	12.5	14.9	14.1 (5.0)
		25.0	35.9	33.1 (7.7)
		50.0	50.7	49.0 (3.4)
		100.0	63.0	65.4 (-3.8)
Expt 2				
	A 20+HSV-1	5.0	10.3	4.1 (60.1)
		10.0	17.0	9.2 (46.1)
		20.0	25.8	15.7 (39.3
		40.0	42.1	20.5 (51.3)
	EMT6+HSV-1	5.0	9.2	8.2 (10.1)
		10.0	19.0	17.2 (9.6)
		20.0	27.0	23.6 (12.6)
		40.0	35.7	32.1 (1.9)

[a] Spontaneous release did not exceed 20%.

[b] Targets were preincubated with 6.25 μg of anti-IA or 1/10 dilution of normal mouse serum.

evaluated for the amount [^{51}Cr] released.

Whereas anti-CD8 treatment of effectors abrogates the response to EMT6 targets it only partially inhibits lysis of the A 20 target population (Table VII). Lysis of EMT6 cells was reduced by over 83%,compared to an average reduction of approximately 60% in the response against A 20 cells. Moreover, the residual lysis of HSV-1 treated EMT6 targets only marginally exceeds that measured against uninfected controls.

Effect of Anti-CD4, or a Combination of Anti-CD4 and Anti-IA on CTL Function.

To evaluate the relative contribution of the CD4$^+$ T cell population in the overall CTL response to HSV-1, primary lymphocyte preparations were treated with anti-CD4 (12.5 μg) at 3 days post initiation of culture. Furthermore, A 20 and EMT6 cells were preincubated with or without anti-IA^d (6.25 μg per 1 x 10^4 targets). Effectors and targets were then combined at an E/T ratio of 80/1 and the mixture incubated for 4 hours prior to measuring radioactivity released.

As both experiments presented in Table VIII indicate, anti-CD4 treatment inhibits CTL function against targets expressing a combination of class I and class II MHC determinants. On the other hand its effect on class I histocompatibble cells is marginal at best. The inhibitory effects of anti-CD4 were more pronounced in the presence of anti-IA treated A 20 cells, whereas similarly treated EMT6 targets exhibited essentially no increased sensitivity to inhibition. Whether anti-CD4 and anti-IA act cumulatively or synergistically cannot be determined at the present time.

Table VII

Effect of anti-CD8 [a] on the Recognition of HSV-1 Treated Targets

		% [^{51}Cr]-release (% Inhibition) [b]		
			HSV-1 +/- anti-CD8	
Targets	E/T	uninfected	-	+
A 20	6.3	3.1	44.9	12.2 (72.8)
	12.5	3.7	49.8	17.6 (64.7)
	25.0	3.8	61.1	24.1 (61.0)
	50.0	7.2	73.7	32.6 (55.8)
EMT6	6.3	1.2	10.6	0.8 (92.5)
	12.5	2.2	25.5	3.7 (85.5)
	25.0	5.8	39.8	6.3 (84.2)
	50.0	8.1	75.7	12.6 (83.4)

[a] Anti-CD8 was used at 12.5 µg/well of culture.

[b] Standard deviation ranged between 1 and 4.

Table VIII

Effect of anti-CD4, or a Combination of anti-CD4 and anti-IA^d on CTL Recognition of HSV-1 Treated Cells

		% [^{51}Cr]-release (% Inhibition) [a]		
		Antisera		
Expt No	Targets	n.s.	anti-CD4 [b]	anti-CD4+anti-IA^d [c]
Expt 1				
	A 20+HSV-1	70.0	49.2 (29.5)	37.1 (46.7)
	EMT6+HSV-1	75.5	68.7 (9.0)	66.2 (12.2)
Expt 2				
	A 20+HSV-1	68.6	42.7 (37.8)	38.6 (43.7)
	ENT+HSV-1	69.1	64.2 (7.1)	59.4 (14.1)

[a] The E/T ratio usedwas 80/1.

[b] Anti-CD4 was used at 12.5 μg per effector population.

[c] Cultures treated with anti-CD4 and anti-IA received 12.5μg and 6. 25 μg of the antibodies respectively.

Dose Response Kinetics of Anti-CD4.

Considering the relatively small concentration of anti-CD4 adequate in inhibiting the CTL response by as much as 38%, it was of interest to examine the dose response kinetics and thus determine the amount of Ab required for maximal inhibition. Hence, various concentrations of anti-CD4 were preincubated with 5 x 10^5 effectors and the mixture was subsequently incubated with 1 x 10^4 HSV-1 treated targets.

The data presented in Table IX indicate that as little as 1.56 µgs of anti-CD4 results in approximately 27% inhibition of lysis against A 20 targets. A plateau is reached at 12.5 µgs of the Ab. Surprisingly, the effect appears to be reversible, inasmuch as concentrations in excess of 50 µg per 5 x 10^5 effectors has no effect on CTL lysis. In contrast to the CTL response against A 20 cells, the response to EMT6 targets does not appear sensitive to any of the anti-CD4 concentrations examined.

Requirement for Cognate Effector-Target Interactions.

To determine the means by which a lethal CTL signal is directed, innocent bystander assays were performed. A 1/1 ratio of [^{51} Cr]-labelled H-2^k and unlabeled H-2^d targets was added to various numbers of effectors. Unlabeled targets were divided into groups of HSV-1 treated and untreated controls.

In none of the experiments performed did lysis of H-2^k, MHC incompatible targets exceed 17% at the highest E/T ratios tested (Table X). Moreover, the difference between A 20 and EMT6 stimulated effectors was minimal. Similarly, HSV-1 pulsing of the H-2^d targets appears to be of no significance in CTL lysis of H-2^k [^{51}Cr]-labeled targets. Therefore the CTL response studied thus far is presumed to occur via cognate, cell to cell contact interactions.

Table IX

Dose Response Kinetics of anti-CD4

		% [^{51}Cr]-release (std. dev.)	
		Targets	
Expt No	[anti-CD4] [a]	A 20+HSV-1	EMT6+HSV-1
Expt 1			
	0.0	58.7 (5.0)	55.7 (3.2)
	1.6	43.1 (2.2)	53.5 (2.5)
	3.1	38.9 (3.6)	50.5 (3.6)
	6.3	33.5 (3.2)	53.0 (2.7)
	12.5	31.6 (3.7)	53.3 (4.1)
	25.0	33.1 (1.4)	52.6 (2.4)
	50 0	30.1 (2.3)	52.9 (3.3)
Ept 2			
	0.0	67.4 (2.4)	44.1 (3.9)
	12.5	41.3 (2.3)	43.9 (2.5)
	25.0	42.7 (1.2)	40.2 (1.9)
	50.0	45.6 (3.7)	41.1 (1.0)
	100.0	65.1 (2.6)	42.8 (1.3)

Effectors were pretreated with anti-CD4 at 4° for 30 minutes before addition of targets.

[a] In μg per 5 x 10^5 effectors.

Table X

Innocent Bystander Assay [a]

		% [^{51}Cr]-release (std. dev.) LT-A (H-2^{k}) labelled Targets E/T ratio			
Cold Targets	Ag	12.5	25.0	50.0	100.0
A 20	none	2.4 (1.1)	6.3 (2.0)	6.2 (1.2)	12.6 (1.5)
	HSV-1	6.6 (0.6)	8.1 (1.9)	12.3 (0.8)	16.9 (1.3)
EMT6	none	2.3 (0.7)	2.2 (0.4)	2.9 (0.1)	7.4 (0.4)
	HSV-1	0.6 (0.6)	3.6 (0.5)	4.6 (1.2)	10.3 (1.9)

Cold H-2^{d}, HSV-1 treated or untreated targets were incubated with effectors (E/T: 100/1) in the presence of [^{51}Cr]-labeled allogeneic LT-A cells (ratio of cold/hot: 1/1).

[a] Data are reprenentative of 3 experiments.

Discussion

CTL inhibition assays were performed to dissect the relative contribution of $CD4^+$ and $CD8^+$ T lymphocytes in the overall CTL response directed against HSV-1 infected cells. The target populations consisted of cells expressing either class I or a combination of class I and II MHC determinants. It is concluded that a considerable number of HSV-1-specific CTL express CD4 and are restricted by IA^d.

Although the functional significance of the CD4 molecule was not examined, its participation in the CTL recognition of class II bearing targets is amply demonstrated. As much as 40% of the total CTL response is liable to anti-CD4 treatment. It is not known whether this percentage represents actual CTL numbers involved, and it would be unwise to assume as such.

In more than one instance the effect of CD4 perturbation attests to the variable significance of the protein in T cell function. Anti-CD4 treatment of T lymphocytes has produced results ranging from complete inhibition of function(5), to enhanced CTL activity (18), or no observable effects whatsoever (116). To explain this phenomenon two complementary models have been proposed. The "accessory model" hypothesis states that CD4 binds to class II MHC determinants in a manner independent of the TcR, thus strengthening conjugate formation between targets and effector T cells (10, 72). Alternatively, evidence exists to suggest that the role of CD4 transcends mere adhesion properties and may actually involve signal transduction (11, 101, 134).To compensate for the apparent dichotomy between the two hypotheses Janeway (51) has proposed the "co-receptor" model of CD4 function. Thus, cells that require only low levels of Ag, otherwise regarded as highly reactive, possibly due to a requirement for fewer TcR signals and thus of a low activation threshold, may only minimally be

inhibited by anti-CD4 (10, 78, 80, 124).Therefore, it cannot at the present time be deduced whether percent inhibition of lysis reflects actual numbers of CTL involved, inasmuch as the effector population tested consists of cells presumably of variable specificities, and perhaps of significant differences in their affinities to Ag.

Unfortunately the results obtained with anti-CD4 lacked consistency. It is possible that Ag specificity may also dictate liability to CD4 perturbation, in that Ag-TcR affinities may vary considerably among individual cells. Therefore, inasmuch as the CTL populations studied were derived from primary bulk lymph node preparations, it would be fallible to assume homogeneity. Paradoxically, anti-CD4 concentrations in excess of 50 μg per 5 x 10^5 effectors diminished the inhibitory effects of the Ab. It is conceivable that anti-CD4 already bound to T cells may actually engage A 20 cells via interactions with Fc receptors, thereby stabilizing effector-target conjugates. Concomitantly, anti-CD4 bound to Fc receptors becomes for all practical purposes solid phase Ab, and may consequently promote cross linking of CD4 and TcR, believed to be a potent stimulus in T cell activation (101). This hypothesis appears attractive based on the requirement for Ia expression. Thus, whereas 100 μg of anti-CD4 renders CTL just as effective as untreated controls against Ia^+, HSV-1 infected A 20 cells, similar concentrations of the Ab does not affect the response to HSV-1 treated EMT6 cells. Consequently, the effect of high doses of anti-CD4 cannot be due to random activation of $CD4^+$ CTL, as observed elsewhere in the absence of Ia (18).

Treatment of cultures with anti-IA^d added further support to the suspected role of the $CD4^+$ T cell population in HSV-1 specific CTL responses. The amount of anti-IA administered was based on the dose response kinetics of the Ab in proliferation assays. The assumption was that anti-IA concentrations adequate to inhibit T cell

proliferation over a period of 12 hours should suffice to interfere with the effects of IA-restricted CTL over 4 hours of incubation. Anti-IA^d specifically inhibits the CTL response against A 20 cells, whereas the response to HSV-1 infected EMT6 fibroblasts remains virtually unperturbed. Therefore, it is not plausible that inhibition of CTL lysis is due to non-specific effects of the Ab on the lymphocyte population. Furthermore, non-specific reduction of target lysis is accounted for by normal serum controls,inasmuch as they represent the reference point for maximal CTL activity.

Inhibition was more pronounced at low E/T ratios, perhaps as a reflection of the respective numbers of class I- and class II-restricted CTL, and/or the relative susceptibilities of the targets to lysis. As E/T ratios begin to approach the 100/1 level, the number of class I-restricted CTL may rise significantly to compensate for the IA-restricted population being inhibited by anti-Ia.

As several reports with $CD4^+$ CTL indicate an optional requirement of cell-cell contact for delivery of the lethal hit (127, 128, 129), it was of interest to examine the possibility of bystander killing in the present study. As is shown in Table X lysis of targets is not caused by soluble mediators. Neither A 20 nor EMT6 HSV-1 treated, MHC compatible targets sensitize CTL for lysis of $H\text{-}2^k$ incompatible, [^{51}Cr]-labeled cells to any degree above that of the uninfected controls

The data presented herein provide strong evidence for a significant HSV-1-specific, $CD4^+$, IA-restricted CTL population. Both anti-CD4 and anti-IA treatments considerably reduce the degree of cytotoxicity against HSV-1 infected A 20 cells. On the other hand anti-CD8 only partially inhibits the response against A 20 cells, whereas it completely abrogates recognition of EMT6, HSV-1 treated targets.

VI. Evaluation of the Importance of Protein Synthesis Mechanisms and Acidified Intracellular Compartments in the Processing Step of HSV-1 Presentation.

List of Abbreviations

APC: antigen presenting cells

CTL: cytotoxic T lymphocytes

HSV-1: herpes simplex virus type 1

gD: glycoprotein D

HA: haemagglutinin

TCGM: T cell growth medium

Introduction

Presentation of Ag to class II MHC-restricted T cells is an active and time dependent process involving the acidified intracellular environment of the endosomes(141). Furthermore, processing of Ag in the endosomal compartment is shown to be chloroquine sensitive (84). These requirements suggested that Ag seen by T cells restricted by class II MHC determinants is indeed degraded forms of the proteins. The assumption was confirmed by demonstrating that proteins enzymatically degraded through in vitro manipulations can be presented by cells bearing Ia molecules and otherwise metabolically inactive (110).

The model of endogenous versus exogenous processing, initially proposed by Morrison et. al. (84), was subsequently modified to account for similarities between

class I- and class II-restricted T lymphocytes in studies with influenza. Thus, work by Townsend et al. (130) convincingly demonstrated that, not unlike Ia-restricted CTL, T cells exhibiting class I MHC restrictions may recognize synthetic peptides generated in vitro, therefore arguing against the presumed necessity for de novo synthesis of proteins.

With the exception of the work by Schmid (104), very little evidence exists to support the in vivo significance of $CD4^+$, Ia-restricted HSV-1 specific CTL. Much inference has relied on clonal situations (138, 139). Moreover, practically no murine correlate has been established, primarily due to lack of appropriate research, inasmuch as most assays performed neglect to include Ia^+ APC or targets. A notable exception was the demonstration of gD specific,Ia-restricted CTL in C_3H/HeJ mice in a study by Martin et. al. (73).

The work presented hereafter was designed to supplement the results of the preceding sections. Chloroquine as well as emetine alone or in conjunction with anti-CD4 treatments were performed to evaluate the presumed roles of endosomal processing and de novo protein synthesis as they relate to HSV-1 specific recognition of targets.

Materials and methods

Animals.

Female Balb/c ($H\text{-}2^d$) mice were obtained from Jackson Laboratories and used at 4 to 8 weeks of age.

Viruses.

The KOS strain of HSV-1 was propagated on Vero cell monolayers and stored as infectious culture supernatant fluid. The virus was used at an MOI of 5 during a 4 hour infection period.

Cell Lines and Antisera.

The B lymphoma line A 20 was originally characterized by K J. Kim (56).and provided by D. W. Horohov. The cells were maintained in RPMI 1640 supplemented with 1% pen/strep and L-glutamine, 10% each of NCTC-109 medium and 10% HI FBS, and 5 x 10^{-5} mM 2-mercaptoethanol. EMT6 and LT-A.cell lines were maintained in DMEM supplemented with 1% pen/strep and L-glutamine, and 10% HI FBS.

The antiserum GK 1.5 (IgG_{2b}) at 12.5 µg per culture was used as a source of anti-CD4.

Mouse Immunizations and Preparations of Lymph Node Cultures.

Four to 8 week old Balb/c mice were injected with 5 x 10^6 $TCID_{50}$ of HSV-1 per site by various routes of inoculation. HSV-1 immune mice received a single injection in each of the hind leg footpads and the base of each ear in 0.04 ml volume. At 5 days post-immunization the popliteal and retropharyngeal lymph nodes were excised and single cell suspensions of 4 x 10^6 cells/ml were cultured in six-well plates (Corning Glass Works) in a total volume of 5 ml of TCGM. The medium consisted of RPMI 1640 (Gibco-BRL) and NCTC-109 (Gibco-BRL) in a 1/1 ratio, supplemented with 1% pen/strep, 5 x 10^{-5} mM 2-mercaptoethanol (2-ME) and 10% HI FBS. The cell suspensions were incubated in a humidified 37° atmosphere, containing 5% CO_2,

for 3 days in the absence of any restimulation The effectors were subsequently used in CTL assays.

Determination of CTL Activity.

Targets were labelled with [^{51}Cr] (μCi amounts as per assay) and treated with or without HSV-1 in serum free RPMI 1640 for 4 hours at 37°.Controls consisted of uninfected cells. Following infection the various target groups were washed three times and resuspended in TCGM.

Lymph node suspensions of 3 day cultures were pooled in TCGM and used as the effector population at various numbers (as per assay) in 96 well V-bottom plates to which 1 x 10^4 targets were added. The plates were then centrifuged (200g for 5 minutes) and incubated for an additional 4 hours. Radioactivity released into the medium was measured, and specific cytotoxic activity determined using the formula:
% specific release = (experimental - spontaneous) / (total - spontaneous) x 100%

Each determination was performed in replicates of 4, with spontaneous release values between 10% and 20%. The results are expressed as the mean values of replicate wells.

Cytotoxicity Assays Performed in the Presence of Chloroquine.

A 20 and EMT6 targets removed from culture flasks were washed once in serum free RPMI 1640 in the presence or absence of 50 μM chloroquine (Sigma Chemical Company). Subsequently targets were treated with or without HSV-1 at an MOI of 5 and incubated in the presence of 250 μCi [^{51}Cr] with or without 50 μM chloroquine at 37°. At 4 hours post infection targets were washed 3 times in serum free RPMI 1640 alone or containing 50 μM chloroquine The targets were then counted

and resuspended in TCGM alone or in the presence of 20 μM chloroquine at a concentration of 2 x 10^5 cells/ml. Fifty μl of target cell suspensions were then added to V-bottom plates and mixed with various numbers of effectors to yield a final volume of 200 μl/well. The 1/4 dilution of the targets in the assay wells resulted in a final chloroquine concentration of 5 μM. Thus targets to be treated with chloroquine were maintained in the presence of the agent from the onset of infection, thereon.

Anti-CD4 inhibitions were performed by pretreating effectors with 12.5 μg of the Ab for 30 minutes at 4^o, before addition of targets.

The Role of Protein Synthesis in Target Sensitization.

For experiments designed to elucidate the effect of protein synthesis in the recognition of targets by CTL A 20 and EMT6 cells were washed once in serum free RPMI 1640 alone or in medium containing 50 μM emetine dihydrochloride (Sigma Chemical Company). The targets were then infected with HSV-1 (MOI:5) and labelled with 300 μCi [^{51}Cr].Emetine treated targets were maintained in 50 μM concentration of the agent throughout the infection period. All targets were subsequently washed 3 times in serum free RPMI 1640 devoid of emetine and resuspended in TCGM at 2 x 10^5 cells/ml. Fifty μl of the cell suspensions were finally mixed with various numbers of effectors in a final volume of 200 μl/well of 96 well V-bottom plates. Radioactivity released was measured as previously described. Anti-CD4 treatment of effectors was performed as described above. All results are expressed as the mean of 4 replicate wells

Results

Effect of Chloroquine on Target Recognition.

To study the possible involvement of the endosomal compartment in the generation of HSV-1 CTL epitopes, targets were pretreated with chloroquine throughout the assay and subsequently the various target groups were incubated with CTL effectors for 4 hours. Uninfected targets were used as negative controls.

As the results in Table XI indicate, chloroquine treatment of A 20 cells partially inhibits the CTL response. The degree of inhibition represents approximately 37% of the maximal response attainable at E/T ratios below 20/1, and is maintained at no less than 25%. At no time is inhibition of lysis complete.

Contrary to the results obtained with A 20 cells, chloroquine appears to have no effect on CTL recognition of HSV-1 treated EMT6 cells regardless of the E/T ratio.

The Role of Protein Synthesis on CTL Recognition of Class I or Class I and II Expressing Targets.

To evaluate the significance of protein synthesis in the CTL recognition of HSV-1 infected targets A 20 and EMT6 cells were treated with the virus in the presence or absence of emetine. Due to the permanent effects of emetine on protein synthesis, target cells were treated with the agent in the presence or absence of HSV-1 and subsequently diluted in emetine free medium, before addition to the effectors.

Whereas emetine treatment of EMT6 cells reduces HSV-1 specific lysis to the level of the uninfected controls, it only partially inhibits the CTL response against A 20 cells (Table XII). Moreover, as with chloroquine, inhibition of HSV-1 specific

Table XI

Effect Of Chloroquine on Target Cell Sensitization
by Infectious HSV-1 [a]

		% [^{51}Cr]-release (% Inhibition)		
			chloroquine	
			-	+
Targets	E/T	uninfected	HSV-1	
A 20	5.0	4.2	32.6	20.8 (36.6)
	10.0	7.0	40.2	26.0 (35.4)
	20.0	10.3	43.6	32.9 (24.7)
	40.0	12.8	50.5	36.7 (27.4)
EMT6	5.0	0.6	13.9	14.1 (-1.5)
	10.0	5.2	22.1	25.2 (-14.3)
	20.0	6.7	38.2	40.7 (-6.5)
	40.0	9.3	50.1	49.7 (0.0)

[a] Targets treated with chloroquine were maintained in the presence of the agent from the onset of infection, thereon.
Data are representative of 3 experiments.

Table XII

Effect of Inhibition of Protein Synthesis
on the Sensitization of Targets [a]

		%[^{51}Cr]-release (% Inhibition)		
			emetine	
			-	+
Targets	E/T	uninfected	HSV-1	
A 20	5.0	4.2	33.0	14.2 (56.9)
	10.0	10.5	40.1	30.8 (23.1)
	20.0	9.4	48.3	37.2 (23.0)
	40.0	12.9	50.9	40.0 (21.4)
EMT6	5.0	0.0	21.8	1.7 (92.0)
	10.0	4.7	30.6	5.3 (82.7)
	20.0	5.6	38.0	8.1 (78.8)
	40.0	7.3	55.9	10.7 (80.8)

[a] Emetine was used at 50 μM during infection. Thereafter, targets were washed free of any residual amounts of the agent and incubated with the effectors.
The data shown are representative of 3 differnt experiments.

lysis of A 20 cells is more pronounced at low E/T ratios, whereas the effect of emetine on the recognition of EMT6 cells remains of a similar magnitude regardless of the E/T ratio examined.

The Combined Effect of Anti-CD4 and Emetine or Chloroquine.

Due to the polyclonality of the CTL examined in the present study, it is not surprising that neither chloroquine nor emetine completely inhibits the response to HSV-1 treated A 20 B lymphomas. Therefore, in order to optimize the inhibitory effects anti-CD4 treatment was performed in conjunction with either emetine or chloroquine. Treatment of targets with either emetine or chloroquine was carried out as already described. Anti-CD4 was included at 12.5 μgs/well where applicable. Untreated, HSV-1 pulsed targets constituted the positive controls for maximal levels of [^{51}Cr] released.

It is clearly evident from the data compiled in Table XIII that anti-CD4 in conjunction with emetine abolishes any CTL activity against A 20 cells. On the contrary, its effect on chloroquine treated A 20 targets is minimal if any. Similarly, anti-CD4 has no effect on the response to HSV-1 infected EMT6 cells be it alone or in combination with either chloroquine or emetine. In agreement with the results in Tables XI and XII chloroquine and emetine only partially inhibit the lysis of A 20 cells. Whereas emetine completely abrogates the response to EMT6 fibroblasts, chloroquine does not appear to have any effect.

Table XIII

Synergistic Effect of anti-CD4 and Emetine,or Chloroquine in CTL Recognition of Targets

		% [^{51}Cr]-release (% Inhibition)			
		E/T			
Targets	inhibitors [b]	5.0	10.0	20.0	40.0
A 20+HSV-1	none	51.7	63.3	69.2	70.3
	emetine	25.6 (50.6)	28.4 (55.1)	40.8 (41.1	48.1 (31.5)
	chloroquine	23.5 (55.0)	32.5 (48.8)	41.3 (40.2)	35.6 (49.3)
	emtn+α-CD4	8.0 (84.5)	14.8 (76.7)	18.1 (73.8)	19.7 (72.0)
	chlrqn+α-CD4	23.3 (54.9)	27.7 (56.3)	34.2 (50.5)	46.1 (34.4)
EMT6+HSV-1	none	27.9	33.4	53.4	60.3
	emetine	1.2 (95.8)	5.5 (83.6)	9.7 (81.9)	18.1 (70.0)
	chloroquine	24.4 (12.5)	34.0 (-1.6)	48.4 (9.3)	59.9 (0.0)
	emtn+α-CD4	4.1 (85.3)	9.8 (70.6)	10.9 (79.4)	15.7 (74.0)
	chlrqn+α-CD4	24.2 (13.4)	32.4 (3.3)	51.3 (3.8)	60.8 (0.0)
A 20	none	4.6	8.7	8.4	12.8
EMT6	none	6.0	8.6	16.1	15.9

[a] Emetine was used only during infection. Chloroquine was used from the onset of infection , thereon.
Anti-CD4 was used at 12.5 μg per culture.

Discussion

The preceding section examines presentation of viral Ags for target cell recognition by HSV-1 specific CTL. The targets used comprised a panel of cells expressing either class I (EMT6) or a combination of class I and II MHC determinants (A 20). All target groups were tested against primary lymph node preparations of acutely infected Balb/c mice.

Encouraged by the inhibitory effects of anti-CD4 and anti-IA on target cell lysis, an attempt was made to further characterize the two apparently distinct effector populations on the basis of their requirements for endogenously or exogenously HSV-1 sensitized targets. Pioneering work with helper T cells by Ziegler et al. (141) and Chesnut et al. (19) demonstrated the significance of low pH endosomes in Ag presentation. Similarly research with influenza suggested a possible dichotomy between $CD4^+$ class II MHC restricted CTL and their $CD8^+$ counterparts regarding Ag processing requirements for recognition of targets (15). The $CD4^+$ population of CTL was shown to lyse targets in a manner independent of viral infectivity. Importantly, targets irreversibly treated with the protein synthesis inhibitor emetine were unaffected in their respective capacities to present influenza HA epitopes to Ia-restricted CTL clones (84). In contrast, chloroquine completely inhibited any CTL response. Significantly, class I-restricted CTL were rendered ineffective in the presence of emetine treated targets, whereas their function was virtually unperturbed when stimulated with cells previously treated with chloroquine.

The general applicability of these observations was confirmed with subsequent research (45, 63), in spite of the fact that the model for class I-restricted CTL has been revised to accommodate Ags either not synthesized de novo (17, 82, 126, 131), or

ordinarily not expressed on the surface of cells (9, 75). A notable exception perhaps, is work with measles virus specific, Ia-restricted CTL, whereby chloroquine does not appear to prevent Ag presentation, presumably due to the mechanics involved in fusion of the virus with phospholipid membranes (87, 108).Taken together, the data presented in the preceding two sections strongly suggest that the Balb/c CTL response to HSV-1 is indeed the concerted effect of two CTL populations distinct not only in phenotypic profiles but their respective requirements for Ag processing pathways as well. Whereas chloroquine treated A 20 B lymphomas elicit only a partial CTL response to HSV-1, similarly treated EMT6 cells retain their full stimulatory capacity.

Although the mechanism by which chloroquine affects the intracellular environment is not completely understood, its effect on the pH of endosomes is adequately defined (133). Thus, it appears that a substantially significant CTL population of Balb/c mice recognizes HSV-1 Ags only as a consequence of endosomal degradation. Any increase in the pH of endosomes neutralizes the ability of a portion of the general CTL population to recognize and kill HSV-1 sensitized targets. Results with emetine are perhaps even more instructive, inasmuch as the phenotype of CTL restricted by IA may be deduced. Although emetine treated class I bearing targets are virtually null in presenting Ag, cells expressing proteins coded for by the entire MHC region are only modestly perturbed. However, in the presence of anti-CD4 emetine treated A 20 cells can no longer support CTL function, and the response is thus reduced to the level of HSV-1 uninfected controls.

It is possible that the concentration of emetine used is only marginally effective against A 20 lymphomas yet sufficient to completely ablate HSV-1 replication in EMT6 cells. However, two arguments render this possibility improbable. Thus, although emetine acts as a non-specific protein synthesis inhibitor,it is its inhibition of

although emetine acts as a non-specific protein synthesis inhibitor,it is its inhibition of viral replication which is most crucial for CTL responses. To assume that the agent preferentially interferes with HSV-1 replication in one cell (EMT6) and not another (A 20) seems rather illogical. Secondly the concentration of emetine employed in the assays performed herein was five times the amount commonly used (84), and sufficient in abolishing any cellular proliferation (data not shown). Similar arguments may be raised to support the non-specific effects of chloroquine. Hence, regardless of which proteins are inactivated in the treatment as a consequence of interference with the pH, the fact remains that an acidified intracellular compartment is indeed involved in the processing and presentation of HSV-1 CTL target Ags.

More importantly, the present observations suggest that $CD4^+$ and presumably class II MHC-restricted CTL recognize virion associated proteins, not necessarily de novo synthesized. Failure to consistently implicate any of the late HSV-1 glycoproteins as CTL targets may reflect limitations posed by experimental designs, inasmuch as class II-restricted CTL responses are ordinarily overlooked.

In summary, a significant proportion of HSV-1 specific Balb/c derived CTL appear to express CD4 and recognize antigenic determinants generated upon endosomal degradation of the virus. Furthermore, HSV-1 replication is not required for the sensitization of H-2I expressing targets. It is not presently possible to determine the actual number or frequency of the $CD4^+$ CTL population as limiting dilution analyses were not performed.

VII. Unifying Discussion and Speculation

Analysis of surface phenotype and functional characteristics of T lymphocytes has indicated a correlation between MHC determinants recognized and the expression of CD4 or CD8 molecules. Although the initial definition of T cell requirements for recognition of Ag are still highly regarded, many presumptions are continually being scrutinized and revised.

T cell subpopulation distinctions are becoming increasingly difficult to decipher, and functional as well as phenotypic diversity is more frequently reported than initially anticipated. Thus, class II-restricted, $CD4^+$ CTL are no longer considered in vitro artifacts (129, 44, 68, 104).

The present study was implemented to expand on the current model of the HSV-1 specific CTL response in mice. Lymphocyte preparations isolated from the draining lymph nodes of acutely HSV-1 immunized Balb/c mice were tested against a panel of cells in proliferation and CTL assays. Target and/or stimulator populations consisted of cells expressing class I (EMT6) or II (RT 2.3, RT 10) MHC determinants, in addition to Balb/c derived B lymphomas with effector cell histocompatibility spanning the entire MHC region. All responses examined were of a primary nature. Collectively, the results presented herein are in support of a biphasic response effected by two phenotypically distinct T cell populations, namely $CD4^+$ and $CD8^+$ CTL. The two subsets are restricted by class II and class I MHC determinants respectively, as demonstrated by Ab inhibition experiments.

Results on lymphoproliferation assays strongly indicate that the responder population includes cells restricted by IA and IE MHC elements in an HSV-1 dependent manner. Both IA^+ RT 2.3 and IE^+ RT 10 L cell transfectants elicit a

proliferative response significantly above that supported by either allogeneic or uninfected controls, with stimulation indexes ranging between 5 and 6 at suboptimal conditions. Maximal response against RT 2.3 cells occurs at 1 x 10^6 stimulators per 3 x 10^5 responders. The uptake of [^{3}H]-TdR is almost entirely abolished in the presence of anti-IA in a dose dependent manner, with maximal inhibition occurring at 6 μg of Ab per 5 x 10^5 stimulator cells. Interestingly, as little as 0.5 μg of anti-IA is sufficient to reduce the proliferative response by 20%. The response against A 20 cells, whereas HSV-1 specific, is only partially inhibited by identical concentrations of the Ab, presumably due to class I restricted proliferating T cells. Although class I-restricted proliferation was not directly demonstrated, the fact that the equivalent CTL response is consistently shown provides ample evidence, indirect as it were, to suggest it. Alternatively the level of IA expression, and/or permissiveness to HSV-1 replication between RT 2.3 and A 20 cells may vary significantly in favor of the latter. However, based on the dose response kinetics of anti-IA inhibition experiments, whereby a plateau is reached at identical Ab concentrations, it appears that the actual responsive elements, that is IA determinants already in complex with HSV-1 T cell epitopes, are of quantitatively very similar numbers. If indeed HSV-IA aggregates on the surface of A 20 cells exceed those found on RT 2.3 cells, one would predict a requirement for higher anti-IA concentrations in order to effect maximal levels of inhibition. Additionally, a factor that must be considered is the turnover rate of IA molecules of individual cells. For instance, it has been documented that IA proteins of murine B lymphomas are maintained at practically stable levels, primarily through recycling between the plasma membrane and the endosomal compartment, in a manner resistant to cycloheximide treatment. In marked contrast, peritoneal exudate cells exhibit significant sensitivity to cycloheximide treatment, suggesting a requirement for

continuous translation of mRNA transcripts for IA maintenance (42). Considering the disparate origins of RT 2.3 and A 20 cells it is not improbable to conceive of a similar difference in the regulation of IA. However, in view of the dose response curve, whether the pool of IA determinants remains constant or is rapidly regenerated or recycled is immaterial. It is unlikely that the TcR responsive elements fluctuates significantly in the duration of the assays, otherwise a plateau of inhibition could not be attained.

The principal evidence suggestive of a class II-restricted, $CD4^+$ T cell dependent CTL response against HSV-1 infected targets is provided by Ab inhibition experiments. Both anti-IA^d and anti-CD4 treatments result in partial inhibition of CTL function directed against A 20 cells. In contrast, the CTL response against EMT6 targets remains unperturbed. On the other hand, anti-CD8 almost entirely abrogates the response against HSV-1 sensitized EMT6 cells, inhibition exceeding 80%, yet as with anti-CD4 it only partially prevents recognition and lysis of A 20 cells. The inhibition caused by anti-CD4 is Ab dose dependent at concentrations below 50 μg of ascites fluid. Concentrations upwards of 50 μg per 4×10^5 effectors are ineffective in diminishing CTL activity, presumably due to Fc receptor interactions of the Ab with the targets. A possible means of confirming the latter assumption may rely in the use of F(ab')2 fragments.

Somewhat unexpectedly, RT 2.3 IA transfectants failed to elicit a CTL response in spite of their apparent capacity to induce proliferation of the lymphocyte cultures. It is conceivable that lack of a CTL response may reflect relative target susceptibilities to the signals mediating lysis and emanating from the CTL. Unfortunately the issue was not resolved at the present time. Certainly resistance to CTL lysis is not a newly encountered phenomenon (44, 129). Assuming that class I-

and class II-restricted CTL lyse targets in a similar fashion, and moreover, taking advantage of the original MHC genotype of RT 2.3 cells (H-2^k), may permit experimentation with effector populations derived from H-2^k mice.

Alternatively, the answer to the discrepancy may lie in a deficiency in endosomal enzymes required for appropriate processing of CTL epitopes capable of associating with IA. Although this interpretation presupposes a demand for enzymes with sequence specificity, an assumption somewhat physiologically unsound, it nevertheless lends itself to further examination. Thus, synthetic peptides or in vitro derived fragments of IA specific CTL epitopes should theoretically bind to IA determinants, hence bypassing the requirement for intracellular degradation of antigenic proteins. As with OVA specific CTL (17), one would predict that whole or predigested HSV-1 Ags may sensitize cells, and consequently predispose them for CTL recognition. Unfortunately however, neither gD nor gC appears to contain appropriate CTL epitopes. Although inability to detect gC specific CTL restricted by IA may be attributed to the purity of the vaccinia construct, inasmuch as class II-restricted CTL do not recognize de novo synthesized proteins (82, 84), the VgD recombinant used during infection was stored and utilized as cellular extracts containing soluble as well as cell associated viral proteins synthesized during propagation of the virus. It is therefore unlikely that gD represents a suitable target Ag for either CTL populations, certainly amply demonstrated for class I MHC-restricted effectors (73, 98).

Even more puzzling is the inability of UV-inactivated HSV-1 to provide adequate antigenic stimulation for a CTL response. In view of the results obtained subsequent to emetine treatment of A 20 cells it was indeed expected that UV-inactivated HSV-1 may elicit a response by the class II-restricted CTL population.

Although negative results are not in themselves conclusive, the data presented regarding the role of non-infectious virus in sensitizing A 20 targets seem to directly contradict the apparent requirement for protein synthesis. Thus, although protein synthesis within Ia^+ targets is not essential for CTL recognition, preventing viral replication appears to adversely affect Ag presentation. An interpretation of the phenomenon may rest in the assumption that none of the virion associated glycoproteins constitute appropriate target Ags recognized by Ia-restricted CTL. However, this remains unlikely considering the results of the emetine experiments, whereby viral replication appears to be at best optional, implicating CTL specific for input Ags rather than proteins de novo synthesized.

An alternate highly speculative hypothesis, is that UV-treated HSV-1 loses its capacity to fuse with cell membranes at physiological pH, a requirement for HSV-1 penetration and infectivity (46). Additionally, UV-irradiation may alter the fusogenic potential of the virus, thereby establishing a UV induced requirement for conditions other than neutral pH. Stated otherwise, whereas infectious HSV-1 fuses with cell membranes at neutral pH (46, 102), UV-inactivated virus may only retain partial fusion activity or none at all. The hypothesis therefore predicts that UV-treated HSV-1 may enter cells primarily or exclusively via endocytosis, a process entirely dictated by the cells themselves and presumably dependent on cellular growth characteristics and/or genotypic potential (45, 63, 113). On the contrary, infectious HSV-1 becomes intracellularly available via direct fusion with cell membranes, as well as cell mediated endocytosis. Consequently, the amount of infectious HSV-1 present within a cell at any one time may theoretically exceed that of UV-treated, fusion inactive virus. At its most absolute this hypothesis may only account for a partial loss in processed Ag. It nonetheless fails to justify the reason endocytosed virions are not processed, unless it

is additionally assumed that the number of viral particles lies below a threshold required for CTL recognition. Consequently the dilemma becomes a question of relative numbers of HSV-1 Ags accessible to the endosomal pathway. Theoretically, resolution of the paradox may lie in the amount of UV-inactivated HSV-1 used in pulsing targets.

An additional factor that perhaps need be considered is the limited capacity of B lymphomas to internalize proteins, hence the requirement for either excess Ag (65), or direct ligand interactions between surface immunoglobulin and Ag (113). It is possible that fusion inactive HSV-1 is not internalized in amounts significant for presentation. To circumvent these limitations it may be necessary to increase the viral titer used in pulsing A 20 cells, or alternatively, use Ab heteroconjugates specific for HSV-1 and A 20 surface proteins in order to enhance viral uptake.

Perhaps the most significant observation reported in the present study concerns the respective pathways in the presentation of HSV-1 Ags. The inability of UV-inactivated HSV-1 to sensitize targets notwithstanding, it is obvious that $CD4^+$, class II-restricted CTL recognize Ags independently of viral infectivity and replication . Not surprisingly, $CD8^+$, class I-restricted CTL recognize newly synthesized HSV-1 proteins in a manner resistant to chloroquine. Although glycoprotein specificity with respect to either gC or gD was not demonstrated experiments with emetine treated targets strongly imply that a considerably significant population of HSV-1 specific CTL of the CD4 phenotype recognize virion associated proteins, perhaps envelope glycoproteins.

Clearly MHC haplotype as well as "holes" in the CTL repertoire may determine the overall immune status (8, 103). However, it is no longer adequate to confine research interests to a particular effector population at the expense of another.

Negative data are literally of very little value or biological salience, unless all possible venues towards a response are considered. Therefore, although much information has accumulated regarding HSV-1 specific, class I MHC-restricted CTL, disappointingly little pertains to Ia-restricted responses. It is puzzling that a field of research that has offered so much insight to the general knowledge of T lymphocyte responses should continue to remain in the backround in HSV-1 research. Perhaps questions that have eluded explanation may find the answers hidden within the $CD4^+$ CTL population. For instance, whereas HSV-1 envelope glycoproteins, once believed to constitute the principal CTL targets (38, 66) have more or less become of secondary importance for vaccine development. Although the investigation is by no means complete, it may receive a boost once alternate approaches are considered. For example, based on the apparent fortuitous requirement for protein synthesis it is assumed that one or a number of HSV-1 envelope associated glycoproteins may indeed elicit class II-restricted CTL responses. Although the results with UV-inactivated HSV-1 provided no conclusive evidence as to its immunogenic potential, the work is by no means exhaustive. Manipulations aimed at improving viral uptake by targets may provide crucial information on the actual role of envelope glycoproteins in CTL induction and function. It is our belief that the $CD4^+$, Ia-restricted CTL population described in the body of the present study recognizes virion associated proteins, with envelope glycoproteins comprising the prime candidates. Therefore, further work with this CTL population may offer long sought after information directly applicable to medical approaches in vaccine development.

Aside from implications for the development of suitable HSV-1 vaccines, the present work may intimate a role for $CD4^+$, Ia-restricted CTL in immunoregulatory processes. Stemming from a general lack of information on the finer functional and

molecular characteristics of suppressor T cells as an entity in themselves, models are being proposed implicating class II MHC-restricted CTL in suppressive mechanisms (52, 62, 63). It is not at all unlikely that Ia^+ cells, be they B cells, macrophages, or endothelial cells may become targets for CTL, thus diminishing their roles in immune responses. For instance, the role of B cells as Ag presenting cells (APC) amply documented (19, 20, 44, 127), it is theoretically possible that they may be reduced to the status of targets recognized by either $CD8^+$ or $CD4^+$ CTL in an Ia-restricted manner (55, 111, 113). Similarly, classical APC, such as macrophages and dendritic cells pulsed by either soluble or viral Ags may be eliminated, thereby counteracting any otherwise beneficial potential inherent to these cells. Furthermore, in diseases such as AIDS, a deficiency in $CD4^+$ helper T cells may directly reflect suppression through lysis of uninfected gp120 sensitized cells (63). Whatever the finer details of such a model for suppression, the potential for class II-restricted killing has been acknowledged, and renewed enthusiasm may provide valuable information in containing otherwise debilitating afflictions.

List of References

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1. Allison, J. P., L. L. Lanier. 1985. Identification of antigen receptor-associated structures on murine T-cells. Nature (London). 404: 107.

2. Andrew, M. E., B. E. H. Coupar, D. B. Boyle, and R. V. Blanden.1987. Recognition by major histocompatibility complex class I-restricted cytolytic T lymphocytes of cells expressing vaccinia-encoded viral and class I proteins.Eur. J. Immunol. 17: 1515.

3. Andrus, L., A. Granelli-Piperno, and E. Reich. 1984. Cytotoxic T cells both produce and respond to interleukin 2. J. Exp. Med. 159: 646.

4. Babbit, B., P. Allen, G. Matsueda, E. Haber, and E. Unanue. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. Nature (London). 307: 349.

5. Bank, I., and I. Chess. 1985. Perturbation of the T4 molecule transmits a negative signal to T cells. J. Exp. Med. 162: 1294.

6. Beckoff, M., T. Kakiuchi, and H. M. Grey. 1985. Accessory cell function in the ConA response: role of Ia-positive and Ia-negative accessory cells. J. Immunol. 134: 1327.

7. Benacerraf, B. 1978. A hypothesis to relate the specificity of T lymphocytes and the activity of I region-specific Ir genes in macrophages and B lymphocytes. J. Immunol. 120: 1809.

8. Bennink, J., and J. W. Yewdell. Murine cytotoxic T lymphocyte recognition of individual virus proteins. High frequency of nonresponder MHC class I alleles. 1988. J. Exp. Med. 168: 1935.

9. Bennink, J. R., J. W. Yewdell, and W. Gerhard. 1982. A virus polymerase involved in recognition of influenza virus-infected cells bu a cytotoxic T cell clone. Nature (London). 286: 75.

10. Biddison, W. E., P. E. Rao, M. A. Talle, G. Goldstein, and S, Shaw. 1982. Possible involvement of the OKT4 molecule in T cell recognition of class II HLA antigens. Evidence from studies of cytotoxic T lymphocytes specific for SB antigens. J. Exp. Med. 156: 1065.

11. Blanchard, D., C. Van Els, J. P. Aubry, J. E. De Vries, and H. Spits.1988. CD4 is involved in a post-binding event in the cytolytic reaction by human CD4+ cytotoxic T lymphocyte clones. J. Immunol. 140: 1744.

12. Blue, M. L., K. A. Craig, P. Anderson, K. R. Branton, Jr., and S. F. Schlossman. 1988. Evidence for specific association between class I major histocompatibility antigens and the CD8 molecules of human suppressor/cytotoxic cells. Cell 53: 413.

13. Blue, M. L., J. F. Daley, H. Levine, K. R. Branton, Jr., and S. F. Schlossman. 1989. Regulation of CD4 and CD8 surface expression on human thymocyte subpopulations by triggering through the CD2 and the CD3-T cell receptor. J. Immunol. 142: 364.

14. Bourgault, I., A. Gomez, E. Gomrad, F. Picard, and J. P. Levy. 1989. A virus specific CD4+ cell-mediated cytolytic activity revealed by CD8+ cell elimination regularly develops in uncloned human antiviral cell lines. J. Immunol. 142: 252.

15. Braciale, T. J., and K. L. Yap. 1978. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells. J. Exp. Med. 147: 1235.

16. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. Generation of functionally distinct T cell subclasses in a differentiative process independent of antigen. J. Exp. Med. 141: 1376.

17. Carbone, F., M. W. Moore, L. M. Sheil, and M. J. Bevan. 1988. Induction of cytotoxic T lymphocytes by primary in vitro stimulation with peptides. J. Exp. Med. 167: 1767.

18. Carrel, S., A. Moretta, G. Pantaleo, G. Tambussi, P. Isler, B. Perussia, and J. C. Cerottini. 1988. Stimulation and proliferation of CD4+ peripheral blood T lymphocytes induced by an anti-CD4 monoclonal antibody. Eur. J. Immunol. 18: 333.

19. Chesnut, R. W., S. M. Colon, and H. Grey. 1982. Requirements for the processing of antigens by antigen-presenting B cells. I. Functional comparison of B cell tumors and macrophages. J. Immunol. 129: 2382.

20. Ciavarra, R. P., and D. H. Burgess. 1988. Antigen-presenting B cells : efficient uptake and presentation by activated B cells for induction of cytotoxic T lymphocytes against Vesicular Stomatitis Virus. Cell. Immunol. 114: 27.

21. Collins, T., A. J. Korman, C. T. Wake, J. M. Boss, D. J. Kappes, W. Fiers, K. A. Ault, M. A. Gimbrone, J. L. Strominger, and J. C. Rober. 1984. Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc. Natl. Aca. Sci. USA 81: 4917.

22. Dennert, G., S. Weiss, and J. Warner. 1981. T cells may express multiple activities: specific allohelp, cytolysis, and delayed-type hypersensitivity are expressed by a cloned T-cell line. Proc. Natl. Aca. Sci. USA. 78: 4540.

23. Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Harrann, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch. 1983. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK 1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen reactivity. Immunol. Rev. 74: 29.

24. Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus-immune effector T cells for H-2K and H-2D compatible interactions: implication for H antigen diversity. Transplant. Rev. 29: 89.

25. Dower, S. K., and D. M. Segal. 1985.Interaction of monoclonal antibodies with MHC class I antigens on mouse spleen cells. II. Levels of expression of H-2K, H-2D, and H-2L in different mouse strains. J. Immunol. 133: 431.

26. Engleman, E. G., C. J. Benike, E. Glickman, and R. L. Evans. 1981. Antibodies to membrane structure that distinguish suppressor/cytotoxic and helper T lymphocyte subpopulations block the mixed leukocyte reaction in man. J. Exp. Med. 154: 193.

27. Evans, R. L., T. J. Faldetta, R. E. Humphreys, D. M. Pratt, E. J. Yunis, and S. F. Schlossman. 1978. Peripheral human T cells sensitized in mixed lymphocyte culture synthesize and express Ia-like antigens. J. Exp. Med. 147: 1430.

28. Fishwild, D. M., C. J. Benike, and E. G. Engleman. 1988. Activation of HLA-restricted EBV-specific cytotoxic T cells does not require CD4+ (helper) T cells or exogenous cytokines. J. Immunol. 140: 1994.

29. Fleischer, B. 1984. Acquisition of specific cytotoxic activity by human T4+ T lymphocytes in culture. Nature (London). 308: 365.

30. Fleischer, B., H. Schrezenmeir, and H. Wagner. 1986. Function of the CD4 and CD8 molecules on human cytotoxic T lymphocytes: regulation of T cell triggering. J. Immunol. 136: 1625.

31. Fleischer, B., and H. Wagner.1986. Significance of T4 or T8 phenotype of human cytotoxic T-lymphocyte clones. Cur. Top. Microbiol. Immunol. 126: 101.

32. Flomenbeg, N., V. Naito, E. Duffy, R. W. Knowles, R. L. Evans, and B. Dupont. 1983. Allocytotoxic T cell clones: both Leu2+3- and Leu2-3+ T cells recognize class I histocompatibility antigens. Eur. J. Immunol. 11: 905.

33. Germain, R. N. 1985. The ins and outs of antigen processing and presentation. Nature (London). 312: 687.

34. Germain, R. N., J. D. Ashwell, R. I. Lechler, D. H. Margulies, K. M. Nickerson, G. Suzuki, and J. Y. L. Tou. 1985. "exon shuffling" maps control of antibody- and T-cell-recognition sites to the NH2-terminal domain of the class II major histocompatibility polypeptide Aβ. Proc. Natl. Aca. Sci. USA. 82: 2940.

35. Germain, R. N., and H. Quill. 1986. Unexpected expression of a unique mixed-isotype class II MHC molecule by transfected L-cells. Nature (London). 320: 72.

36. Gething, M., U. Koszinowski, and M. Wakerfield. 1978. Fusion f Sendai virus with the target cell membrane is required for T cell cytotoxicity. Nature (London). 274: 689.

37. Glasebrook, A. L., A. Kelso, and H. R. McDonald. 1983.Cytotoxic T lymphocyte clones that proliferate autonomously to specific alloantigen stimulation. II. Relationship of the Lyt-2 molecular complex to cytolytic activity, proliferation, and lymphokine secretion. J. Immunol. 130: 1545.

38. Glorioso, J., V. Kees, G. Hume, H. Kirchner, and P. Krammer. 1985. Identification of herpes simplex virus type 1 (HSV-1) glycoprotein C as the immunodominant antigen for HSV-1-specific cytotoxic T lymphocytes. J. Immunol. 135: 575.

39. Gooding L. R., and K. A. O'Connell. 1983. Recognition by cytotoxic T lymphocytes of cells expressing fragments of the SV40 tumour antigen. J. Immunol. 131: 2580.

40. Greenstein, J. L., J. Kappler, P. Marrack, and S. J. Burakoff. 1984. The role of L3T4 in recognition of Ia by a cytotoxic, H-2Dd-specific T cell hybridoma. J. Exp. Med. 159: 1213.

41. Greenstein, J. L., B. Malissen, and S. J. Burakoff. 1985. Role of L3T4 in antigen driven activation of a class I-specific T cell hybridoma. J. Exp. Med. 162: 369.

42. Harding, C. V., and E. R. Unanue. 1989. Antigen processing and intracellular Ia; possible roles of endocytosis and protein synthesis in Ia function. J. Immunol. 142: 12.

43. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983 The major histocompatibility complex-restricted antigen receptor on T cells. J. Exp. Med. 157: 1149.

44. Hayward, A. R., O. Pontesilli, M. Herberger, M. Laszlo, and M. Levin. 1986. Specific lysis of Varicella Zoster virus-infected B lymphoblasts by human T cells. J. Virol. 58: 179.

45. Hewitt, C. R. A., and M. Feldman. 1989. Human T cell clones present antigen. J. Immunol. 142: 1429.

45. Holland, T. C., and S. Person. 1977. Ammonium chloride inhibits cell fusion induced by syn mutants of herpes simplex virus type 1. J. Virol. 23: 313.

47. Horohov, D. W. 1985. The regulation of herpes simplex virus specific cytotoxic T lymphocyte induction by a suppressor cell circuit. University of Tennessee, Knoxville. Dissertation.

48. Howes, E. L., W. Taylor, N. A. Mitchinson, and E. Simpson. 1979. MHC matching shows that at least two T cell subsets determine resistance to HSV. Nature (London). 277: 66.

49. Jacobson S., J. R. Richert, W. E. Biddison, A. Satinsky, R. J. Hartzman, and H. F. McFarland. 1984. Measles virus-specific T4+ human cytotoxic T cell clones are restricted by class II HLA antigens. J. Immunol. 132: 754.

50. Jandinski, J. H. Cantor, T. Tadakuma, D. L. Peavy, and C. W. Pierce. 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: suppressor and helper activities are inherent properties of distinct T cell subclasses. J. Exp. Med. 143: 1382.

50. Janeway, C. A. Jr.1989. The role of CD4 in T-cell activation; accessory molecule or co-receptor. Immunol. Today. 10: 234.

51. Janeway, C. A. Jr., P. J. Conrad, E. A. Lerner, J. Babich, P. Wettstein, and D. B. Murphy. 1984. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells: possible role of T cell bound Ia antigens as targets of immunoregulatory T cells. J. Immunol. 132: 662.

52. Janeway, C. A. Jr., J. Rojo, K. Saizawa, U. Dianzani, P. Portoles, J. Tite, S. Haque, and B. Jones. 1989. The co-receptor function of murine CD4. Immunol Rev. 109: 77.

54. Jefferies, W. A., U. Ruther, E. F. Wagner, and S. Kvist. 1988. Cytolytic T cells recognize a chimeric MHC class I antigen expressed in influenza A infected transgenic mice. EMBO J. 7: 3423.

54. Kaplan, D. R., R. Grffith, V. L. Braciale, and T. J. Braciale. 1984. Influenza virus-specific human T cell clones: heterogeneity in antigenic specificity and restriction by class II MHC products. Cell. Immunol. 88: 193.

56. Kim, K. J., C. Kanellopoulos-Langevin, R. M. Merwin, D. H. Sachs, and R. Asofsky. 1979. Establishment and characterization of Balb/c lymphoma lines with B cell receptors. J. Immunol. 122: 549.

57. Koszinowski, U. H., M. J. Reddehase, G. M. Keil, and J. Schikedanz. 1987. Host immune response to cytomegalovirus products of transfected viral immediate early genes are recognized by cloned cytolytic T lymphocytes. J. Virol. 61: 2054.

58. Krensky, A. M., C. Clayberger, J. L. Greenstein, M. Grimmins, and S. J. Burakoff. 1983. A DC-specific cytotoxic T lymphocyte line is OKT8+. J. Immunol. 131: 2777.

59. Krensky, A. M., C. Clayberger, C. S. Reiss, J. L. Strominger, and S. J. Burakoff. 1982. Specificity of OKT4+ cytotoxic T lymphocyte clones. J. Immunol. 129: 2001.

60. Krensky, A. L., C. Reiss, J. W. Mier, J. L. Strominger, and S. J. Burakoff. 1982. Long-term human cytotoxic T-cell lines allospecific for HLA-DR6 antigen are OKT4+. Proc. Natl. Aca. Sci. USA. 79: 2365.

61. Krieger, J. I., S. F. Crammer, H. M. Grey, and R. W. Chesnut. 1985. Antigen presentation by splenic B cells: resting B cells are ineffective, whereas activated B cells are effective accessory cells for T cell responses. J.Immunol. 135: 2937.

62. Lanzavecchia, A. 1989. Is suppression a function of class II-restricted cytotoxic T cells? Immunol. Today. 10: 157.

63. Lanzavecchia, A., E. Roosnek, T. Gregory, P. Berman, and S. Abrignani. 1988. T cells can present antigens such as HIV gp120 targeted to their own surface molecules. Nature (London). 334: 530.

64. Larsen, H. S., M-F. Feng, D, W, Horohov, R. N. Moore, and B. T. Rouse. 1984. Role of T lymphocyte subsets in recovery from herpes simplex virus infection. J Virol. 50: 56.

65. Lasky, L. A., G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon. 1987. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell. 50: 975.

66. Lawman, M. J., R. J. Courtney, R. Eberle, P. A. Schaffer, M. K. O'Hara, and B. T. Rouse. 1980. Cell-mediated immunity to herpes simplex virus: specificity of cytotoxic T cells. Infect. Immun. 30: 451.

67. Lawman, M. J., B. T. Rouse, R. J. Courtney, and R. D. Walker. 1980. Cell-mediated immunity against herpes simplex virus: induction of cytotoxic T lymphocytes. Infect. Immun. 27: 133.

68. Lindsley, M. D., D. J. Torpey III, and C. R. Rinaldo. 1986. HLA-DR-restricted cytotoxicity of cytomegalovirus-infected monocytes mediated by Leu-3-positive T cells. J. Immunol. 136: 3045.

69. Long, E. O.1989. Intracellular traffic and antigen processing. Immunol. Today. 10: 232.

70. Macphail, S.,and O. Stutman. 1987. L3T4+ cytotoxic T lymphocytes specific for class I H-2 antigens are activated in primary mixed lymphocyte reactions. J. Immunol. 139: 4007.

71. Maddon, P. J., D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, and R. Axel. 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. Cell. 42: 93.

72. Marrack, P., R. Endres, R. Shimonkevitz, A. Zlotnik, D. Dialynas, F. Fitch, and J. Kappler. 1983. The major histocompatibility complex-restricted antigen receptor in T cells. II. Role of the L3T4 product. J. Exp. Med. 158: 1077.

73. Martin, S., E. Cantin, and B. T. Rouse. 1988 Cytotoxic T lymphocytes. Their relevance in herpesvirus infections. Cytotoxic T Cells.Annals of the New York Academy of Sciences. 532: 257.

74. Martin, S., and B. T. Rouse. 1987. The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: clearance of local infection. J. Immunol. 138: 3431.

75. Martin, S., R. J. Courtney, G. Fowler, and B. T. Rouse. 1988. Herpes simplex virus type 1 -specific cytotoxic T lymphocytes recognize virus nonstructural proteins. J. Virol. 62: 2265.

76. Maurer, D. H., J. H. Hanke, E. Michelson, R. R. Rich, and M. S. Pollack. 1987. Differential presentation of HLA-DR, DQ, and DR restriction elements by interferon-γ-treated dermal fibroblasts. J. Immunol. 139: 715.

77. Meuer, S. C., J. C. Hodgdon, R. E. Hussey, J. P. Potentis, S. F. Schlossman, and E. L. Reinherz. 1983. Antigen-like effects of monoclonal antibodies directed at receptors on human T cell clones. J. Exp. Med. 158: 988.

78. Meuer, S. C., R. E. Hussey, J. C. Hodgdon, T. Hercend, S. F. Schlossman, and E. L. Reinherz. 1982. Surface structures involved in target recognition by human cytotoxic T lymphocytes. Science. 218: 471.

79. Meuer, S. C., R. E. Hussey, A. C. Penta, K. A. Fitzgerald, B. M. Stadler, S. F. Schlossman, and E. L. Reinherz. 1982. Cellular origin of interleukin 2 (IL-2) in man: evidence for stimulus-restricted IL 2 production By T4+ and T8+ lymphocytes. J. Immunol. 129: 1076.

80. Meuer, S. C., S. F. Schlossman, and E. L. Reinherz. 1982. Clonal analysis of human cytotoxic T lymphocytes: T4+and T8+ effector T cells recognize products of different major histocompatibility complex regions. Proc. Natl. Aca. Sci. USA. 79: 4395.

81. Mingari, M. C., A. Moretta, E. Maggi, G. Pantaleo, S. Romagnani, and L. Moretta. 1984. Frequent coexpression of cytolytic activity and lymphokine production among human T lymphocytes. Production of B cell growth factor and inetrleukin 2 by T8+ and T4+ cytolytic clones. Eur. J. Immunol. 14: 1066.

82. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell. 54: 777.

83. Moretta, L., M. C. Mingari, P. R. Sekaly, A. Moretta, B. Chapuis, and J. C. Cerottini. 1981. Surface markers of cloned human T cells with various cytolytic activities. J. Exp. Med. 154: 569.

84. Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan, and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. J. Exp. Med. 163: 903.

85. Moss, D. J., L. E. Wallace, A. B. Rickinson, and M. A. Epstein. 1981. Cytotoxic T cell recognition of Epstein-Barr virus-specific B cells. I. Specificity and HLA restriction of effector cells reactivated in vitro. Eur. J. Immunol. 11: 686.

86. Mustafa, A. S., and T. Godal. 1987. BCG induced CD4+ cytotoxic T cells from BCG vaccinated healthy subjects: relation between cytotoxicity and suppression in vitro. Clin. Exp. Immunol. 69: 255-262.

87. Nagai, Y., M. Hamaguchi, T. Toyoda, and T. Yoshida. 1983. The uncoating of paramyxoviruses may not require a low pH mediated step. Virology. 130: 263.

88. Newman, W., D. Pious, and P. Gladstone. 1980. HLA variants of human lymphoblastoid cell lines as targets for cytotoxic T cells: analysis of CTL reactivity against 2 HLA-DR variants. J. Immunol. 125: 2515.

89. Oettgen, H. C., J. Kappler, W. J. M. Tax, and C. Terhorst. 1984 Characterization of the two heavy chains of the T3 complex on the surface of human T lymphocytes. J. Biol. Chem. 259: 12039.

90. Patel, S. S., A. D. Duby, D. L. Thiele, and P. E. Lipsky. 1988. Phenotypic and functional characterization of human T cell clones. J. Immunol. 141: 3726.

91. Pfizenmaier. K., H. Jung, A. Starzinsky-Powitz, M. Rollinghoff, and H. Wagner. 1977. The role of T cells in anti herpes simplex virus immunity. I. Induction of antigen-specific cytotoxic T lymphocytes. J. Immunol. 119: 939.

92. Pfizenmaier, K., A. Starzinsky-Powitz, M. Rollinghoff, D. Falke, and H. Wagner. 1977. T cell mediated cytotoxicity against herpes simplex virus infected target cells. Nature (London). 265: 630-632.

93. Reinherz, E. L., P. C. Kunz, G. Goldstein, and S. F. Schlossman. 1979. Separation of functional subsets of human T cells by a monoclonal antibody. Proc. Natl. Aca. Sci. USA. 76: 4061.

94. Reinherz, E. L., S. C. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. Cell. 30: 735.

95. Reinherz, E. L., S. C. Meuer, and S. F. Schlossman. 1983. The human T cell receptor: analysis with cytotoxic T cell clones. Immunol. Rev. 74: 83.

96. Reinherz, E. L., and S. F. Schlossman. 1980. The function and differentiation of human T cells. Cell. 19: 121.

97. Roehm, N. W., H. J. Leibson, A. Zlotnik, J. Kappler, P. Marrack, and J. C. Cambier. 1984. Interleukin-induced increase in Ia expression by normal mouse B cells. J. Exp. Med. 160: 179.

98. Rosenthal, K. L., J. R. Smiley, S. South, and D. C. Johnson. 1984. Cells expressing herpes simplex virus glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes. J. Virol. 61: 2438.

99. Rottveel, F. T. M., I. Kokkelink, R. A. W. Van Lier, B. Kuenen, A. Meager, F. Miedema, and C. J. Lucas. 1988. Clonal analysis of functionally distinct human CD4+ T cell subsets. J. Exp. Med. 168: 1659.

100. Rouse, B. T., and H. Wagner. 1984. Frequency of herpes simplex virus-specific cytotoxic T lymphocyte precursors in lymph node cells of infected mice. Immunology. 51: 57.

101. Saizawa, K., J. Rojo, and C. A. Janeway Jr. 1987. Evidence for a physical association of CD4 and the CD3: α:β T-cell receptor.Nature (London). 328: 260.

102. Sarmiento, M., M. Haffey, and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B_2) in virion infectivity. J. Virol. 29: 1149.

103. Schaeffer, E. B., A. Sette, D. L. Johnson, M. C. Bekoff, J. A. Smith, H. M. Grey, and S. Buus. 1989. Relative contribution of "determinant selection" and " holes in the T cell repertoire" to T cell responses. Proc. Natl. Aca. Sci. USA. 86: 4649.

104. Schmid, D. S. 1988. The human MHC-restricted cellular response to herpes simplex virus type 1 is mediated by CD4+, CD8- T cells and is restricted to the DR region of the MHC complex. J. Immunol. 140: 3610.

105. Schmid, D. S., and B. T. Rouse. 1983. Cellular interactions in the cytotoxic T lymphocyte response to herpes simplex virus antigens: differential antigen activation requirements for the helper T lymphocyte and cytotoxic T lymphocyte precursors. J. Immunol. 131: 479.

106. Schmitt-Verhulst, A. M., C. B. Pettinelli, P. A. Henkart, J. K. Lunney, and G. M. Shearer. 1978. H-2-restricted cytotoxic effectors generated in vitro by the addition of trinitrophenyl-conjugated soluble proteins. J. Exp. Med. 147: 352.

107. Schwartz, R. H. 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Ann. Rev. Immunol. 3: 237.

108. Sekaly, R. P., S. Jacobson, J. R. Richert, C. Tonnelle, H. F. McFarland, and E. O. Long. 1988. Antigen presentation to HLA class II-restricted measles virus-specific T-cell clones can occur in the absence of the invariant chain. Proc. Natl. Aca. Sci. USA. 85: 1209.

109. Shaw, S., G. Goldstein, T. A. Springer, and W. E. Biddison. 1985. Susceptibility of cytotoxic T lymphocyte (CTL) clones to inhibition by anti-T3 and anti-T4 (but not anti-LFA-1) monoclonal antibodies varies with the "avidity" of CTL-target interaction. J. Immunol. 134: 3019.

110. Shimonkevitz, R., J. Kappler, P. Marrack, and H. M. Grey. 1983. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. J. Exp. Med. 158: 303.

111. Shinohara, N. 1987. Class II antigen-specific murine cytolytic T lymphocytes (CTL). I. Analysis of bulk populations and establishment of Lyt-2+L3T4- and Lyt-2-L3T4+ bulk CTL lines. Cell. Immunol. 107: 395.

112. Shinohara, N., N. Hozumi, M. Watanabe, J. A. Bluestone, R. Johnson-Leva, and D. H. Sachs. 1988. Class II antigen-specific murine cytolytic T lymphocytes (CTL). II. Genuine class II specificity of Lyt-2+ CTL clones. J. Immunol. 140: 30.

113. Shinohara, N., M. Watanabe, D. H. Sachs, and N. Hozumi. 1988. Killing of antigen-reactive B cells by class II-restricted, soluble antigen-specific CD8+ cytolytic T lymphocytes. Nature (London). 336: 481.

114. Simpson, E. 1988. Suppression of the immune response by cytotoxic T cells. Nature (London). 336: 426.

115. Spits, H., J. Borst, W. Tax, P. J. A. Capel , C. Terhorst, and J. E. De Vries. 1985. Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T-cell receptor for antigen. J. Immunol. 135: 1922.

116. Spits, H., J. Borst, C. Terhorst, and J. E. De Vries. 1982. The role of T cell differentiation markers in antigen-specific and lectin-dependent cellular cytotoxicity mediated by T8+ and T4+ human cytotoxic T cell clones directed at class I and class II MHC antigens. J. Immunol. 129: 1563.

117. Sprent, J., and S. Webb. 1987. Function and specificity of T cell subsets in the mouse. Adv. Immunol. 41: 39.

118. Steeg, P. S., R. N. Moore, H. M. Johnson, and J. J. Oppenheim. 1982. Regulation of murine Ia antigen expression by a lymphokine with immune interferon activity. J. Exp. Med. 156: 1780.

119. Sterkers, G., Y. Henin, V. Lepage, D. Fradelizzi, C. Hannoun, and J. P. Levy. 1984. Influenza A hemagglutinin-specific T cell clones strictly restricted by HLA-DR1 or HLA-DR7 molecules. Eur. J. Immunol. 14: 125.

120. Strassman, G., and F. H. Bach.1984. OKT4+ cytotoxic T cells can be blocked by monoclonal antibody against T4 molecules. J. Immunol. 133: 1705.

121. Swain, S.L. 1981. Significance of Lyt phenotypes: Lyt2 antibodies block activities of T cells that recognize class I MHC antigens regardless of their function. Proc. Natl. Aca. Sci. USA. 78: 7101.

122. Swain, S. L. 1983. T cell subsets and the recognition of MHC class. Immunol. Rev. 74: 129.

123. Swain, S. L., G. Dennert, S. Wormsley, and R. W. Dutton. 1981. The Lyt phenotype of a long-term allospecific T cell line. Both helper and killer activities to IA are mediated by Ly-1 cells. Eur. J. Immunol. 11: 175.

124. Swain, S. L., D. P. Dialynas, F. W. Fitch, and M. English. 1984. Monoclonal antibody to L3T4 blocks the function of T cells specific for class 2 major histocompatibility complex antigens. J. Immunol. 132: 1118.

125. Swain, S. L., and P. R. Panfili. 1979. Helper cells activated by allogeneic H-2K or H-2D differences have an Ly phenotype distinct from those responsive to I differences. J. Immunol. 122: 383.

126. Tevethia, S., M. Tevethia, A. Lewis, V. Reddy, and S. Weissman. 1983. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). IX. Analysis of TrAg in mouse cells synthesizing truncated SV40 large T antigens. Virology. 128: 319.

127. Tite, J. P., and C. A. Janeway Jr. 1984. Cloned helper T cells kill B lymphoma cells in the presence of specific antigen: Ia restriction and cognate vs. noncognate interactions in cytolysis. Eur. J. Immunol. 14: 878.

128. Tite, J. P., B. Jones, M. E. Katz, and C. A. Janeway Jr. 1986 Generation, propagation , and variation in cloned, antigen-specific, Ia-restricted cytolytic T-cell lines. Curr. Top. Microbiol. Immunol. 126: 93.

129. Tite, J. P., M. B. Powell, and N. H. Ruddle. 1985. Protein-antigen specific Ia-restricted cytolytic T cells: analysis of frequency, target cell susceptibility, and mechanism of cytolysis. J. Immunol. 135: 25.

130. Townsend, A. R. M., R. M. Goth, and J. Davey. 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. Cell. 42: 457.

131. Townsend, A. R. M., A. J. McMichael, N. P. Carter, J. A. Huddleston, and G. G. Brownlee. 1984. Cytotoxic T cell recognition of the influenza nucleoprotein and hemagglutinin expressed in transfected mouse L cells. Cell. 39: 13.

132. Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahabur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell. 44: 959.

133. Unanue, E. R. 1984. Antigen-presenting function of the macrophage. Ann. Rev. Immunol. 2: 395.

134. Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. Nature (London). 338: 257.

135. Vidovic, D., A. Juretic, Z. A. Nagy, and J. Klein. 1981. Lyt phenotypes of primary cytotoxic T cells generated across the A and E region of the H-2 complex. Eur. J. Immunol. 11: 489.

136. Wassmer, P., C. Chan, L. Logdberg, and E. M. Shevach. 1985. Role of the L3T4 antigen in T cell activation. II Inhibition of T cell activation by monoclonal anti-L3T4 antibodies in the absence of accessory cells. J. Immunol. 135: 2237.

137. Yamada, a., J. F. Young, and F. A. Ennis. 1985. Influenza virus subtype-specific cytotoxic T lymphocytes lyse target cells coated with a protein produced in *E. coli*. J. Exp. Med. 162: 1720.

138. Yasukawa, M., A. Inatsuki, T. Kobayashi. 1988. Helper activity in antigen-specific antibody production mediated by CD4+ human cytotoxic T cell clones directed against herpes simplex virus. J. Immunol. 140: 3419.

139. Yasukawa, M., and J. M. Zarling. 1984. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. I. Lysis restricted by HLA class II MB and DR antigens. J. Immunol. 133: 422.

140. Yu, D. T. Y., R. J. Winchester, S. M. Fu, A. Gibofsky, H. S. Ko, and H. G. Kunkel. 1980. Peripheral blood Ia-positive T cells; increases in certain diseases and after immunization. J. Exp. Med. 151: 91.

141. Ziegler, K., and E. R. Unanue. 1982. Decrease in macrophage catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. Proc. Natl. Aca. Sci. USA. 79: 175.

142. Zinkernagel, R. M., and P. C. Doherty. 1975. H-2 compatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded for in the H-2K or H-2D. J. Exp. Med. 141: 1427.

VITA

Gerasimos Kolaitis was born in Athens Greece on February 21, 1962, the year of the tiger. He attended elementary schools in Montreal, Canada and his native city. High school education was completed primarily in Greece. In the summer of 1979 Gerry came to the United States of America for the first time as a high school exchange student and was graduated from West Monona High School, Iowa, in June of 1980. The following summer he attended seminars in West Germany as a representative of AFS student exchange programs. In 1982 he arrived in Knoxville where he attended the University of Tennessee for a Bachelors degree in Microbiology and a minor in Psychology. Gerry began his graduate work in the fall of 1986, whereon he accepted a teaching assistantship. The present work was defended on September, 1989 and the degree of Master of Science in Microbiology awarded in May of the following year.

No sooner was this work completed than Gerry left Tennessee and proceeded to pursue a Ph. D. degree in the same field at the University of British Columbia in Vancouver, Canada.