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Cytotoxic T Cell Response to Influenza Vaccination in Older Adults

Caroline R. Letter
Old Dominion University

CYTOTOXIC T CELL RESPONSE TO INFLUENZA VACCINATION IN OLDER ADULTS

by

Caroline R. Letter
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Approved by:

Janet McElhaney (Director)

Patricia Pleban (Member)

Ann C

Ann Campbell (Member)

Stefan Gravenstein (Member)

ABSTRACT

CYTOTOXIC T CELL RESPONSE TO INFLUENZA VACCINATION IN OLDER ADULTS

Caroline Reeve Letter
Old Dominion University, 2000
Director of Advisory Committee: Dr. Janet McElhaney

Influenza infections result in activation of cellular and humoral immune responses, leading to stimulation of cytotoxic T cells (CTL) and helper T cells with subsequent viral clearance. The increased morbidity and mortality from influenza infections are associated with a decline in cellular mediated immunity. Influenza vaccination is recommended to prevent serious illness for all persons over the age of 50 and is a cost-saving medical intervention. Although animal models and cloned cell lines have been used to study the cellular immune response to influenza, there are still uncertainties about protective mechanisms against influenza in people due to the effect of aging on the CTL response.

While the duration of the CTL response observed in older adults is similar to that of young adults, the magnitude of the response is decreased. This decrease in magnitude of the response is believed to occur due to a functional change in $CD8^+$ T cells, which may be associated with one of the CTL mechanisms resulting in lysis of virus-infected cells – perforin-mediated killing and fas-mediated killing. Perforin–mediated killing has been shown to be particularly important for influenza virus-specific immunity and a very sensitive measure of functional CTL.

In this research, the ex vivo T cell response was characterized using live influenza A (H_3N_2) virus-stimulated ex vivo peripheral blood lymphocytes from

healthy young and older adult populations. By using an assay of granzyme B activity, the in vivo cellular-mediated immune response of old adults was found to be significantly decreased in comparison to young adults. The effect of aging on the CTL response was further investigated via enzyme-linked immunospot technique. A significant increase in number of virus-specific T cells occurred in older adults after vaccination, although the magnitude of the CTL response decreased in comparison to young adults. These data suggest that a mechanistic defect in the CTL response caused the decreased response of the $CD8^+$ T cells in older adults; and provides a basis for further investigation of age-related changes in the mechanism of the CTL response to influenza vaccination in older adults.

This work is dedicated to my parents and my husband,

who always gave much needed love and support.

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TABLE OF CONTENTS

Page

Chapter

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Influenza A and B viruses are negative-strand RNA viruses belonging to the orthomyxovirus family. Both influenza viruses contain a segmented genome consisting of eight single-stranded RNA molecules. The segmented genome of influenza virus facilitates the development of new strains of influenza A and B viruses through the reassortment of genetic segments of the influenza virus. This genetic instability leads to the emergence of new virus strains that evade the host immune system, and is responsible for the annual epidemics of influenza infection.

Influenza infections result in the activation of the cellular and humoral immune response, leading to antibody production and stimulation of cytotoxic T cells (CTL) and helper T cells with subsequent viral clearance. Due to increased susceptibility to influenza complications in the older adult population, influenza vaccination is recommended to prevent illness and potential medical complications. Both animal models and cloned cell line systems have been used to study influenza, allowing the cellular immune system response to influenza to be better understood. There are still uncertainties about the response to influenza in humans due to the differences in the cytotoxic T lymphocyte memory and the effect of virus strain and aging on the CTL response compared to similar experiments completed in animal models. CTL response

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The model for this thesis is Journal of Immunological Methods.

is similar to the young adult population. The decline in cell mediated immunity in older adults may be associated with a defect in one of the CTL cytolytic mechanisms.

Epidemiology of influenza

Acute respiratory illnesses, such as influenza, are believed to have afflicted humans as early as the year 412 B. C. . Retrospective studies of influenza epidemics in humans in the 1890s (Webster et al., 1980; Cox et al., 1989) have shown that changes within influenza viral proteins occurred, enabling the emergence of new influenza virus strains that evade the host immune system. The respiratory illness occurs mainly in the winter months of each year and disappears after several weeks of circulation.

Influenza illness occurs more often in the elderly population than the young adult population. Influenza and pneumonia represent the fourth leading cause of death in the elderly population (Couch et al., 1986; Kohn, 1986; Ferruci et al., 1997). Influenza is estimated to cause in excess of 170,000 hospital admissions, resulting in excess of 40,000 influenza–associated deaths at a cost of $10 billion per year in the United States (Read et al., 2000; Drinka et al., 1997; Brammer et al., 2000).

Influenza illness begins one to four days after virus infection of the host. An initial headache can lead to the onset of fever, nonproductive cough, and postnasal drip. The influenza illness persists typically for approximately three days, while many have symptoms for weeks. Although in the event of complication(s) from influenza, recovery is completed after seven to ten days (Douglas, 1975). Complications of influenza illness include pneumonia, which can lead to secondary bacterial pneumonia and bronchitis.

Influenza vaccination is recommended for all persons over 50 years of age as it is a cost saving medical intervention (Centers for Disease Control, 2000). The current

vaccines are only 50-60% effective in preventing illness but at least 80-90% effective for preventing serious illness in the healthy elderly population and may offer little protection for the institutionalized older adult (Gross et al., 1995). Antiviral drugs are able to prevent or treat influenza infections, however the emergence of drug-resistant strains of influenza virus, adverse drug reactions and cost of antivirals limit the use of these drugs to individualized treatment (Hayden and Palese, 1997).

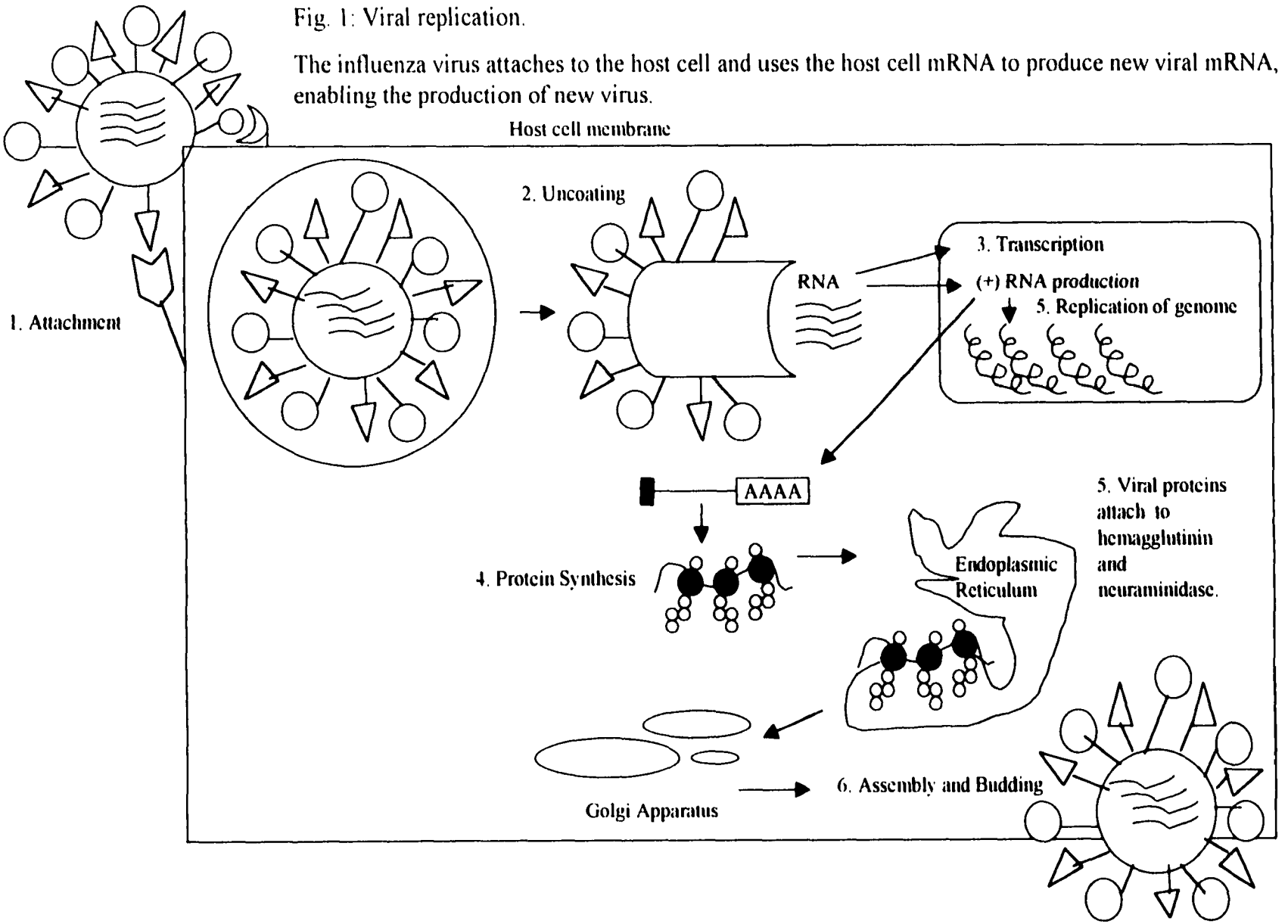
Viral replication of influenza

As with all viruses, influenza virus depends on the host cell enzymes to replicate. The influenza viral particle surface contains glycoproteins, hemagglutinin and neuraminidase. The binding of hemagglutinin to the sialic acid residues located on the host epithelial cell surface results in attachment of the viral particle to the host cell (Rogers et al., 1985). Upon receptor-mediated endocytosis into the host cell (Marsh and Helenius, 1989), the virion is internalized into endocytic vesicles (Figure 1). The influenza M_2 protein within the virion cause the pH to decrease in secondary endosomes, resulting in a conformational change in the hemagglutinin protein and uncoating of the virus, releasing the viral genome into the cytoplasm of the host cell (Wharton et al., 1990). The eight RNA genomic segments of influenza virus from the core of the virus travel to the nucleus of the host cell.

Ribonucleoproteins, composed of basic protein 1 (PB1), basic protein 2 (PB2), acidic protein (PA), and nucleocapsid protein (NP), are encoded on one of the eight influenza virus RNA segments. The nucleocapsid protein is believed to interact with many regions of the PB1, PB2 and PA RNA. NP is known to contain antigenic determinants of the influenza virus. Thus, the conserved NP sequence is the major

Fig. 1: Viral replication.

The influenza virus attaches to the host cell and uses the host cell mRNA to produce new viral mRNA, enabling the production of new virus.



target of cross-reactive cytotoxic T cell generated against all influenza virus subtypes.

Another type-specific antigen of influenza virus is the matrix 1 protein (M1), which is encoded on one of the influenza virus RNA segments. The amino acid sequence of M1 is highly conserved among the influenza A virus subtypes. M1 interacts with the ribonucleoprotein structures. An interaction of M1 and NP has been proposed to be required for the production of the ribonucleoproteins (Yasuda et al., 1993).

Transcription into viral mRNA requires capped (m^7GppNm-containing) RNA fragments derived from host cell RNA polymerase II transcripts (Bouloy et al., 1978) (Plotch et al., 1981), which are generated by a viral cap dependent endonuclease. Viral mRNA chains are elongated by the addition of a fifteen to twenty-two base stretch of uridine residues. Transcription is then completed by the addition of a polyadenylated (poly A) tail (Hay et al., 1977; Robertson et al., 1981). The production of full-length viral mRNA transcripts is necessary for replication of influenza virus. The viral template RNAs or full-length copies of cellular RNA are initiated without a primer and do not terminate with a poly A tail. Template RNAs are copied into virus-specific mRNAs in the nucleus of the host cell and are translated into proteins in the cytoplasm. Hemagglutinin and neuraminidase are synthesized on membrane bound ribosomes of the endoplasmic reticulum and translocated across the membrane of the endoplasmic reticulum into the smooth endoplasmic reticulum for maturation (Elder et al., 1979). Once the glycoproteins have been correctly assembled within the endoplasmic reticulum, the proteins are transported from the endoplasmic reticulum to the Golgi apparatus. The viral RNAs and glycoproteins then form new influenza viral particles, which bud from the surface of the host cell. Completion of the influenza viral process occurs by pinching off the budding particle at the host cell surface.

Measurement of immune responses to influenza

Humoral immune responses to influenza vaccines are measured via the hemagglutination inhibition assay (Verbonitz et al., 1978). Studies in the older adult population have shown a decline in the cell-mediated immune response in comparison to the young adult population, while there was little or no decline in the humoral immune response (Ferry et al., 1979; Gross et al., 1989; Gross et al., 1997; Dorrell et al., 1997). Although the efficacy of influenza vaccination has been measured using antibody responses to influenza, the antibody responses often do not predict protective immunity in the older adults (Hobson et al., 1973). In young adults, the humoral immune response to influenza vaccination has been found to fluctuate, and do not correspond with severity of influenza illness (Fries et al., 1993). These antibody assay studies suggest protection from influenza infection may be more reliably measured using assays of the cell-mediated immune response in the older adult population.

The humoral and cell-mediated immune responses to infection with influenza virus result in the production of a variety of cytokines by virus-activated T-cells. The cytokines stimulate the B-cells and T-cells. The activated B-cell population differentiates and produces antibodies specific for the strains of influenza (A/H_1N_1, A/H_3N_2, B) within the vaccine (Mitchell et al., 1985; Virelizier et al., 1974). The antibodies bind to the glycoproteins, neuraminidase and hemagglutinin, on the surface of the viral particle resulting in the neutralization of the viral particle. The virus is able to evade the immune response induced from prior infection with related influenza viruses, by naturally slightly altering the peptide sequences of the viral glycoproteins (termed antigenic drift) leading to outgrowth of new influenza virus strains. The changes of influenza virus resulting from antigenic drift necessitate annual modifications of the

virus strains contained in the influenza vaccine if it is to remain antigenically similar in the subsequent season to the predicted circulating strains of influenza.

Humoral and cellular immune responses to influenza

Influenza virus is initially taken up by antigen-presenting cells such as macrophages and dendritic cells (Figure 2). Dendritic cells capture influenza virus or influenza viral antigen and migrate into the draining lymph nodes where they stimulate T-cell responses. Dendritic cells are the most potent inducers of T-cell responses (Bender et al., 1996; Romani et al., 1996). The activated macrophages present influenza viral proteins and produce proinflammatory cytokines to the T cells. The production of cytokines by the influenza virus-activated T cells results in stimulation of B cells to differentiate and produce influenza strain-specific antibodies (Goidl et al., 1976).

Precursor cytotoxic T cell ($CD8^+$) maturation towards lytic effector cells involves a multi-step inductive process, composed of a variety of regulatory events. Activation of precursor cytotoxic T cell requires co-stimulation through accessory and adhesion molecules, as well as by receptors for lymphokines (Weiss and Littman, 1994). $CD4^+$ T cells and major histocompatability complex class II T cells regulate effector cytotoxic T cell responses by providing stimulatory or inhibitory signals (Bretscher, 1992). The production of interleukin 2 by T helper type I cells and interleukin 4 by T helper type II cells has been reported to be essential for the maturation of precursor cytotoxic T cells (Widmer et al., 1987). A secondary stimulation of the effector cytotoxic T cells with influenza virus results in an immune response in the regional lymph nodes, resulting in a significant increase in the number of effector cytotoxic T cells (Flynn et al., 1988). The effector cytotoxic T cells migrate to the spleen, where they become either terminally

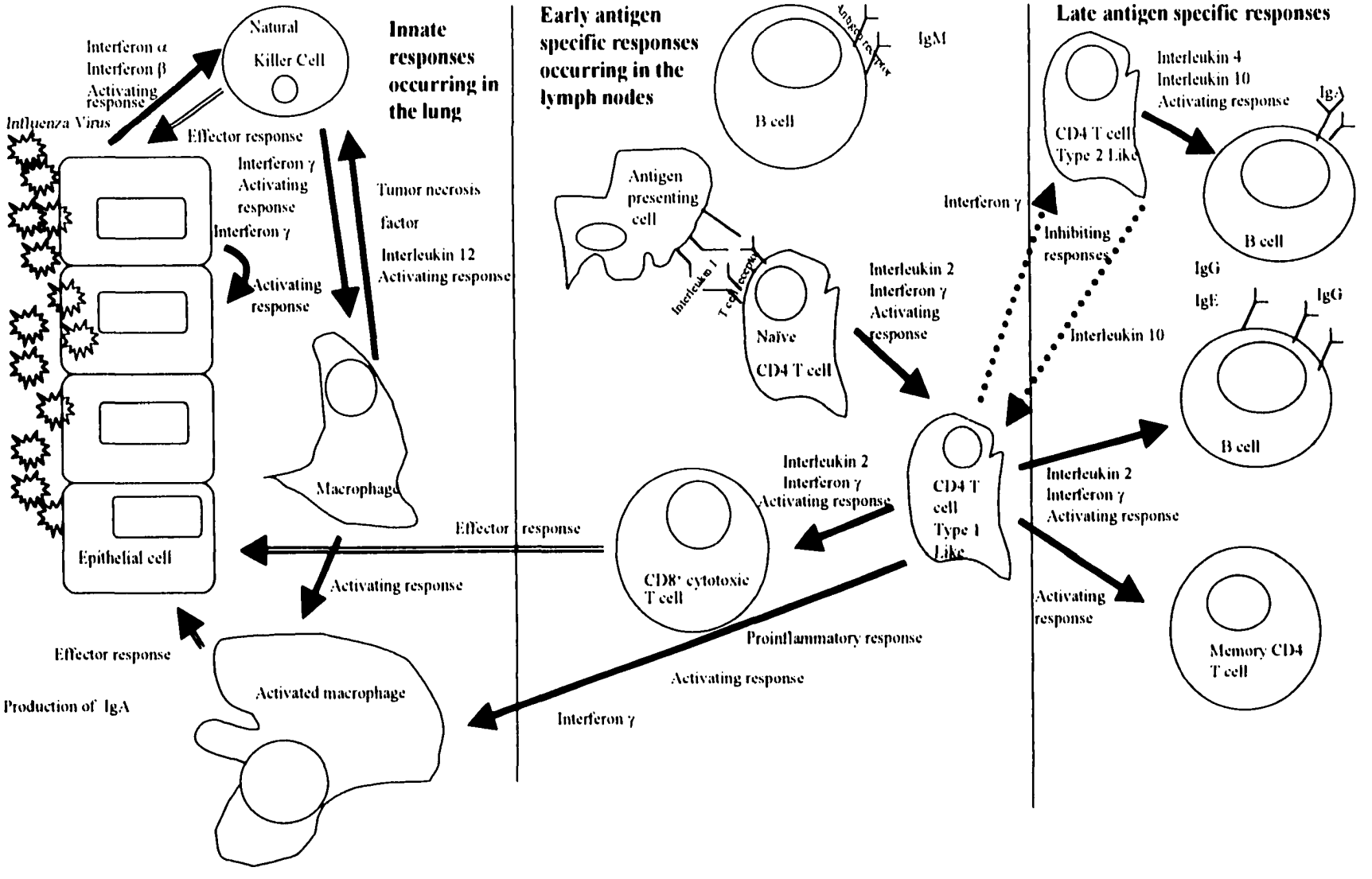


Fig. 2: Immune Responses to Influenza Virus.

differentiated effector cytotoxic T cells or resting precursor memory cytotoxic T cells. Upon presentation of influenza virus antigen, precursor memory cytotoxic T cells respond to internal influenza viral proteins (matrix protein, nucleocapsid proteins), which are more conserved amoung influenza viral subtypes than highly variable surface glycoproteins, and differentiate into effector memory cytotoxic T cells. Effector memory cytotoxic T cells in mice respond to several influenza viral antigen peptides, thus ensuring the ability to clear influenza virus infected cells despite the reassortment in influenza glycoproteins (Van Bleek and Nathenson, 1990; Vitiello et al., 1996).

Influenza viral proteins are processed and presented as peptide antigens by the major histocompatability complex to activate T cells. In contrast to cytotoxic T cells, T helper cells recognize antigens presented by major histocompatability complex class II, which is expressed almost exclusively on cells of the immune system (Hackett and Eisenlohr, 1990; Demotz et al., 1990). Cytotoxic T cells recognize influenza viral peptides on major histocompatability complex class I (Ennis et al., 1981). While the influenza viral peptides presented with major histocompatability complex class I are generally believed to be products of viral replication within antigen presenting cells, the presentation of peptides on major histocompatability complex class I occur via non-classical pathways. One of these non-classical pathways of activation occurs after the presentation of major histocompatability complex class I attached to antigen (Morrison et al., 1986). Thus, T helper cells and cytotoxic T cells have different requirements in terms of the form of influenza virus required for stimulation.

Inactivated influenza virus vaccines stimulate cytotoxic T cells, as well as T helper cells (Lamb et al., 1982; Askonas et al., 1982). Stimulation with live attenuated influenza virus vaccines induce a better humoral immune response in serum or nasal

mucosa than stimulation with inactivated influenza vaccines in children and young adults while little or no response is observed in the older adult population (Clements et al., 1984; Johnson et al., 1986). Studies completed with live attenuated influenza virus vaccines have resulted in a broader immune response to influenza, including the production of secretory antibodies (Wright et al., 1982). The increased immune response after stimulation with live attenuated influenza vaccines is believed to result from the presence of the influenza nucleoproteins (fragments of non-transmemebrane proteins presented in their native confirmation rather than as a denatured protein fragment to the cytotoxic T cell), which stimulate the cellular immune response (Townsend and McMichael, 1985). The cellular immune response to live attenuated influenza vaccine induce influenza virus specific cytotoxic T cells (Daisy et al., 1981; Ennis et al., 1981), resulting from six influenza RNA genes (PB1, PB2, PA, NP, M, NS) and two genes encoding influenza surface glycoproteins (hemagglutinin and neuraminidase) within the live vaccine.

The cell types involved in the humoral and cellular mediated immune response have been shown to vary in specificity to influenza. However, the humoral immune response changes in response to antigenic drift of the various types of influenza (Russell and Liew, 1979; Butchko et al., 1978). The antigenic determinants of the cell mediated immune response (helper T cells and cytotoxic T cells) are more conserved within the various types of influenza (A/H_3N_2 vs. A/H_1N_1). The peptides of neuraminidase and hemagglutinin serve as antigenic determinants of the helper T cell response and are cross-reactive within influenza subtypes H_3N_2 vs. H_1N_1 (Mitchell and Callard, 1983). The nucleoproteins of influenza virus are conserved within the types of influenza (A/H_3N_2 vs. A/H_1N_1). Cytotoxic T cell responses are stimulated by the internal viral

proteins (nucleocapsid proteins, matrix proteins) and are cross-reactive for all strains within influenza types A vs. B. While B cell memory for influenza is life-long, subtype and strain-specific, T cell memory is more cross-reactive within various types of influenza due to the genetic similarities of the nucleoproteins of the influenza subtype A viruses (Fischer et al., 1982). The duration of T cell memory in response to influenza vaccination has been shown to persist over periods from months to years in the healthy young adult population (Ennis et al., 1981; McMichael et al., 1983). Understanding of the changes in magnitude of T cell memory after influenza vaccination, especially in older adults, should enable a better prediction of the severity of influenza illness.

Destruction of influenza infected cells

Virus-specific cytotoxic T cells recognize and destroy virus-infected host cells. Virus-infected cells expressing viral proteins on the major histocompatibilty complex I on the cell surface are recognized by and activate the cytotoxic T cells (Ennis et al., 1981). Once the activation of cytotoxic T cells has occurred, perforin-mediated killing pathway (Podack et al., 1985; Masson and Tschopp, 1985; Pasternack and Eisen, 1985; Bleackley et al., 1988; Jenne and Tschopp, 1988) and fas-mediated killing pathway (Itoh et al., 1991; Rouvier et al., 1993) are activated, as well as an increase in the production of interferon-γ (Ennis and Meager, 1981; Taylor et al., 1985; Yamada et al., 1986). The lytic properties of perforin-mediated killing suggest this pathway play an important role in the control of viral influenza infection. Fas-mediated killing has been shown to be an alternate pathway of cell lysis (Doherty et al., 1997).

Cytotoxic T cells contain pore-forming proteins (perforins) and granzymes (granzyme A through H) within the granules of cytotoxic T cells. Activation of

cytotoxic T cells, through contact with the viral infected target cell, results in the mobilization of granules to the surface of the cytotoxic T cell (Shi et al., 1992; Shi et al., 1997). The precursor form of granzyme B is then processed by a dipeptidyl peptidase I, enabling the removal of an amino-terminal peptide, resulting in the activated form of granzyme B (Smyth et al., 1995; Caputo et al., 1993; McGuire et al., 1993). Granzyme B can enter the cytoplasm of the target cell, where it remains until perforin reaches the target cell (Jans et al., 1996; Pinokoski et al., 1996; Trapani et al., 1996). Through an amphipathic pore complex created by 20-perforin monomers (Young et al., 1986), the granzymes are also able to traffic across and enter the cytoplasm of the target cell. The presence of perforin within the target cell enables granzyme B to enter the nucleus of the target cell (Trapani et al., 1996). The granzymes then initiate an enzymatic cascade, which will lead to DNA fragmentation and cell death (apoptosis) (Helgason et al., 1993; Darmon et al., 1995). One fundamental function of granzyme B is to mimic the cleavage specificity of the caspase family of pro-apoptotic cysteine proteases. Thus, granzyme B is able to cleave the caspase precursors, resulting in the autocatalytic activation of the caspases which cause apoptosis (Fernandes-Alnemri et al., 1996; Darmon et al., 1996).

Fas-mediated killing results from the interaction of fas ligand on the surface of activated cytotoxic T cell and fas receptor on the target cell. Fas ligand expression on the cytotoxic T cell is up-regulated via signaling through the T-cell receptor (TcR)-CD3 complex, resulting in the expression of fas ligand on the surface of the cytotoxic T cell (Anel et al., 1994; Vignaux et al., 1995). Fas ligand expressed on the cytotoxic T cell surface rapidly signals target cell apoptosis upon engagement of the membrane bound fas receptor present on target cells (Hanabuchi et al., 1994; Suda et al., 1993; Ramsdell et al., 1994) by activating a family of caspases ("death domain") in the intracellular terminus of

the fas receptor. The "death domain" contains many of the caspases activated by granzyme B, suggesting a convergence of the two distinct pathways involved in apoptosis (Depraetere and Goldstein, 1997; Atkinson et al., 1998). The suggested primary role of fas-mediated killing is the downregulation of the immune response by eliminating autoreactive T cells in the periphery (Kagi et al., 1994; Mueller et al., 1998). There is evidence that the dysregulation of the fas/fas ligand regulatory mechanism and perforin-mediated killing results in the acceleration of lymphoproliferative disease and mortality (Peng et al., 1998). Thus, the expression of fas ligand must be tightly controlled because the presence of fas on a number of tissues within and outside the immune system could make uncontrolled expression deadly (Ogasawara et al., 1993).

A comparison of perforin-mediated killing with fas-mediated killing (measured by cytotoxic T cell cytotoxicity assays in the presence of calcium) showed that perforin-deficient cytotoxic T cells have significantly less cytolytic activity against influenza infected cells (Lee et al., 1996). Once the lethal hit of granzyme B is delivered to a target cell via the perforin pore; the target cell will recognize the presence of perforin and granzyme B; although it has also been signaled to apoptose through the fas receptor. Thus, perforin cytotoxicity in the presence of calcium may mask fas-mediated killing.

The ability to clear influenza infected cells has been associated with an increase in granzyme B production. The granzyme B gene is located at the 5' flanking end on chromosome 14; the genes for granzyme H, casthepsin G and mast cell chymase located on chromosome 14 as well (Hanson and Ley, 1992). Granzyme B synthesis is believed to be regulated by a locus-control-like element upstream of the serine protease gene cluster on chromosome 14. The locus-control-like element interacts with tissue-specific elements near each gene to permit high-level, linear-specific expression of the gene

(Grosveld et al., 1987; Ley, 1991). The expression of granzyme B increases within activated cytotoxic T cells, suggesting it plays a vital role in cytotoxic T cell-mediated apoptosis (Mueller et al., 1998; Meier et al., 1990; Lobe et al., 1986). The genetic deletion of perforin in mice resulted in the inability to clear influenza virus-infected target cells, illustrating the necessity of perforin for the mechanism of cytotoxic T cell-mediated killing (Kagi et al., 1994). A study in double knockout mice for granzyme A and granzyme B showed granzyme B is required for the initial stages of DNA fragmentation in influenza virus infected cells, suggesting it plays a key role in viral target cell lysis (Simon et al., 1997). These studies suggest that levels of granzyme B activity may be a critical component of influenza specific viral immunity.

Effect of aging on cytotoxic T cell response to influenza

The magnitude of the cytotoxic T-cell response to influenza decreases in the aging-mouse model (Bender et al., 1995). Both the mouse model and in vitro human peripheral blood mononuclear cell cultures, provide evidence of the decline in cytotoxic T-cell activity with age (Effros and Walford, 1983; Powers, 1993; Thomas and Weigle, 1989). The effect of aging on the cytotoxic T-cell response to influenza in human beings has been shown to result in a decreased peak cytotoxic T-cell activity, while the duration of the cytotoxic T-cell response appears similar to the young adult population (Powers and Belshe, 1993; Mbawuike et al., 1993). While inactivated influenza vaccines are able to stimulate the cytotoxic T cell in people (older and chronically ill adults), the use of live-attenuated vaccines increases the cross-reactivity of the cytotoxic T-cell responses for the subtypes of influenza (Gorse and Belshe, 1990). The variations in the response to

influenza in older adults may occur due to the type of vaccine and ultimately, the way the influenza antigens are presented to the immune cells.

The older adult population may have an increased susceptibility to complication of influenza infections due to a decreased T-cell response (Gottesman et al., 1985; Powers and Belshe, 1993), rather than the primary B-cell failure (Goidl et al., 1976). Older healthy adults have decreased antibody responses to influenza due to the fact that antibody responses are T-cell dependent (Levine et al., 1987; Gross et al., 1988). The variation in the immune response to influenza vaccination within the older adult population may be due to chronic disease, prior exposure to influenza, and the aging process (McElhaney et al., 1998). Studies regarding age-related changes of the T cell response to influenza vaccination have reported that the influenza virus-specific cellular immune responses in older adults to be significantly reduced prior to vaccination in comparison to the young adult population. The older adult population's cytotoxic T-cell response after influenza vaccination increased to the level observed in the young adult population prior to vaccination, while the cytotoxic T cell response of the young adult population after vaccination remained similar to their response prior to vaccination (Powers, 1993; McElhaney et al., 1994). Although studies by Ennis, Gorse and Belshe, and Plebanski have determined the cytotoxic T cell response to influenza vaccination, none of these studies have compared the responses between different age groups. The regulation of the cytotoxic T cell mechanisms which respond to influenza vaccination are likely to be an important component of protection from influenza, especially for the older adult.

Studies of the cytotoxic T cell responses in people have been limited by the small number of participants and few have included older adults. This has been due to the

labor-intensive techniques required to complete chromium (^{51}Cr) release assays for measuring cytolytic activity. The inter-assay variability of the ^{51}Cr-release assay also has prevented the direct comparison of data from one study to the next. ^{51}Cr-release assays require a six-day culture of T-cells populations, such that proliferation and differentiation under in vitro conditions may not reflect the in vivo in the response to vaccination. By measuring granzyme B responses to influenza vaccination instead of ^{51}Cr release assays, larger study groups can be used (McElhaney et al., 1996), and the inter-assay variability of the vaccine response measure reduced. Granzyme B is a measure of cytotoxic T cell activity, which has substrate specificity due to an acetic acid side chain located at the aspartyl residue (termed P1 site) (Poe et al., 1991; Caputo et al., 1993; Odake et al., 1991), allowing it to be measured via colorimetric detection of the cleaved substrate. The hydrolysis of the preferred substrate of granzyme B (N-t-butyloxycarbonyl-L-alanyl-L-alanyl-L-aspartyl-paranitroanilide) after the aspartic acid amino acid provides a specific assay of granzyme B (Odake et al., 1991). A comparison study of cytotoxic T cell response to influenza showed that measurement of granzyme B activity is as sensitive an indicator of cytotoxic T cell activity as the ^{51}Cr-release assay (Butchko et al., 1978; McElhaney et al., 1996). Increased granzyme B activity have been found in virus-stimulated peripheral blood mononuclear cells from institutionalized older adults after influenza illness with lower respiratory tract symptoms (McElhaney et al., 1994). The granzyme B activity of older adults appears to have decreased in response to the more recently circulating compared to older strains of influenza (McElhaney et al., 1998). The observed variations in magnitude of the cytotoxic T cell response in older adults may result from age-related changes in the cytotoxic T cell mechanisms.

Research objectives

The cell-mediated immune response is affected by the aging process and results in changes in the magnitude of the cytotoxic T cell response. The decreased cytotoxic T cell response in healthy older people may result from decreases in levels of granzyme B activity. The long-term objective of this research is to identify components of cytotoxic T-cell killing (perforin-mediated killing and fas-mediated killing) which decline with aging. This research will accomplish the following specific aims:

1. To characterize the cytotoxic T cell response to influenza vaccination in ex vivo virus-activated peripheral blood mononuclear cells, as a measure of the in vivo response. Our hypothesis was that ex vivo virus-activated peripheral blood mononuclear cells would respond similarly to the *in vitro* virus-activated peripheral blood mononuclear cells in the young adult population. Using *ex vivo* and *in vitro* peripheral mononuclear blood cells stimulated with three different live H_3N_2 strains of the influenza subtype A virus, the *ex vivo* and *in vitro* levels of granzyme B activity assay and virus-specific cytotoxic T cells were measured. The number of influenza virus-specific $CD8^+$ T cells were monitored using enzyme-linked immunospot (ELIspot) assay, quantitating the number of virus-specific memory cytotoxic T cells within the total cell population. We expected the number of influenza virus-specific T cells to correlate with the levels of granzyme B activity due to the fact granzyme B is produced within the cytotoxic T cells. We compared the levels of granzyme B activity in *ex vivo* and *in vitro* cultures for each strain of influenza virus to determine if there were independent effects from the *in vitro* cell cultures, which may have affected the levels of granzyme B activity. The number of virus-specific cytotoxic T cells and granzyme B levels were measured to distinguish

between a qualitative and a quantitative difference in the response to influenza vaccination.

2. *To determine the effect of age on the T cell response in ex vivo virus-stimulated peripheral blood mononuclear cells as a measure of the in vivo response.* Our hypothesis was that the levels of granzyme B activity are lower in vaccinated healthy older adults in comparison to vaccinated healthy young adults. We determined the effect of age on the magnitude of the cytotoxic T cell response to influenza in healthy vaccinated older adults (<65 years). Using *ex vivo* and *in vitro* peripheral mononuclear blood cells from healthy older people stimulated with three different live H_3N_2 strains of influenza subtype A virus, *ex vivo* and *in vitro* levels of granzyme B activity and virus-specific cytotoxic T cells were measured. The *ex vivo* granzyme B levels were compared to the *in vitro* granzyme B levels in each of the age groups. We expected the granzyme B activity in the healthy older adults to be less than the young adults. We expected the number of influenza virus-specific cytotoxic T cells to correlate with the *ex vivo* granzyme B activity within the age groups. The determination of the *ex vivo* cytotoxic T cell response to various H_3N_2 strains of influenza type A virus stimulation in older adult people illustrated that the in vitro response to vaccination likely reflects the in vivo influenza virus specific response.

CHAPTER II

MATERIALS AND METHODS

Preparation of live influenza virus

Sixteen dozen fertilized chicken eggs were incubated for ten days in a high hatch egg incubator (Catalog Number 2GHHI) (Murray McMurray Hatchery, Inc., Webster City, IA) at 101°F. By holding the embryonated chicken eggs to a narrow light source, the eggs were determined to be alive or dead in the presence or absence of chicken embryo movement. The eggs containing a live embryo were divided into three separate groups and inoculated with influenza A/H_3N_2 seed viruses (A/Sydney/05/97, or A/Nanchang/933/95, or A/Johannesburg/33/94, which had been generously donated by Dr. Nancy Cox at the Center of Disease Control, Biological Products Division) and 2500 mg/1000 units of penicillin/streptomycin. The inoculation site on the chicken eggs was sealed with a drop of Elmer's glue. Several of the live chicken eggs were not inoculated with influenza seed virus, enabling the production of allantoic fluid, which was used as a negative control in the following experiments. The chicken eggs were incubated for seventy-two hours in the egg incubator at 101°F and determined to be alive or dead in the presence or absence of chicken embryo movement. Then, the live chicken eggs were incubated overnight at 4°C (to kill the embryo).

The influenza virus was harvested as described by the viral harvesting protocol from the University of Wisconsin (a standard methodology). By opening of the egg shell housing the air sac around the chicken embryo, the shell membrane and chorio-allantoic

membrane were able to be peeled away, enabling the removal of allantoic fluid containing live influenza virus. The influenza virus was sterile filtered through 0.45 micron filtering units and stored at -80°C.

Titration of influenza virus

The 50% tissue culture infective dose ($TCID_{50}$) of live influenza virus was determined as described by Cottey et al. (1997). In a sterile round-bottomed 96-well plate, 90 µL of Dulbecco's minimum essential medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 5% fetal bovine serum, 50 µg/mL gentamicin, 100 units/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B was added into each well. A 200 µl aliquot of Madin-Darby canine kidney cells (concentration of 2 x 10^5 cells/mL) was added to each well of the plate. A 100 µL aliquot of a 1:10 dilution of the stock influenza A/H_3N_2 virus was pipetted into the first well. The influenza A/H_3N_2 virus was serially diluted across the 96-well plate (wells numbered 2 to 11). 100 µL of DMEM supplemented with 5% fetal bovine serum, 50 µg/mL gentamicin, 100 units/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B was added to the well numbered 12 of each row of the plate, serving as the negative control. The 96-well plate was covered and incubated overnight at 37°C, 5% CO_2. Following the incubation, the supernatant (90 µL) contained in each well was aspirated and discarded. A 200 µL aliquot of DMEM supplemented with 0.0002% trypsin was added to each well of the plate. The plate was covered and incubated for 72 hours at 37°C, 5% CO_2. The plate was removed from the incubator and 50 µL of 0.5% chicken red blood cell (Treslow Farms, Chester Town, MD)/phosphate buffered saline suspension was added to

each well. After the 96-well plate had incubated for one hour at 4°C, the agglutination patterns for the influenza A/H_3N_2 virus was recorded. The 50% tissue culture infectious dose ($TCID_{50}$) for the stock influenza A/H_3N_2 was calculated using the method of Reed and Muench (1938).

Isolation of peripheral blood mononuclear cells from venous blood

Peripheral blood mononuclear cells were isolated as described by McElhaney et al. (1994) with minor modifications. 30 mL of venous blood was collected in heparinized tubes from the subject volunteers at several time points (prior to vaccination, four weeks post vaccination, twelve weeks post vaccination, and thirty-six weeks post vaccination). The venous blood sample was layered on a Ficoll-Paque (Sigma, St. Louis, MO) and centifuged at 250 x g for 30 minutes. Peripheral blood mononuclear cells were collected from the interface and washed in phosphate buffered saline solution and centrifuged at 250 x g for 12 minutes. The peripheral blood mononuclear cell pellet was washed a second time in phosphate buffered saline solution and centrifuged for 250 x g for 12 minutes. The cells were resuspended in 3 mL of Aim V medium (Gibco BRL, Grand Island, NY) and the cell number was calculated using a hemacytometer. The cells were diluted to a final concentration of 1.5×10^6 cells per mL in Aim V medium.

Production of Dynabeads coated with CD56

Dynabeads were coated with purified anti-CD56 antibody (a surface marker for natural killer cells) using the Dynal protocol from the package insert (Product Number 110.22, Dynal, Lake Success, NY), to remove natural killer cells from the peripheral blood mononuclear cell cultures. 450 µL of Dynabeads Pan Mouse IgG was aliquotted

into a 5 mL snapcap test-tube and placed in the Dynal MPC (magnetic device). The test-tube containing the Dynabeads was attached to the magnetic device and the storage solution was removed. The Dynabeads were resuspended in 1 mL of wash solution (phosphate buffered saline/0.1% bovine serum albumin). The Dynabeads were washed twice and resuspended in 450 μL of wash solution. Purified anti-CD56 antibody (1 μg/mL) (PharMingen International, San Diego, CA) was added to 50 μL Dynabeads Pan Mouse IgG (25 μL/1 μg of antibody). Dynabead Pan Mouse IgG/antibody solution was incubated for 30 minutes at 4°C with gentle tilting and rotation. The Dynabeads Pan Mouse IgG/antibody beads were separated from the supernatant using the magnetic device for 2.5 minutes at 22°C. The anti-CD56 coated Dynabeads were washed three times in 1 mL of wash solution and resuspended in 450 μL wash solution. The anti-CD56 coated Dynabeads were used in the procedure for negative selection of $CD8^+$ T cells.

Negative selection for $CD8^+$ T cells

Peripheral blood mononuclear cells were prepared at concentrations of 1 x 10^6cells/mL and were chilled to 4°C. Peripheral blood mononuclear cells were incubated with 144 μL of Dynabeads $CD4^+$ (which contain antibodies to CD4) (Dynal, Lake Success, NY) and anti-CD56 coated Dynabeads (50 μL/mL of cells) for 30 minutes at 4°C with gentle tilting and rotation. The peripheral blood mononuclear cells/Dynabeads solution was placed into the magnetic device for 2.5 minutes at 22°C, to remove the natural killer (NK) cells and $CD4^+$ T cells and ensure the only interferon-γ producing cells within the cultures would be $CD8^+$ T cells. The peripheral blood mononuclear cell

supernatant was transferred into a fresh test-tube. The number of $CD56^-$ $CD4^-$ peripheral blood mononuclear cells was determined for use in the ELIspot assay.

Depletion of $CD8^+$ T cells

Peripheral blood mononuclear cells were prepared at concentrations of 1 x 10^6cells/mL, chilled to 4°C, and incubated with 144 µL of Dynabeads $CD8^-$ (Dynal, Lake Success, NY) for 30 minutes at 4°C with gentle tilting and rotation. The peripheral blood mononuclear cells/Dynabeads solution was placed into the magnetic device for 2.5 minutes at 22°C, which allowed the collection of the $CD8^-$ coated Dynabeads. The peripheral blood mononuclear cell supernatant was transferred into a fresh test-tube. The number of $CD8^-$ peripheral blood mononuclear cells obtained was determined for use in the ELIspot assay.

***Ex vivo* stimulation**

Cell cultures were prepared at concentrations of 2.25 x 10^6cells/well in 24 well plates (Costar, Corning International, Corning, NY). The cells were stimulated with live influenza A/H3N2 strains (A/Sydney/05/97, or A/Nanchang/933/95, or A/Johannesburg/33/94) at a concentration of 10^5 $TCID_{50}$/mL and recombinant human interleukin 7 at a concentration of 25 ng/mL. The cell cultures, which had been stimulated were incubated for 17 hours (37°C, 5% CO_2). The cells were harvested via scraping the well of the plate with a pipette tip and collecting the cells and supernatant into a 1 mL microfuge tube. The cell/supernatant mixture was centrifuged at 170 x g for 10 minutes. The supernatant was then aspirated and discarded. The cell pellets were lysed with 100 µL of a lysis buffer (1% Triton X-100/150mM NaCl/15mM Tris-HCl, pH

8.0). Three freeze-thaw cycles (Incubation in ethanol/dry ice bath for 1 minute and 37°C water bath for 1 minute, followed by through vortexing is the equivalent to one freeze-thaw cycle.) were completed on the cell lysates to ensure complete lysis had occurred. The cell lysates were frozen at -80° C until time of assay for granzyme B activity. Cell nuclei were pelleted from the lysate by centrifugation at 225 x g for 10 minutes at 4°C prior to the assay for granzyme B activity.

***In vitro* stimulation**

Cell cultures were prepared as described for the *ex vivo* stimulation procedure with several modifications. The cell cultures were incubated for 6 days (37°C, 5% CO_2) in the absence of recombinant human interleukin 7.

Determination of protein concentration

Total protein in the peripheral blood mononuclear cell lysates was measured using the bicinchoninic acid (BCA) assay (Pierce Biotec Company, Rockford, IL). Bovine serum albumin (BSA) was used as the standard. To determine the protein concentration of the cell lysates, the 2 mg/mL BSA stock solution was used to make the solutions used in the standard curve. The standard curve consisted of the following dilutions of BSA in lysis buffer (1% Triton X-100/150mM NaCl/15mM Tris-HCl, pH 8.0) – 0 μg/mL BSA, 62.5 μg/mL BSA, 125 μg/mL BSA, 250 μg/mL BSA, 500 μg/mL BSA, 750 μg/mL BSA, 1000 μg/mL BSA, 1500 μg/mL BSA, and 2000 μg/mL BSA. 10 μL of each standard or cell lysate sample was pipetted in triplicate into a 96 well flat bottom plate. 200 μL of BCA working reagent (50 parts BCA Solution A containing

sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.2 N NaOH) and 1 part BCA Solution B (containing 4% $CuSO_4$) was aliquotted into each well. The plate was mixed on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 35 minutes. Following the incubation, the plate was allowed to cool for 5 minutes at 22°C. The absorbance of the samples was read at 562 nm using a microtiter plate reader (PowerWave X, Bio-Tek Instruments, Inc.) using KC4 (Kinetcalc for Windows). The absorbance reading of the blank sample was subtracted from the standards and samples. A standard curve was plotted to determine the protein concentration of the samples in mg of protein.

Preparation of YT lysates

YT (human natural killer leukemia cell line) cells were grown at a concentration of 1 x 10^6 cells/mL in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 mM 2-β-mercaptoethanol, 10 mM hepes (pH 7.2) (Gibco BRL, Grand Island, NY), and 10 mM non-essential amino acids (Gibco BRL, Grand Island, NY). The YT cells were stimulated with 90 units/mL of interleukin 2 for 24 hours at 37°C, 5% CO_2. Following the incubation, the cells were centrifuged at 170 x g for 12 minutes. The supernatant was discarded and the cells were resuspended in 50 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 mM 2-β-mercaptoethanol, 10 mM hepes (pH 7.2), and 10 mM non-essential amino acids. The cells were incubated for 24 hours at 37°C, 5% CO_2. Following the incubation, the cells were centrifuged at 170 x g for 12 minutes. The supernatant was discarded and the cell density was determined. The YT cells were centrifuged at 170 x g for 12 minutes. The supernatant was aspirated and discarded. Using the cell density, the volume of lysis buffer (1% Triton X-100/

150mM NaCl/15mM Tris-HCl, pH 8.0) which was added to the YT cell pellet was determined by the following equation:

[Total live YT cells/2 x 10^6 cells] x 50 = μL of lysis buffer to be added

The YT cell pellet was lysed with the calculated volume of lysis buffer. Two freeze-thaw cycles were completed on the YT cell lysate to ensure complete lysis had occurred. The initial concentration of YT lysate was arbitrarily determined to be 100 units/mL. The cell lysates were aliquotted into microfuge tubes, containing 30 μL volumes.

The YT lysates were calibrated by the completion of a seven point standard curve. The YT lysates were diluted in lysis buffer using two fold dilutions from 50 units/mL to 0 units/mL. 10 μL of the various dilutions of the YT lysate was aliquotted into a 96 well flat-bottom plate. 40 μL of 2.5 mM Boc-Ala-Ala-Asp-paranitroanilide (BACHEM, Torrance, CA) and 150 μL of sterile distilled water were added to each well in the plate. After a 18-hour incubation, absorbance of the samples was read at 405 nm using a microtiter plate reader (PowerWave X, Bio Tek Instruments, Inc.) using KC4 (Kinetcalc for Windows) and a standard curve was plotted. The initial YT preparation standard curve was compared to further YT lysate preparations. Upon completion of the calibration of the YT lysates, the lysates were frozen at -80° C until time of assay for granzyme B activity.

Assay for granzyme B activity

Triplicate samples containing 10 μL of peripheral blood mononuclear cell lysate and 40 μL of 2.5 mM Boc-Ala-Ala-Asp-paranitroanilide was assayed for each sample in

200 μL reaction volumes. Cell-lysates of influenza-stimulated peripheral blood mononuclear cell were prepared as described above. YT lysates as described above were used as the standards for this assay. Unstimulated peripheral blood mononuclear cell (cultured on days 0 and 6) were included as negative controls. After a 12-hour incubation, absorbance of the samples was read at 405 nm using a microtiter plate reader (PowerWave X, Bio-Tek Instruments, Inc.) using KC4 (Kinetcalc for Windows). The absorbance reading of the blank sample was subtracted from the standards and samples. A standard curve was plotted, enabling the granzyme B activity of the samples to be determined as units of absorbance at 405 nm.

The granzyme B activity was reported as the units/mg protein in the lysate. The granzyme B activity was determined in all cellular lysates using the following formulas:

lysate (units of absorbance at 405 nm) –blank (units of absorbance at 405 nm) = z

z/absorption at 405 nm of YT lysate standard = y

y x concentration of YT lysate standard(units) = granzyme B activity of lysate

$$\frac{\text{granzyme B activity of lysate (units of absorbance at 405 nm)}}{\text{protein concentration of the lysate (mg protein)}}$$

ELIspot assay of interferon γ

The number of interferon gamma producing cells in the peripheral blood mononuclear cell cultures was determined as described by Czerkinsky et al. (1983) with minor modifications. Ninety-six well nitrocellulose plates (Millititer, Millipore, Bedford, MA) were coated with 50 μL mouse anti-human interferon γ monoclonal antibody (Mabtech, Stockholm, Sweden) in coating buffer pH 9.5 (15 mM Na_2Co_3/35

mM $NaHCO_3$/3.1 mM NaN_3) overnight at 4°C. The wells were washed with phosphate buffered saline (pH 7.3) (1.9 mM NaH_2PO_4/8.1 mM Na_2HPO_4/154 mM NaCl) supplemented with 0.25% Tween 20 (Sigma, St. Louis, MO) and blocked with phosphate buffered saline supplemented with 5% fetal bovine serum for thirty minutes at 37°C. A 100 µL aliquot of RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% human anti-AB serum, 2 mM L-glutamine, 50 µM 2-β-mercaptoethanol, 100 units/mL penicillin and 100 µg/mL streptomycin was incubated on the plates for ten minutes, diluting any remaining Tween 20. Two fold dilutions of an aliquot of 100 µL/well of peripheral blood mononuclear cells (concentration/well: 5 x 10^6/mL to 5 x 10^5/mL), from which $CD56^+$ (natural killer cells) and $CD4^+$ (T helper cells) had been removed, were stimulated with three different H_3N_2 strains of live influenza A virus (A/Sydney/05/97, or A/Nanchang/933/95, or A/Johannesburg/33/94) (concentration of 10^5 $TCID_{50}$/mL) and 25 ng/mL interleukin-7 in the antibody-coated plates and incubated for 17 hours (37°C, 5% CO_2). Serial dilutions of unstimulated peripheral blood mononuclear cells were added to the antibody-coated plates and incubated for 17 hours (37°C, 5% CO_2), serving as the negative control. Serial dilutions of peripheral blood mononuclear cells stimulated with concanavalin A (10 µg/mL) were added to the antibody-coated plates and incubated for 17 hours (37°C, 5% CO_2), serving as the positive control. The plates were washed thirteen times with phosphate buffered saline supplemented with 0.25% Tween 20. The wells were incubated with 50 µL of biotinylated polyclonal mouse anti-human interferon γ antibody (Mabtech, Stockholm, Sweden) in PBS supplemented with 1% bovine serum albumin for two hours at 22°C. After washing the wells six times with phosphate buffered saline/0.25% Tween 20, 100

μL avidin-horseradish peroxidase (Sigma, St. Louis, MO) (1 μg/mL) was added for two hours at 22°C. The wells were washed six times with phosphate buffered saline/0.25% Tween 20 and then, 50 μL/well of aminoethyl carbazole solution (Sigma, St. Louis, MO) (70.3 μg/mL) was added and incubated for 30 minutes at 22°C. Washing the plates three times with distilled water stopped the color development. After allowing the plates to air-dry, the colored spots were counted using a stereomicroscope.

Handling of biohazardous materials

All tissue culture was completed in Forma Scientific Class IIA/B3 Safety Cabinet. All personnel involved in this project had been approved to handle biohazardous materials by the Biosafety Committee at Eastern Virginia Medical School, Norfolk, Virginia. The protocols involving use of live influenza viruses had been reviewed and approved by the Biosafety Committee at Eastern Virginia Medical School, Norfolk, Virginia. All procedures were in accordance with Eastern Virginia Medical School Bloodborne Pathogens Plan and personnel were appropriately trained by the Biosafety Committee at Eastern Virginia Medical School, Norfolk, Virginia.

Use of human subjects

This study was performed at the Glennan Center for Geriatrics and Gerontology, Eastern Virginia Medical School and was reviewed and approved by the Eastern Virginia Medical School Institutional Review Board.

Recruiting: Subjects eligible for each of the studies (see criteria selection below) were competent to sign an IRB approved consent. Human participation required the individual volunteers to be competent to give their own consent. They were initially asked by a health professional designated by the investigators if they were interested in participating. If so, they were interviewed and those who offered, were enrolled with a written, informed consent. For the studies, subjects were enrolled as follows:

Site: the Hampton Roads residential community and long-term care facilities around the Glennan Center. Norfolk ranks second in population in Hampton Roads, with African Americans comprising 39%. A recent recruitment effort targeted older adults belonging to an ethnic minority group yielded 7% participation.

Criteria Selection: These young (ages 18 to 50, 12 females and 4 males) and older adults (ages 63 to 85, 6 females and 4 males) were screened. Inclusion criteria was based upon the following for "healthy" subjects.

The inclusion criterions for subjects in the young and older adult populations were based upon all of the following points.

1. Ambulatory.
2. In reasonably good health.
3. Must not require legal or medical guardian.
4. Available for duration of the study.

The exclusion criterions for the subjects in the young and older adult populations were based upon any of the following points.

1. An acute febrile illness (temperature >100.4°F) within 72 hours prior to enrollment in the study.
2. Elevated vital signs during initial physical exam.
3. Known or suspected allergy to any components of the vaccine (i.e. eggs, or egg products, thimerosal or contact lens solution, or fomaldehyde).
4. Self-reported history of severe adverse reaction to any vaccination.
5. Known or suspected disease of the immune system.
6. Currently or within 3 months of enrollment taking immunosuppressive or immunomodifying medication (i.e. cancer chemotherapeutic agents, steroids).
7. Diabetes being treated with insulin or oral hypoglycemic agents.
8. Receipt of blood or blood products within 3 months prior to enrollment.
9. If female and of child bearing potential, pregnant as confirmed by urine pregnancy test. Women $\geq$ 60 years of age were considered not of childbearing potential.
10. Receipt of any vaccine in the previous 4 weeks or plans to receive another vaccine during the 5 week study period.
11. Current participation in another clinical trial.
12. Participation in any other experimental drug or vaccine trial within the 30 days prior to enrollment.
13. Self-reported receipt of any influenza vaccine since January 1 of the study year.
14. Previous therapy with cadaveric pituitary derived human growth hormone.

15. Any condition, which, in the opinion of the investigator, would pose a health risk to the subject or interfere with the evaluation of the vaccine (e.g. pulmonary disease, cardiovascular disease, etc).

Influenza vaccine: Vaccination with the current influenza vaccine (0.5 mL dose) was a requirement for participation in each year of the study. Influenza virus vaccine 1998-1999 contained 15 μg of hemagglutinin of A/Sydney/05/97 (H_3N_2)-like strain, A/Beijing/262/95 (H_1N_1)-like strain, and B/Beijing/184/93-like strain. Influenza virus vaccine 1999-2000 contained 15 μg of hemagglutinin of A/Sydney/05/97 (H_3N_2)-like strain, A/Beijing/262/95 (H_1N_1)-like strain, and B/Beijing/184/93-like strain. The split virus representing three difference strains of influenza vaccine may change from year to year as influenza viruses antigenically drift.

Risks: The risks of this study involved those associated with the administration of the influenza vaccine, and phlebotomy and were determined to fall within the "minimal risk" levels as defined by the FDA. The United States Public Heath Service currently recommends people over 65 years to be vaccinated as part of their general health maintenance. From 10 to 30% of such vaccine recipients may have experienced mild myalgias lasting one to two days and up to 5% may have experienced a low-grade fever.

Benefits: Protection from influenza and its complications far outweighed the risks associated with vaccination.

Subjects had venipunctures performed on two to three occasions annually. The risks associated with venipuncture included acute mild pain and a remote chance of bruising or infection at the venipuncture site. There was no foreseeable risk associated with the amount of blood taken and the amount of blood taken fell under the "minimal risk" level as defined by the FDA.

Confidentiality: Every effort was made to maintain subject confidentiality. All subjects' data was coded to protect the anonymity of subjects enrolled in the study. No individually identifiable data will be published. A complete record of the subject's pertinent history, clinical progress, and results of laboratory and diagnostic examination was kept on case report forms (which were coded).

Statistical analysis

All data were processed using a computer program, SPSS 9.0. Granzyme B activity levels within the subject populations were initially analyzed by averaging the granzyme B activity levels and calculating the 95% confidence intervals. The frequency of cytotoxic T cells within the subject populations was initially analyzed by averaging the number of interferon γ producing cells and calculating the 95% confidence intervals. The t distribution for the granzyme B activity levels (difference between 4 week post vaccination value and prior to vaccination value) and probability (p) (significance level set at $\alpha = 0.05$) corresponding to the t distribution was calculated for the three different influenza A/H_3N_2 strains. Although the sample sizes of the subject populations were relatively small, the data was assumed to be normally distributed. Mann-Whitney U test was used to determine the differences between the two age groups with respect to the

different influenza A/H_3N_2 strains on the granzyme B activity levels (difference between 4 week post vaccination value and prior to vaccination value), the frequency of cytotoxic T cells (difference between 4 week post vaccination value and prior to vaccination value), and the ratio of granzyme B activity per influenza virus-specific $CD8^+$ T cell. Probability (p) (significance level set at $\alpha = 0.05$) corresponding to the Mann-Whitney U distribution was determined. Although the sample sizes of the subject populations were relatively small, the data was assumed to be similarly distributed.

CHAPTER III

RESULTS: INVESTIGATION OF GRANZYME B ACTIVITY IN THE YOUNG ADULT POPULATION

The cytotoxic T cell response in vaccinated young adults was measured as levels of granzyme B activity using N-t-butyloxycarbonyl-Lalanyl-L-alanyl-L-aspartyl-paranitroanilide (BAAD-pNA) substrate. Serial measures of the levels of granzyme B activity in virus-stimulated peripheral blood mononuclear cells in vaccinated young adults were completed to determine the changes in granzyme B levels over time in culture following influenza vaccination. In vitro cytotoxic T cell responses were then compared to the ex vivo responses. In order to optimize the conditions for measuring ex vivo cytotoxic T cell levels of granzyme B activity, we used different concentrations of interleukin-7 in virus-activated peripheral blood mononuclear cell cultures and measured granzyme B activity at different time points in the cultures.

Preliminary studies

The initial study measured the kinetics of granzyme B production in peripheral blood mononuclear cell cultures in two types of culture medium. We obtained blood samples from healthy young adult subjects at several time points following vaccination with the 1998-1999 trivalent influenza vaccine. Peripheral blood mononuclear cells isolated from these blood samples at twelve weeks post vaccination were stimulated with live influenza A/Nanchang/933/95 (H_3N_2) (10^5 $TCID_{50}$/mL) and harvested on each day

five to nine in either Aim V culture medium or RPMI 1640 culture medium supplemented with 5% fetal bovine serum. The peak granzyme B activity, using the BAAD-pNA substrate in the granzyme B assay, occurred on day 6 in Aim V tissue culture medium (Figure 3). This data corresponded to earlier studies using N-t-butyloxycarbonyl-L-alanyl-L-alanyl-L-aspartyl-thiobenzyl ester (BAADT) substrate in the granzyme B assay. The peak of granzyme B activity in peripheral blood mononuclear cells stimulated in RPMI medium supplemented with 5% FBS was poorly defined, potentially due to the effects of the fetal bovine serum in these cultures. Thus, Aim V medium was confirmed to be the medium of choice for all of the peripheral blood mononuclear cell cultures.

To test for variability within the assay, blood samples were obtained from healthy young adults, who had been vaccinated with the 1998-1999 trivalent influenza vaccine twelve weeks previously, and stimulated *in vitro* with live influenza A/Nanchang/933/95 (H_3N_2) virus in Aim V medium. Several cell lysates (a minimum of three) were prepared for each young adult subject from this blood collection. The interassay variability in healthy young adults was <10% and within acceptable limits for a biological assay (Figure 4A).

The reproducibility of the granzyme B assay was examined using healthy young adults who had been vaccinated with the 1998-1999 trivalent influenza vaccine thirty-six weeks previously, and stimulated in vitro with live influenza A/Sydney/5/97 (H_3N_2) virus. Blood samples were obtained on three different days within a seven-day period and granzyme B activity levels were measured in three separate experiments. Significant reproducibility of the granzyme B activity levels was apparent in the *in vitro* experiments (Figure 4B).

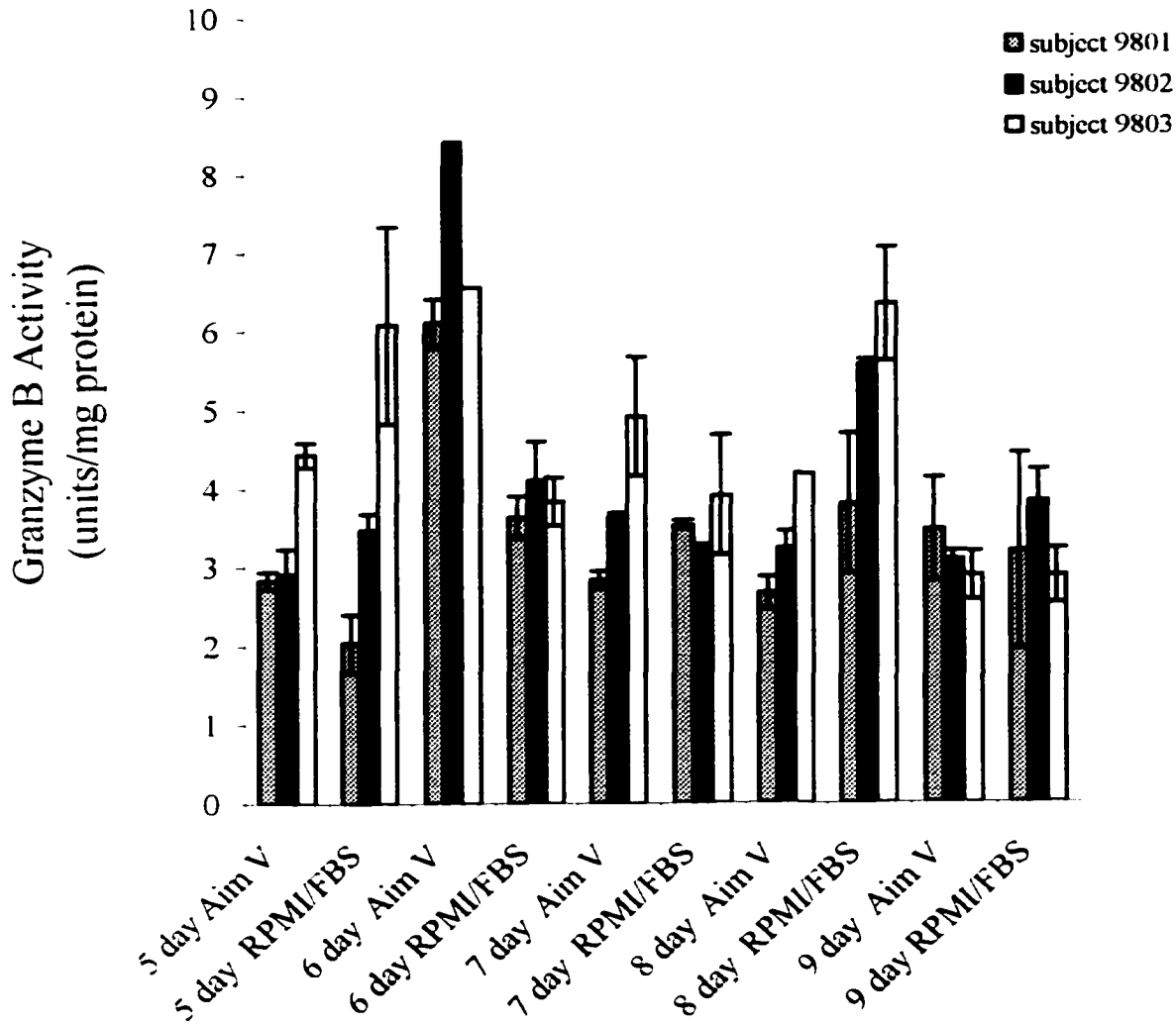


Fig. 3: Determination of peak *in vitro* granzyme B activity (twelve weeks post vaccination) in various culture media. Mean values from three different subject lysates are shown here. Error bars represent the standard deviation of the assay within subjects.

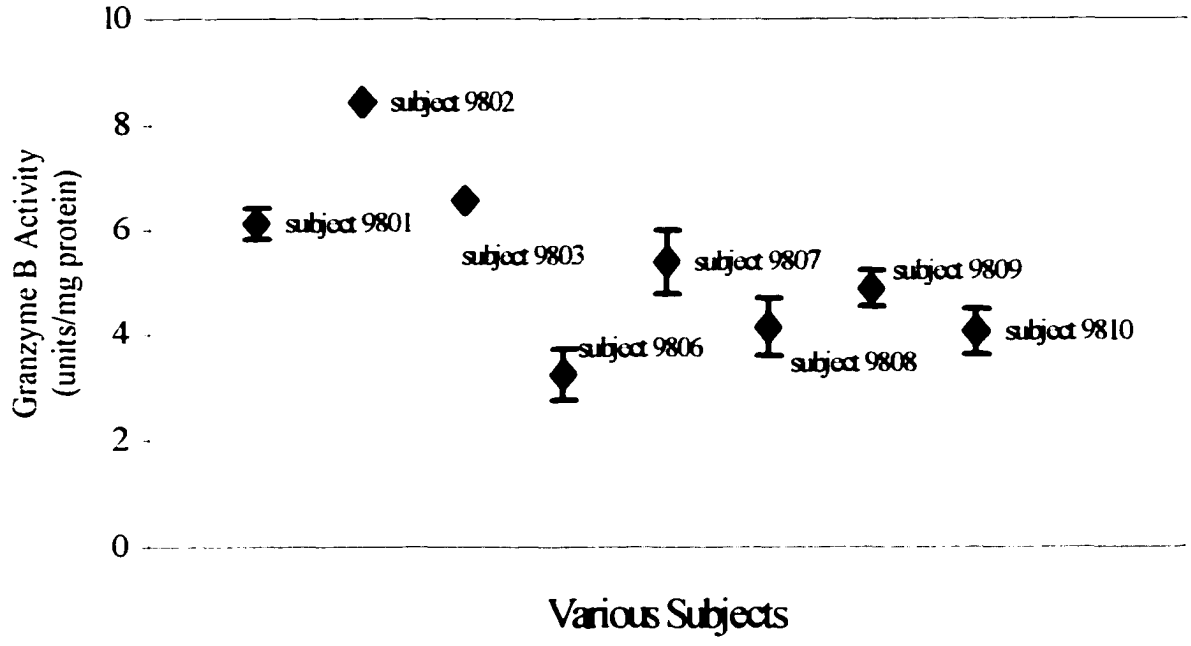


Fig. 4A: Error on the measurement of *in vitro* granzyme B activity assay (twelve weeks post vaccination) in Aim V medium. Mean values from triplicate samples are shown. Error bars represent standard deviation within the assay, which may be too small to be visualized.

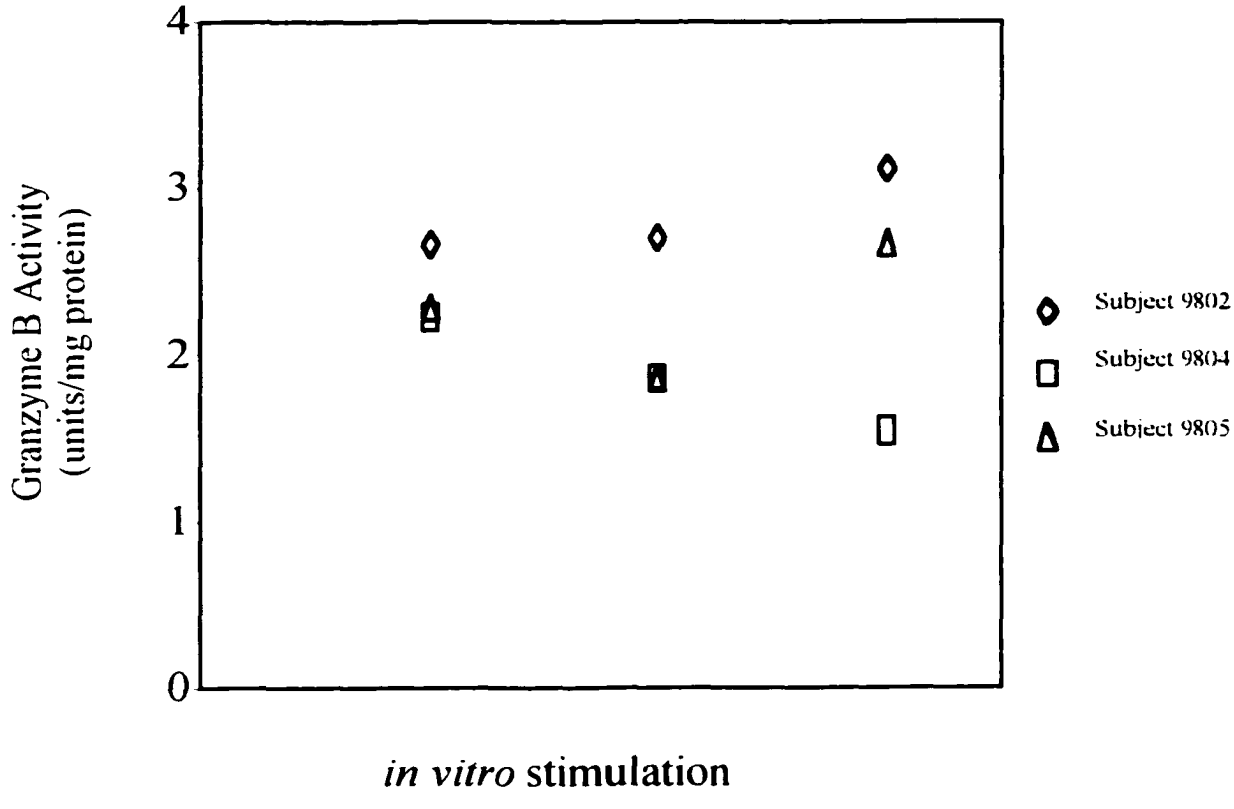


Fig. 4B: Reproducibility of the granzyme B activity assay (using three blood samples thirty-six weeks post vaccination). Mean values from triplicate samples of each blood draw are shown.

In vitro CTL response to influenza vaccination

Granzyme B activity levels were measured in influenza A/Nanchang/933/95 (H_3N_2) stimulated peripheral blood mononuclear cell cultures of healthy vaccinated young adults over a period of thirty-six weeks to determining the sensitivity of the granzyme B activity assay using BAAD-pNA substrate. Peripheral blood mononuclear cells were isolated from the healthy young adult subjects at three different time points – four, twelve and thirty–six weeks post vaccination with the 1998-1999 trivalent influenza vaccine. The granzyme B activity of peripheral blood mononuclear cells of healthy vaccinated young adults at twelve weeks post vaccination (Figure 5) is comparable to earlier studies (McElhaney et al., 1996). This study suggests that cytotoxic T cell memory following vaccination declines after four weeks, suggesting a relatively short duration of cytotoxic T cell memory in young adults. Cytotoxic T cell responses to live influenza virus as measured by *in vitro* restimulation (using ^{51}Cr release or granzyme B assays) are relatively short in duration and may result from a primary as well as a secondary response to influenza vaccination.

Ex vivo CTL response to influenza vaccination

To obtain an assessment of the in vivo response to influenza vaccination, we developed an *ex vivo* assay of virus-activated peripheral blood mononuclear cell cultures, which had been stimulated in culture for different periods of time (six to thirty-one hours) to study the kinetics of granzyme B activity in *ex vivo* cultures. The optimal conditions for measuring granzyme B response to influenza was determined in the *ex vivo* cultures supplemented with interleukin-7.

Figure 6 depicts the study of the dose-response of interleukin-7 over a

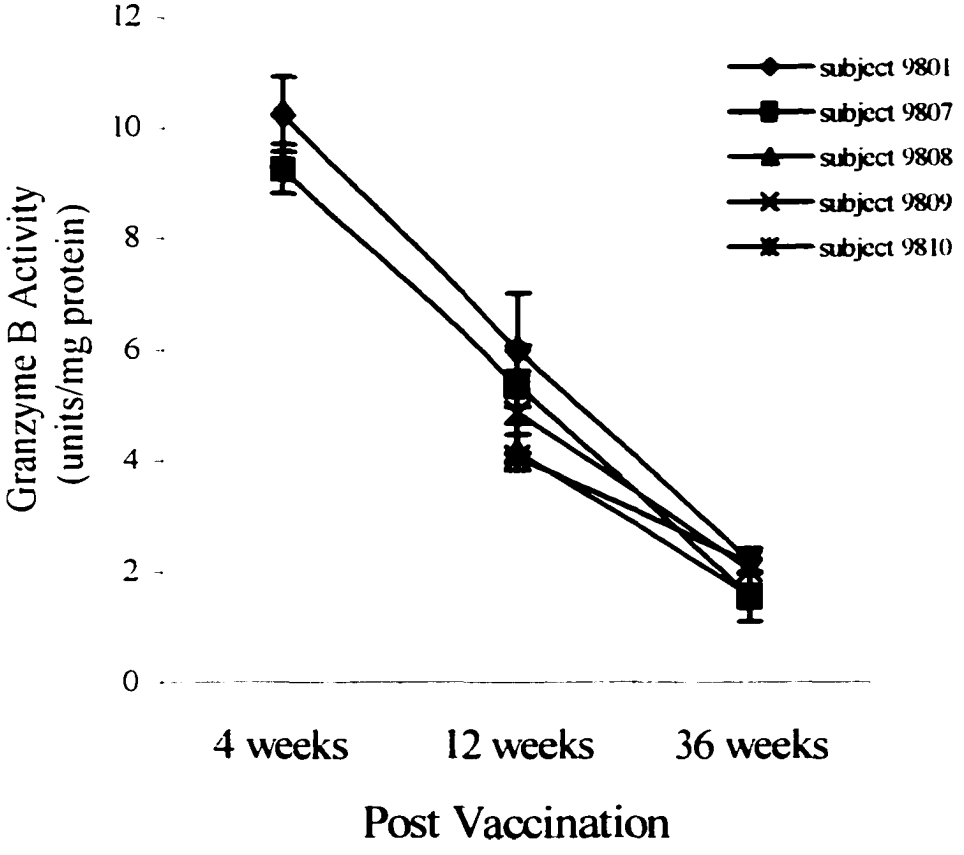


Fig. 5: Cell mediated immune response to live influenza A/Nanchang/933/95 (H_3N_2) virus stimulation, measured via *in vitro* granzyme B activity assay. Mean values from triplicate samples are shown. Error bars represent standard deviation within the assay.

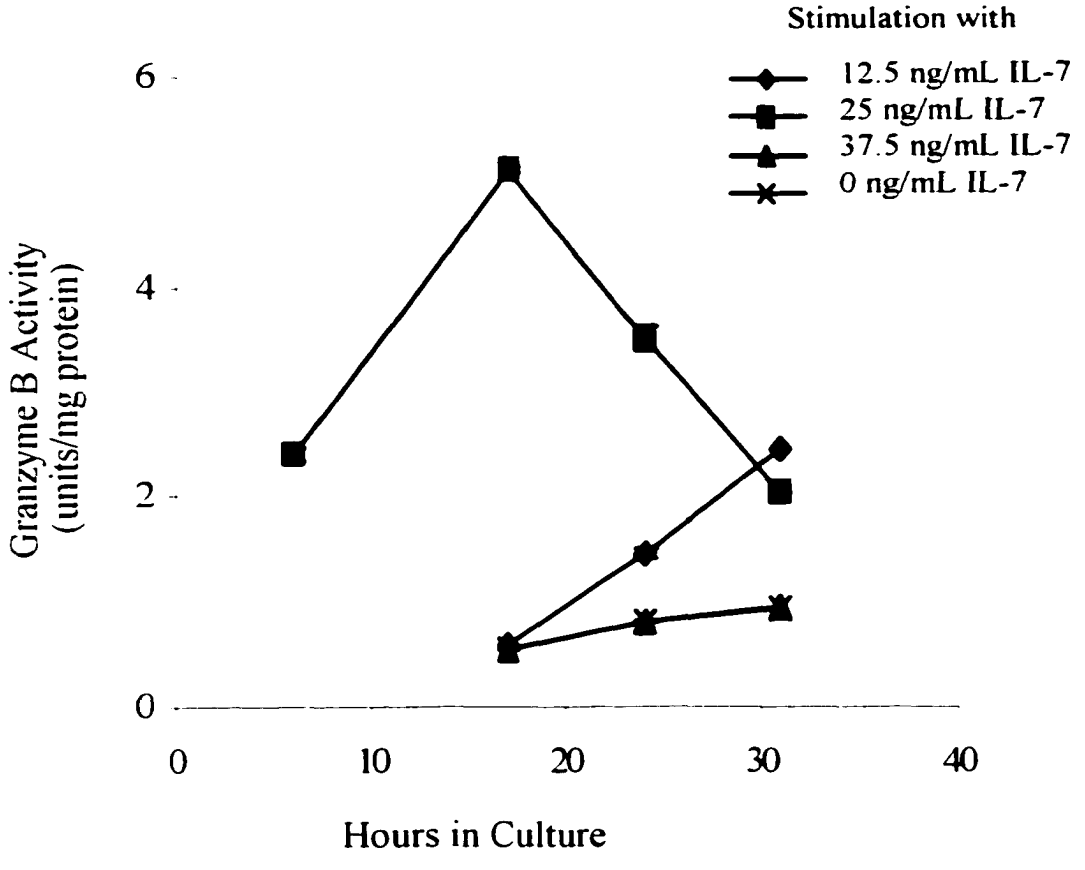


Fig. 6: Effect of interleukin 7 on *ex vivo* CTL response (twelve weeks post vaccination) to live influenza A/Nanchang/933/95 (H_3N_2) virus stimulation. Mean values from triplicate subjects are shown. Error bars represent the standard error of the mean, which may be too small to be visualized.

from 12.5 to 37.5 ng/mL on granzyme B levels in the cultures from healthy young adults who had been vaccinated with the 1998-1999 trivalent influenza vaccine. Peripheral blood mononuclear cells were obtained from blood samples collected from the young adult subjects twelve weeks post-vaccination. The optimum concentration of interleukin-7 for maximum granzyme B activity was 25 ng/mL. The addition of 12.5 ng/mL of interleukin-7 to the peripheral blood mononuclear cell cultures resulted in activation of the $CD8^+$ T cells occurring at approximately the same rate as the peripheral blood mononuclear cell culture that had not been stimulated with interleukin-7. The supplementation of 37.5 ng/mL interleukin-7 to the cultures caused only a sight increase in activation of the $CD8^+$ T cells. The effect of interleukin-7 on the virus-stimulated peripheral blood mononuclear cells after 17 hours appears to be a direct effect on the $CD8^+$ T cells, which results in activation of the cells over a short time period without stimulating a proliferative response, thereby increasing the precursor frequency of virus-specific cytotoxic T cells. These data demonstrates that the frequency of precursor memory cytotoxic T cell can be accurately measured in *ex vivo* peripheral blood cell cultures to determine the cytotoxic T cell response to influenza vaccination.

The reproducibility of the *ex vivo* granzyme B assay was determined at thirty-six weeks post vaccination in healthy young adults. Figure 7 depicts the reproducibility of the assay from measurements of granzyme B activity in three separate blood draws over a seven-day period and identical culture conditions. The data had similar levels of granzyme B activity as determined in the *in vitro* experiments (Figure 4B).

We now needed to determine the frequency of the *ex vivo* response to influenza vaccination. Using the granzyme B assay, the response to influenza vaccination in young adults has been determined by measuring a concentration of cells in culture.

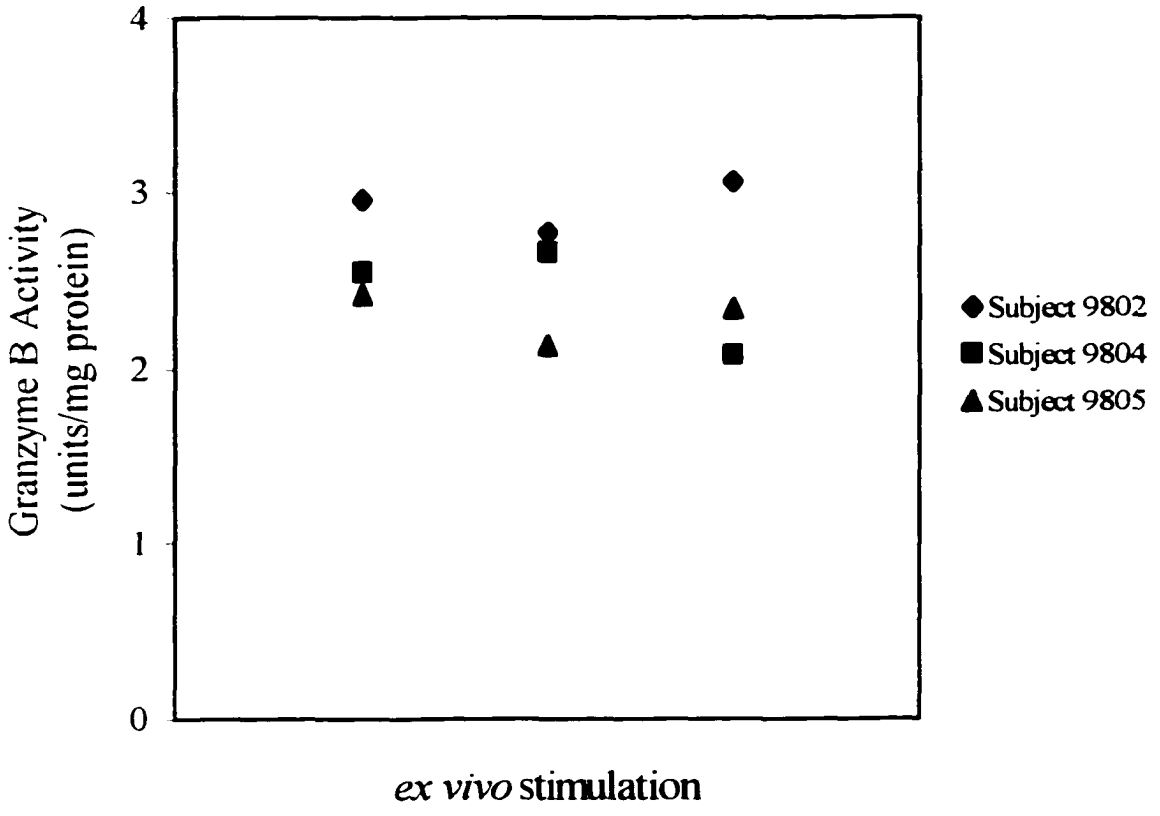


Fig. 7: Reproducibility of the *ex vivo* granzyme B activity assay (using three blood samples thirty-six weeks post vaccination). Mean values from triplicate samples of each blood draw are shown.

Determining the *ex vivo* response to influenza vaccination on a per cell basis will help clarify the relationship between granzyme B and cytotoxic T cell response to influenza vaccination.

CHAPTER IV

RESULTS: MEASUREMENT OF VIRUS-SPECIFIC CTL FREQUENCY IN THE YOUNG ADULT POPULATION

Microbe-derived peptides from the influenza virus are generated by intracellular processing of replicating influenza virus, bound to major histocompatibilty complex class I molecules, and transported to the cell surface of infected cells. Virus-specific cytotoxic T cells recognize the major histocompatibilty complex and upon activation, secrete interferon γ. Using a monoclonal anti-interferon γ antibody and biotinylated monoclonal anti-interferon γ antibody pair, the number of activated virus-specific T cells was determined. This technique is the basis of the enzyme-linked immunospot (ELIspot) assay and is able to directly measure the frequency of epitope-specific T cells (Lalvani et al., 1997; Czerkinsky et al., 1988). The measurement of proliferation of T cells in response to their specific antigen has previously been performed using the limiting dilution technique and chromium-release assay in order to determine the number of antigen specific T cells. The ELIspot assay has the advantage of detecting cytokine release in response to antigen stimulation on a single cell level, thereby permitting direct calculation of T cell frequencies by volume of blood.

Preliminary study

Initially, influenza virus-specific precursor frequency was measured as the number of interferon γ producing cells in the cell cultures in healthy young adults, using

the ELIspot technique. Peripheral blood mononuclear cells were obtained from the healthy young adult subjects twelve-weeks post-vaccination with the 1998-1999 trivalent influenza vaccine and stimulated with live influenza A/Nanchang/933/95 (H_3N_2) virus. One of the young adult subjects donated their blood twice during a one-week period of time. Reproducibility of the ELIspot technique was determined in one healthy adult subject, showing no significant variation in the number of interferon γ ("ELIspots") spots (Figure 8). The frequency of virus-specific precursor T cells within the total cell population of the healthy young adult subjects was determined to be 170.63 $\pm$ 15.45 (SE) interferon γ producing cells within 5 x 10^6 cells in culture.

Effect of interleukin 7 on ELIspot technique

In order to determine if the supplementation of 25 ng/mL of interleukin-7 affected the live influenza A/Nanchang/933/95 (H_3N_2) virus stimulation *of ex vivo* lymphocytes, a study was performed using the ELIspot assay in the presence and absence of interleukin-7. Peripheral blood mononuclear cells were obtained from healthy young adults who had been vaccinated with the 1998-1999 trivalent influenza vaccine twelve weeks previously. The cell cultures were stimulated with live influenza A/Nanchang/933/95 (H_3N_2) virus for 17 hours either in the presence or absence of interleukin-7 supplementation (25 ng/mL). The effects of live influenza A/Nanchang/933/95 (H_3N_2) virus stimulated *ex vivo* lymphocytes supplemented with and without 25 ng/mL interleukin 7 are shown in Figure 9. These results can be interpreted to mean that the precursor frequency of virus-specific cytotoxic T cells do not appreciably change with the addition of interleukin-7, consistent with the observations that a primary response is not stimulated under these conditions.

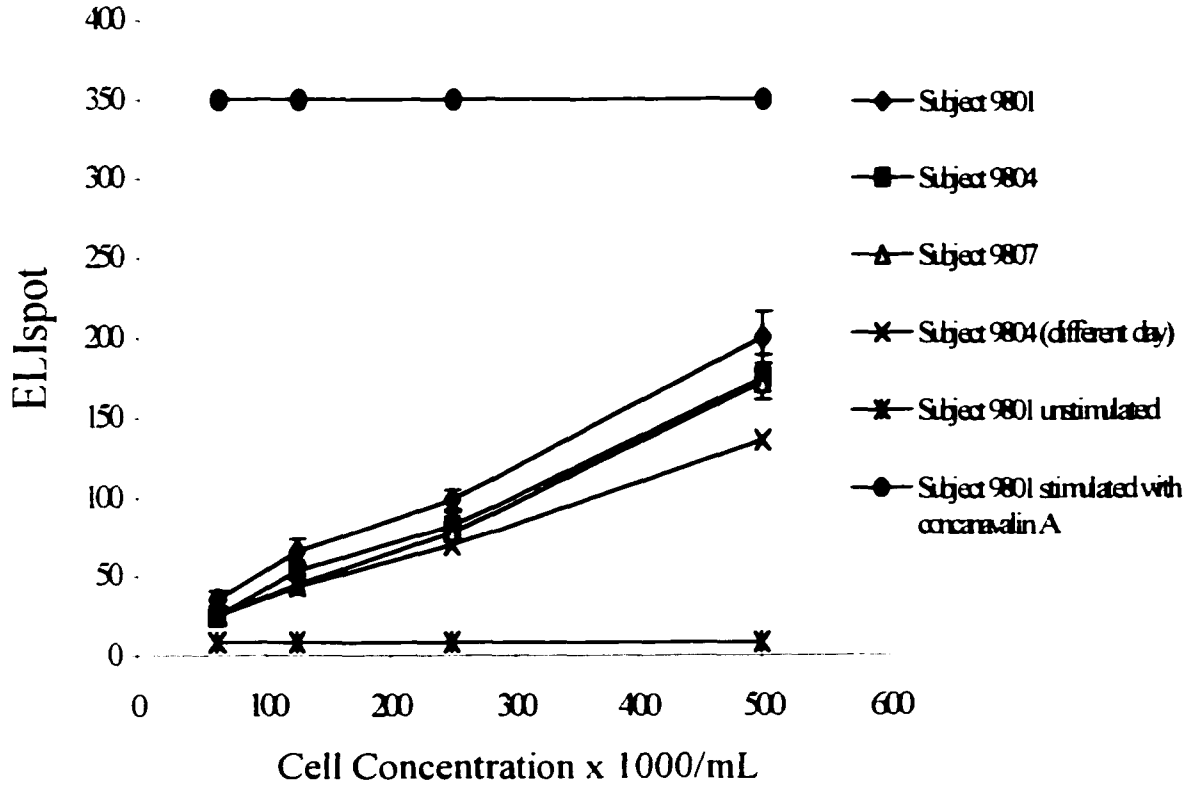


Fig. 8: *Ex vivo* virus-specific CTL response to stimulation with live influenza A/Nanchang/933/95 (H_3N_2) virus 12 weeks post vaccination. Mean ELIspot values from quadruplicate wells are shown. Error bars represent standard deviation within the assay, which may be too small to be visualized.

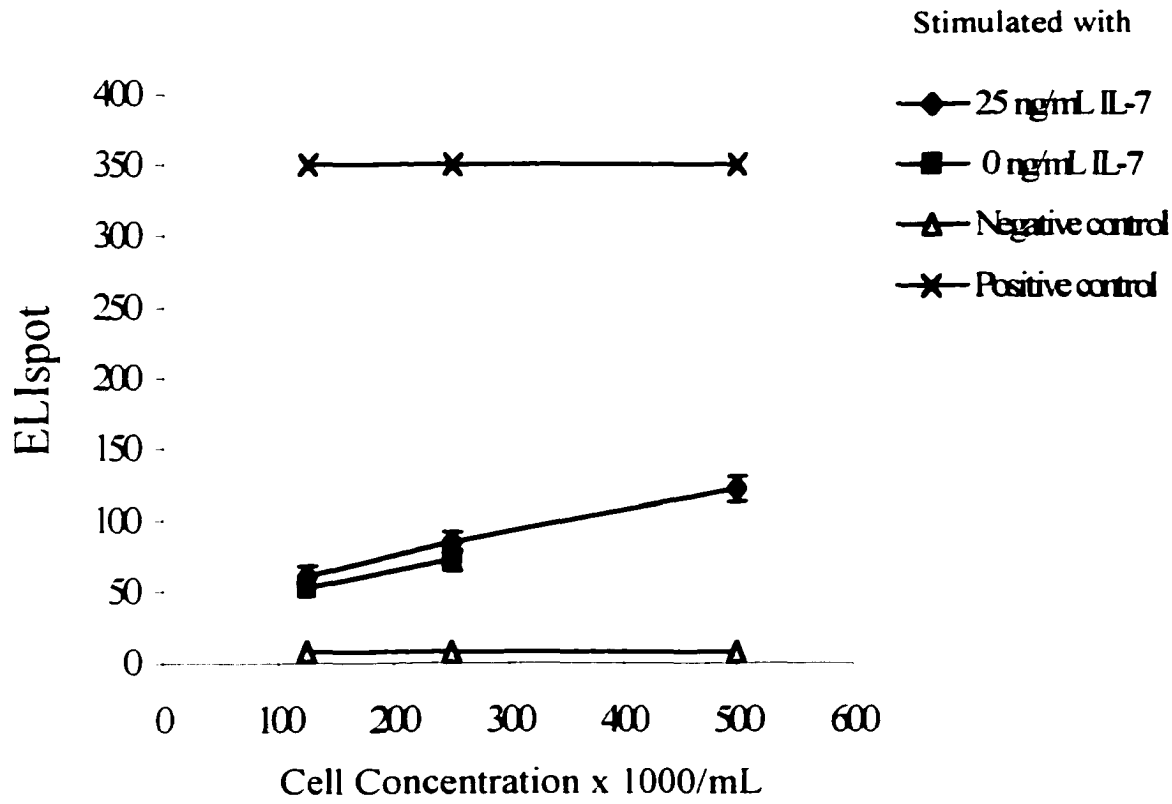


Fig. 9: Effect of interleukin 7 on virus-specific CTL response to live influenza A/Nanchang/933/95 (H_3N_2) virus stimulation 12 weeks post vaccination. The negative control was not stimulated. The positive control was stimulated with concanavalin A. Mean ELIspot values from three subjects are shown. Error bars represent standard error of the mean, which may be too small to be visualized.

$CD8^+$ T cell response to influenza vaccination

We initially investigated the frequency of influenza virus-specific cells within the influenza stimulated *ex vivo* lymphocytes in the absence of $CD8^+$ T cells. Peripheral blood mononuclear cells were obtained from healthy young adults, who had been vaccinated with the 1998-1999 trivalent influenza vaccine twelve weeks previously. $CD8^+$ T cells were removed from peripheral blood mononuclear cell preparations and were compared to non-depleted peripheral blood mononuclear cells in cultures stimulated with live influenza A/Nanchang/933/95 (H_3N_2) virus under *ex vivo* conditions in the ELIspot assay. The frequency influenza virus-specific cells within depleted $CD8^+$ T cell population was decreased by 17% in comparison to the total cell population (Figure 10), suggesting the $CD8^+$ T cell population represent a significant proportion but not all of the interferon γ producing cells in the culture.

The frequency of influenza virus-specific cells within the influenza activated *ex vivo* lymphocytes may have resulted from several cell types - $CD8^+$ T cells, natural killer cells and $CD4^+$ T cells. To determine the precursor cytotoxic T cell frequency in young adults, peripheral blood mononuclear cells were obtained from healthy young adults thirteen weeks post vaccination. Using negative selection techniques, the natural killer cells and $CD4^+$ T cells were removed from the peripheral blood mononuclear cell cultures. Figure 11 can be interpreted to mean that the peripheral blood mononuclear cell population, which had been negatively selected for $CD8^+$ T cells (i.e. $CD4^+$ $CD59^+$ peripheral blood mononuclear cells), contained a higher frequency of influenza virus-specific cells than the total cell population (non-selected peripheral blood mononuclear cells).

The ratio of interferon-γ producing $CD4^+$ T cells and natural killer cells to $CD8^+$

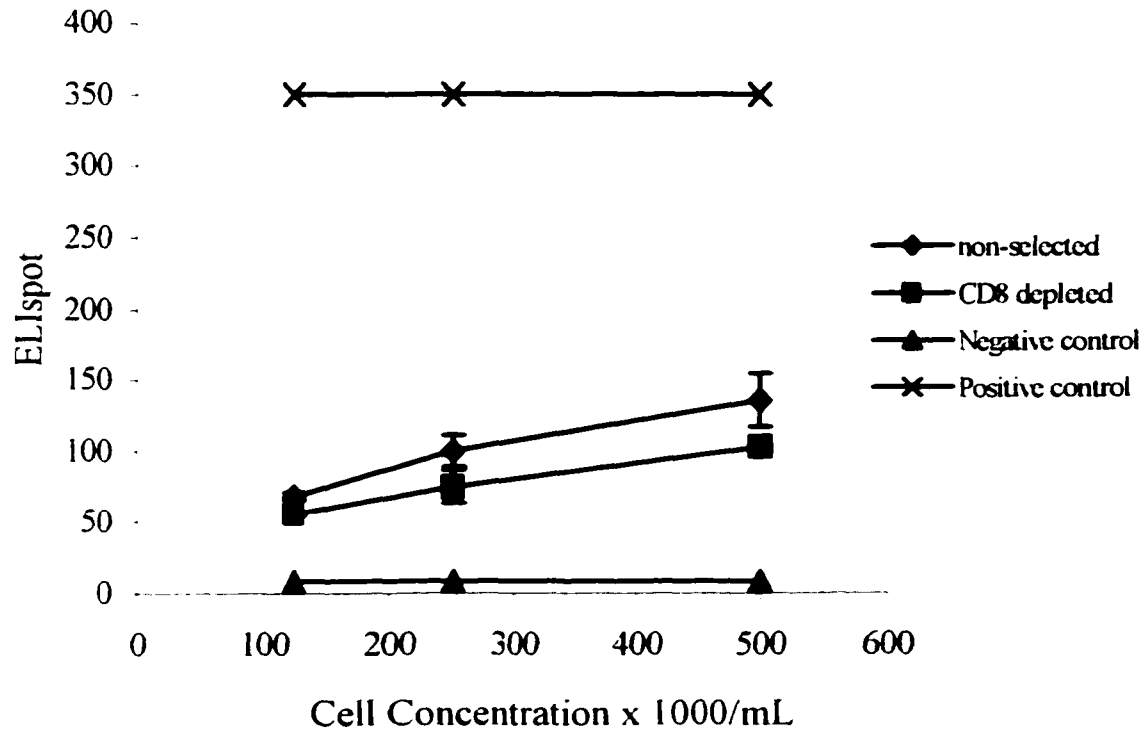


Fig. 10: Virus-specific CTL response to live influenza A/Nanchang/933/95 (H_3N_2) virus stimulation 12 weeks post vaccination in the absence of $CD8^+$ T cells. The negative control was not stimulated. The positive control was stimulated with concanavalin A. Mean ELIspot values from three subjects are shown. Error bars represent standard error of the mean, which may be too small to be visualized.

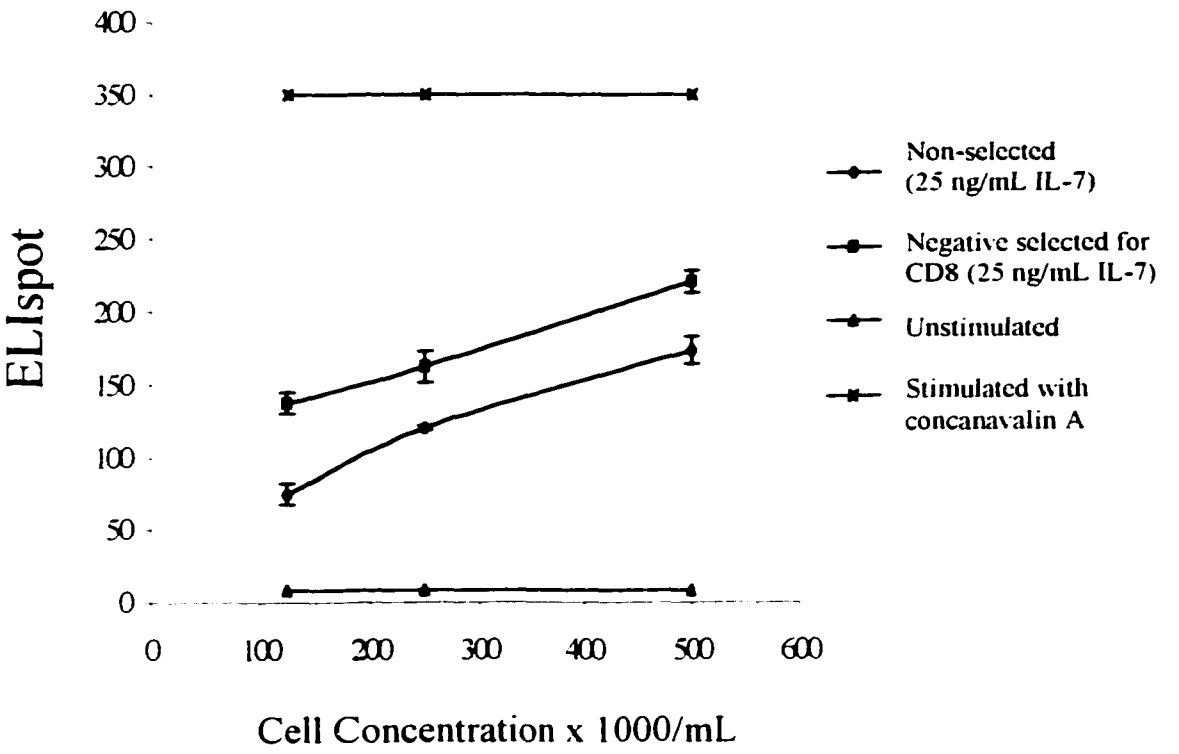


Fig. 11: Identification of virus-specific $CD8^+$ CTL response to live influenza A/Nanchang/933/95 (H_3N_2) virus stimulation 13 weeks post vaccination. Mean ELIspot values from three subjects are shown. Error bars represent standard error of the mean, which may be too small to be visualized.

T cells in the peripheral blood mononuclear cell cultures was 1:46 virus-specific precursor T cells. These data imply that the interferon γ producing cells are largely derived from cytotoxic T cell population and is consistent with previously published work (Taylor et al., 1985).

CHAPTER V

RESULTS: IDENTIFICATION OF THE EFFECT OF AGING ON THE CTL RESPONSE TO INFLUENZA VACCINATION AND CROSS-REACTIVITY BETWEEN VARIOUS INFLUENZA STRAINS

We have demonstrated that a live influenza virus stimulates granzyme B activity under the *ex vivo* and *in vitro* culture conditions in healthy young adult population and can be used to track the response to vaccination. To determine the effect of age on cross-reactivity of the granzyme B response between different influenza A/H_3N_2 strains, we compared healthy young adults to healthy older adults. The *ex vivo* and *in vitro* cytotoxic T cell response to influenza vaccination in young adults has been shown to be comparable in terms of granzyme B activity (Figure 5 and Figure 6). The correlation between the *ex vivo* and *in vitro* granzyme B activity levels and frequency of influenza virus-specific cytotoxic T cell suggested that the *in vitro* restimulation of the response to influenza vaccination was similar to the *ex vivo* measurements. To determine the effects of aging on the granzyme B response to influenza vaccination, we compared healthy young adults to healthy older adults cytotoxic T cell response to influenza vaccination.

CTL response measured via granzyme B assay

In order to determine the effect of age on influenza A/H_3N_2 strain-related changes in granzyme B production, a study containing ten healthy young adults and ten healthy older adults was completed. Blood samples from these 20 subjects were obtained prior to and four weeks following vaccination with the 1999-2000 trivalent influenza vaccine.

Peripheral blood mononuclear cells were isolated from these blood samples and stimulated with live virus preparations of influenza A/Sydney/5/97 (H_3N_2), influenza A/Nanchang/933/95 (H_3N_2), or influenza A/Johannesburg/33/94 (H_3N_2). This study measured the *ex vivo* and *in vitro* granzyme B activity in the healthy young and healthy older adult subjects as well as determining the frequency of influenza virus-specific cytotoxic memory T cells in both populations.

Granzyme B activity measured *ex vivo* in the healthy young and healthy older adult populations groups were increased four weeks post vaccination, as illustrated in Figure 12. The amount of stimulation within the cell cultures differed from one influenza H_3N_2 strain to another. The largest granzyme B response was demonstrated to the vaccinating strain, influenza A/Sydney. The increase in *ex vivo* granzyme B activity from pre- to post-vaccination of the healthy young adult subjects was statistically significant in both age groups ($p < 0.05$, paired student's t test) for the three different H_3N_2 strains of live influenza A virus. The levels of *ex vivo* granzyme B activity in the healthy young adults compared to the healthy older adults was significantly higher ($p < 0.05$, Mann-Whitney U test) for the three different influenza A/H_3N_2 strains, consistent with an age-related decline in the cytotoxic T cell response to vaccination.

Granzyme B activity measured *in vitro* in the healthy young and older adults were increased four weeks post vaccination, as illustrated in Figure 13. The *in vitro* granzyme B activity were determined to be almost identical to the *ex vivo* granzyme B activity in both the healthy young and older adult populations. We had expected the levels of granzyme B activity *in vitro* to have increased in comparison to the *ex vivo* granzyme B activity because the number of cells in the *in vitro* cultures should have increased and therefore produced more granzyme B within these cells. The *in vitro* cell

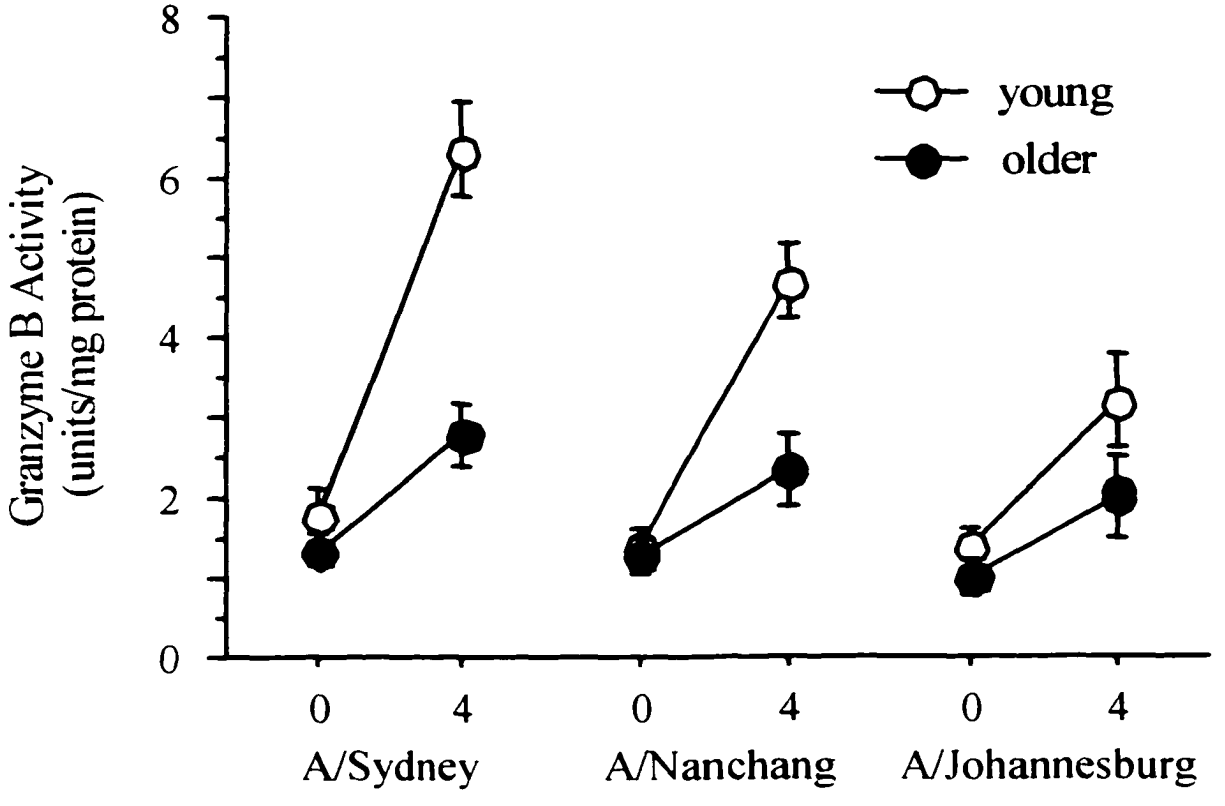


Fig. 12: *Ex vivo* (17 hours in culture) cell-mediated response to three different live H_3N_2 strains of influenza A subtype. Cultures were negatively selected for $CD8^-$ T cells. (10 subjects per population group, 0 = prior to vaccination, 4 = 4 weeks post vaccination). Sample mean values are shown here. Error bars represent 95% confidence intervals.

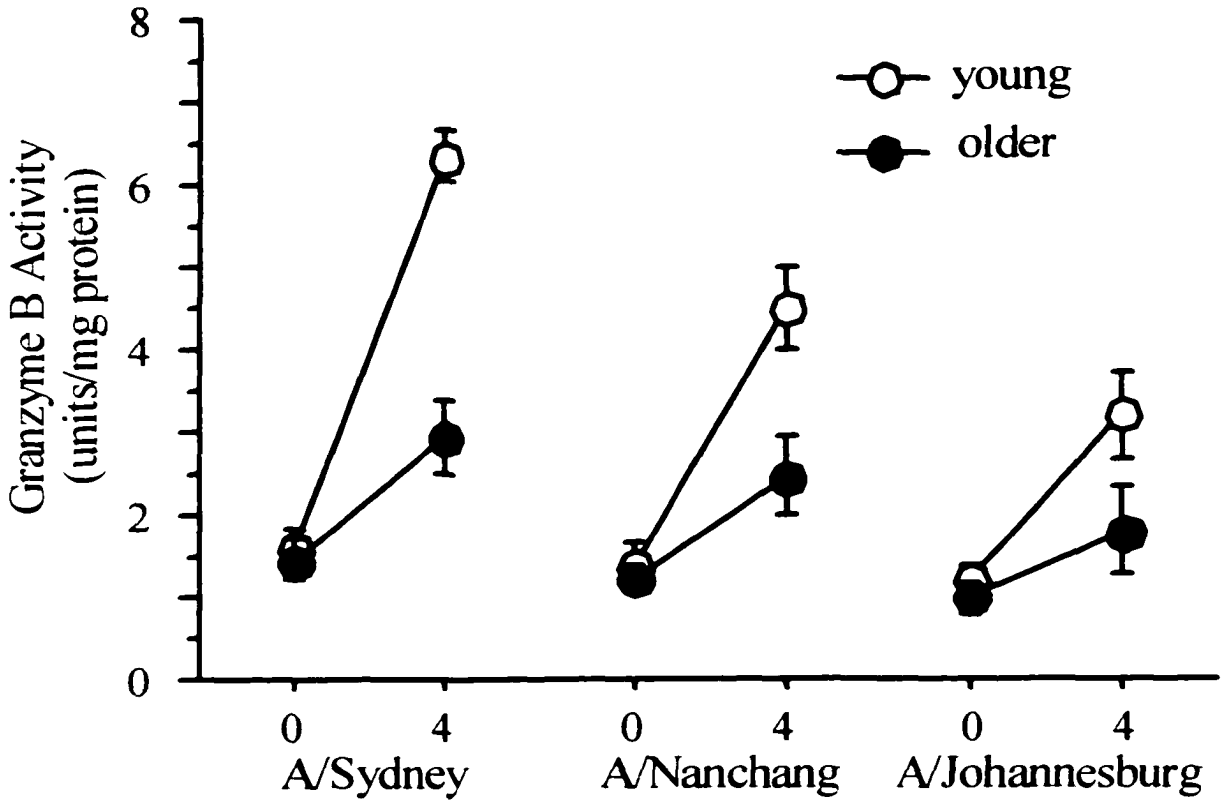


Fig. 13: *In vitro* (6 days in culture) cell-mediated response to three different live H_3N_2 strains of influenza A subtype. Cultures were negatively selected for $CD8^-$ T cells. (10 subjects per population group, 0 = prior to vaccination, 4 = 4 weeks post vaccination). Sample mean values are shown here. Error bars represent 95% confidence intervals.

cultures produced slightly higher protein levels in comparison to the protein level of the *ex vivo* cell cultures. The frequency of influenza-specific cytotoxic T cells in the *in vitro* cultures increases with time in culture in comparison to the *ex vivo* cultures. This increase in protein levels may have occurred due to cell proliferation of the *in vitro* cell cultures over the 6-day culture period. This may consequently have affected the calculation of granzyme B activity in the *in vitro* cultures, yielding apparently similar levels of *in vitro* and *ex vivo* granzyme B activity.

The *in vitro* granzyme B activity in both age groups corresponded to previously reported granzyme B activity (McElhaney et al., 1996). We also observed the granzyme B activity to be greater in the healthy young adult population in comparison to the healthy older adult population similar to McElhaney et al. (1996). The shorter time period (17 hours in culture) for the *ex vivo* procedure provides confidence that the granzyme B activity being measured is not a result of cell proliferation and differentiation in culture over time.

Virus-specific CTL response to influenza vaccination

The number of cytotoxic T cells producing interferon γ was determined via ELIspot detection of influenza virus-specific $CD8^+$ T cells in the healthy young and healthy older adult populations. Influenza vaccination enabled the healthy older adult population to increase their number of influenza virus-specific cytotoxic T cells to a level approximately equal to the healthy young adult population prior to influenza vaccination (Figure 14). The increase in the number of influenza virus-specific memory cytotoxic T cells from pre- to post-vaccination of the healthy young adult population was statistically significant ($p < 0.05$, paired student's t test) for each of the three influenza A/H_3N_2

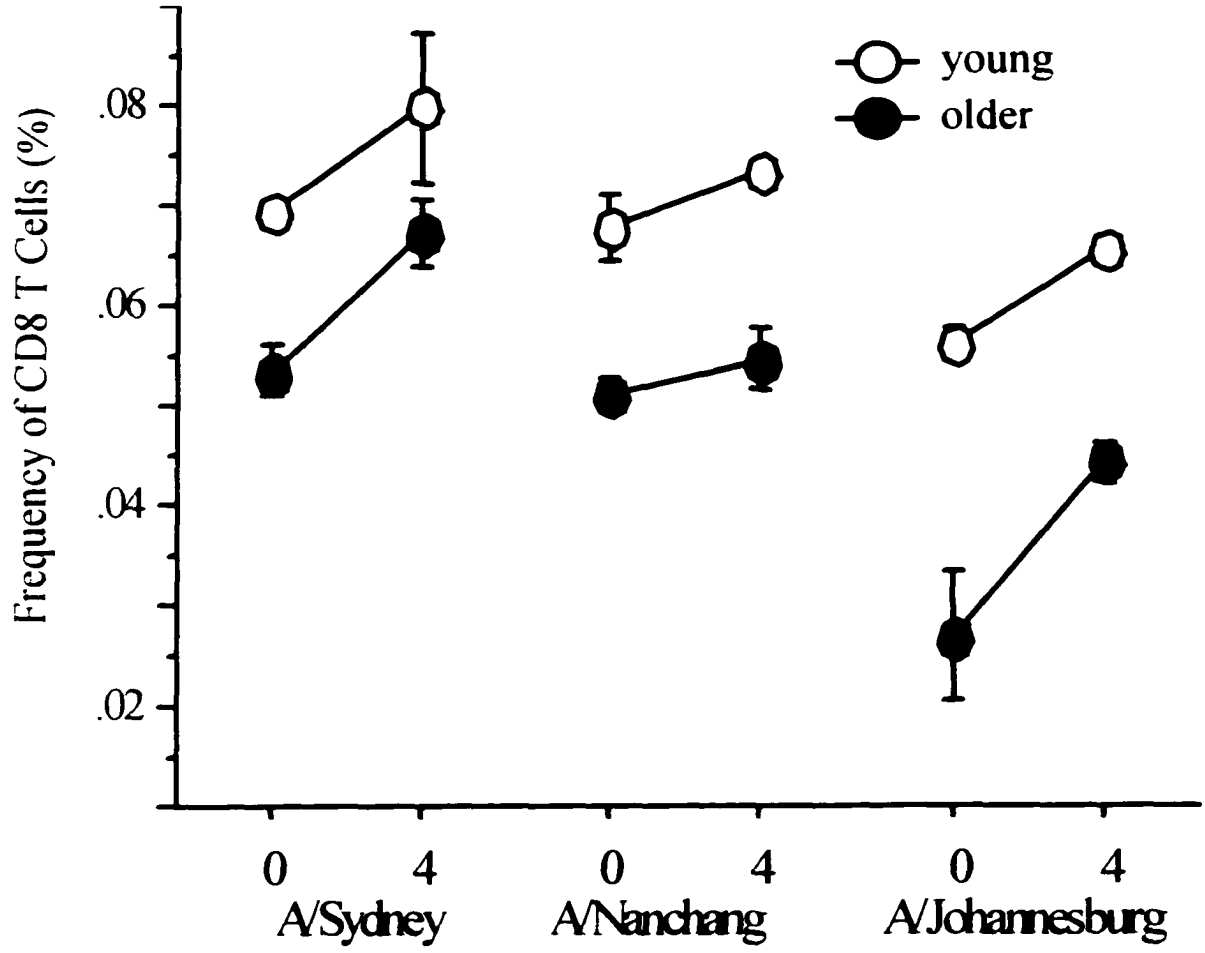


Fig. 14: Virus-specific $CD8^+$ CTL response (17 hours in culture) to live influenza A/H3N2 virus stimulation. Cultures were negatively selected for $CD8^+$ T cells. (10 subjects per population group, 0 = prior to vaccination, 4 = 4 weeks post vaccination). Sample mean values are shown. Error bars represent 95% confidence intervals.

strains. The number of influenza A/Nanchang (H_3N_2) and A/Johannesburg (H_3N_2) virus-specific memory cytotoxic T cells were significantly higher ($p < 0.05$, Mann-Whitney U test) in the young adult population compared to the older adult population across the three different H_3N_2 strains. A similar comparison of influenza A/Sydney (H_3N_2) stimulated memory T cells illustrated no statistical significance, which may have resulted from the small sample size of the young and older adult populations or recent prior exposure to the circulating strain.

The comparison of influenza A/Sydney (H_3N_2) virus-stimulated memory cytotoxic T cells prior to vaccination to post vaccination in the older adult population resulted in statistical significance ($p = 0.015$, paired student's t test). The influenza A/Sydney (H_3N_2) virus-stimulated memory cytotoxic T cells of the healthy young adult population compared pre- to post-vaccination frequencies were not statistically different; this may reflect a Type II error due to the small sample size. Influenza vaccination increases the number of virus-specific cytotoxic T cells by approximately 50% in the healthy older adult population (Figure 14).

Effects of aging on immune response

The ratio of the granzyme B level to the number of influenza virus-specific $CD8^+$ T cells was calculated to determine the amount of granzyme B activity measured on a per cell basis. In the young adult population, there was a four-fold increase in the amount of granzyme B activity produced on a per cell basis, while the level increased only two fold in the older adult population (Table 1). This fold-increase in the granzyme B level per cell was consistent across the three H_3N_2 strains of influenza virus within each age group.

Table 1

Ratio of Granzyme B Activity per Influenza virus-specific $CD8^+$ T Cell

	Stimuli in Culture		
	Influenza A/Sydney	Influenza A/Nanchang	Influenza A/Johannesburg
Young Adults Prior to Vaccination	0.0020	0.0018	0.0016
Older Adults Prior to Vaccination	0.0018	0.0015	0.0012
Young Adults 4 Weeks Post Vaccination	0.0079	0.0056	0.0040
Older Adults 4 Weeks Post Vaccination	0.0036	0.0031	0.0023

This difference was statistically significant ($p = 0.04$, Mann-Whitney U test) for influenza A/Sydney (H_3N_2) stimulated peripheral blood mononuclear cells.

We have determined that the granzyme B levels increase after stimulation with influenza virus as result of the activation of memory cytotoxic T cells. Although the frequency of virus-specific cytotoxic T cells does not linearly correlate with the granzyme B levels, it suggests that the increase in number of virus-specific cytotoxic T cells in response to a killed influenza virus vaccine may occur via a different mechanism than that which increases granzyme B levels. The effect of aging on the cytotoxic T cell response to influenza vaccination is that the granzyme B levels are less effectively stimulated in healthy older adults compared to healthy young adults when using an inactivated influenza vaccine.

CHAPTER VI

CONCLUSIONS

T cell responses to influenza vaccination are affected by numerous variables. The strain of influenza virus within the vaccine as well as age, specific chronic illness, and frailty may contribute to the variations in the T cell response to influenza vaccination. Although the cytotoxic T cell response to influenza vaccination has been studied, a limited number of studies have compared the responses between different age groups (Gorse and Belshe, 1990; Powers, 1993; Powers and Belshe, 1993).

We initially investigated the *in vitro* cytotoxic T cell response to influenza vaccination in healthy young and older adults using the granzyme B assay (*ex vivo* and *in vitro*) and frequency of virus-specific cytotoxic T cells as a measure of the cytotoxic T cell response between these two different age groups. Our results are consistent with previously published results (McElhaney et al., 1996; McElhaney et al., 1998) using a modified version of the granzyme B assay for this study. Our laboratory observed a decreased *in vitro* cytotoxic T cell response to influenza vaccination in the healthy older adult population in comparison to the healthy young adult via the granzyme B assay. The *ex vivo* granzyme B activity were similar to the *in vitro* granzyme B activity. In these *in vitro* stimulated cultures, the observed granzyme B activity levels may have resulted in the stimulation of the primary as well as the secondary response to stimulation with live influenza virus. Thus, we developed an *ex vivo* granzyme B assay to eliminate the effects of proliferation and differentiation of the cells occurring in 6 days of culture.

The *in vitro* cell cultures produced on average slightly higher protein levels in comparison to the ex vivo cell cultures. The increased protein levels may have resulted due to the differentiation of the cultures over time. The increased protein levels may have affected the determination of granzyme B activity in the in vitro samples. The similar levels of granzyme B activity determined *ex vivo* and *in vitro* may have resulted from the manner in which granzyme B activity is calculated; when the protein levels are increased the granzyme B levels will decrease. The increased protein levels may have caused the *ex vivo* and *in vitro* granzyme B levels to appear to be similar, although there is an increase in the frequency of cytotoxic T cells in the *in vitro* cultures, which should have resulted in higher granzyme B levels *in vitro*.

To directly measure the age-related changes in the response to influenza vaccination, *ex vivo* cell cultures of healthy young and older adults were stimulated with live influenza virus and the cytotoxic T cell response to influenza vaccination was measured using the granzyme B assay. The *ex vivo* cytotoxic T cell response to influenza vaccination in healthy young and healthy older adult populations produced levels of granzyme B activity, that were comparable to the *in vitro* granzyme B activity observed in our laboratory. This data validates our previous results measuring granzyme B activity within *in vitro* cultures. However, there may be differences in the mechanism by which granzyme B activity levels are increased and does not necessarily reflect the total amount of granzyme B activity in *ex vivo* cultures compared to the *in vitro* cultures.

The *ex vivo* granzyme B assay differs from the *in vitro* granzyme B activity assay in the time period required for cell culture, as well as the supplementation of interleukin-7 to the cultures. The presence of interleukin-7 increases the level of granzyme B measured at 17 hours by shortening the period of time over which virus-specific

cytotoxic T cells are activated and thus provides a more accurate assessment of the *in vivo* environment.

The effect of age-related changes on the cytotoxic T cell response to influenza vaccination was investigated in *ex vivo* and *in vitro* influenza virus-stimulated cultures of the healthy young and older adult populations. The cultures were stimulated with three different H_3N_2 strains of live influenza subtype A virus. The largest *ex vivo* and *in vitro* granzyme B activity was observed for the most recent circulating H_3N_2 strain of influenza A virus (A/Sydney) in both the healthy young and older adult population. The magnitude of the cytotoxic T cell response to influenza vaccination was decreased in the healthy older adult population when compared to the healthy young adult population, consistent with an age-related decline in the cytotoxic T cell response to vaccination.

In order to determine if the decreased granzyme B response in the healthy older adults compared to the healthy young adults resulted from a change in the frequency of the cytotoxic T cells, the number of the interferon γ producing cells in $CD8^+$ T cell cultures was compared between the two age groups. The frequency of influenza virus-specific $CD8^+$ T cells of the healthy young and healthy older adults demonstrated similar trends to the various influenza A/H_3N_2 stimulation as the *ex vivo* and *in vitro* granzyme B activity levels (i.e. response to A/Sydney being the greatest). The frequency of the cytotoxic T cell response in the older adult population increased after influenza vaccination showing that stimulation of the influenza virus- specific cytotoxic T cells is similar to the young adult population. This observation indicates that the aging process may affect the granzyme B levels within the cytotoxic T cells of older adults. Thus, the function of activating granzyme B within the influenza-infected cells of older adults may be defective in some manner.

The health status in older adults has been shown to impact on the protective ability of influenza vaccination. A decline in cell-mediated immunity in older adults may result due to health status, causing a deficient T cell response to influenza vaccination. T helper cell responses have been determined to be subtype specific to various influenza virus strains, while the cytotoxic T cell response is type-specific between various strains of influenza A (e.g. H_1N_1 and H_3N_2) virus (Mbawuike et al., 1993; Powers and Belshe, 1993; Gorse and Belshe, 1990). These studies conducted in older adult subject populations have shown variations in the cross-reactivity of T cell responses to different strains of killed influenza virus. A broader cytotoxic T cell response after infection with live influenza virus has been previously reported in the murine model (Webster and Askonas, 1980; Askonas and Taylor, 1983). The stimulation of the T cell response with live influenza virus in *ex vivo* cultures appears to indicate that the variations in cross-reactivity to different strains of influenza virus are able to be decreased using live influenza vaccines.

Three different H_3N_2 strains of live influenza subtype A virus were used to stimulate the cross-reactive response of the cytotoxic T cell in both *ex vivo* and *in vitro* peripheral blood mononuclear cell cultures of healthy young and older adults. Stimulation of the cytotoxic T cell response by the nuclear proteins of the live influenza virus has been previously determined to be cross-reactive for all strains within the subtypes of influenza virus. The cross-reactive immune responses to influenza virus of a different subtype than initially encountered is thought to be mediated by the cytotoxic T cells specific for the influenza nucleoprotein (a conserved gene) (Townsend et al., 1984; Yewdell et al., 1985; Taylor et al., 1987; Wraith et al., 1987). The memory cytotoxic T cells would then recognize all strains within a type of influenza virus. Amino acid

changes accumulate in all eight of the influenza RNA segments after formation of the viral RNA segments during viral replication. Influenza A/H_3N_2 virus is able to change on a yearly basis due to subtle nucleotide changes coding for the amino acids within the viral surface proteins.

The cytotoxic T cell response after stimulation with three different influenza A/H_3N_2 strains illustrated the largest increase of memory cytotoxic T cell response to influenza A/Sydney in both the healthy young and older adult populations. The resting memory cytotoxic T cells appear to reside in the immune system for years after exposure to the influenza virus in both healthy young and older adults, as shown by the number of virus-specific cytotoxic T cells in influenza A/Johannesburg/33/94 activated cultures.

The results for this research project serve as the basis for the investigation of the mechanism of decline in cellular-mediated immunity in the healthy older adult population. This project has provided a basis for future characterization of the cytotoxic T cell response to influenza vaccination in the aging population. The ability to measure the cytotoxic T cell response in *ex vivo* cell cultures will result the understanding of the *in vivo* response to influenza vaccination in human subjects.

The information gained from this project has now provided evidence that the cytotoxic T cell response in the older adult population may be affected by a mechanistic defect in one of the cytolytic mediated pathways of killing influenza-infected cells. Future studies addressing these mechanistic questions concerning cytotoxic T cells in healthy young and older adults will need to be completed using larger subject populations to determine if the health or gender have an effect on the cytotoxic T cell response to influenza vaccination. An investigation of the activation of perforin-mediated killing pathway on a biochemical level will enable the determination of a

possible defect in the perforin or granzyme B at the level of messenger RNA production, which would result in an increased production of fas ligand messenger RNA, a compensatory mechanism. The cleavage mechanism of granzyme B from the precursor enzyme to the active form of the enzyme within the cytotoxic T cell may also impair the perforin-mediated killing pathway. An examination of perforin, granzyme B and fas ligand at the levels of transcription and translation will lead to a more complete understanding of the components of the cytolytic mediated pathways of killing influenza-infected cells. The identification of these specific-components of cytolytic killing pathways will result in the determination of the age-related defects that decrease the cytotoxic T cell response to influenza vaccination.

The results from this project can be an explanation of the declining cellular-mediated immune response in healthy older adults. The relatively simple techniques developed to measure cellular-mediated immunity will allow further investigation of the subject- and health-related variables associated with the immunological response to influenza vaccination of the elderly population. The characterization of the *ex vivo* cytotoxic T cell response to vaccination will provide a greater understanding of the *in vivo* response to vaccination and may aide in a better comprehension of the protective mechanisms of vaccination in the elderly population.

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VITA

CAROLINE REEVE LETTER

EDUCATION

M.S. Chemistry, Anticipated December 2000
Thesis Title: Cytotoxic T Cell Response to Influenza Vaccination in Older Adults
Old Dominion University, Department of Chemistry and Biochemistry, Norfolk, Virginia

B.S. Chemistry, May 1991
Thesis Title: Supersaturation Concentrations for Nucleation of Solute Gas Bubbles in Aqueous Solutions
Simmons College, Boston, Massachusetts

EXPERIENCE

Graduate Research Assistant, Department of Internal Medicine, Eastern Virginia Medical School, Norfolk, Virginia, 1998-2000.

Graduate Research Assistant Award, Department of Chemistry and Biochemistry, Old Dominion University, 1996.

Graduate Research Assistant, Department of Biochemistry, Eastern Virginia Medical School, Norfolk, Virginia, 1996-1998.

Graduate Teaching Assistant, Department of Chemistry and Biochemistry, Old Dominion University, 1993.

Research Assistant, Department of Chemistry, Hampton University, Hampton, Virginia 1992-1993.

PUBLICATIONS

Letter, C.R., Gravenstein, S., McElhaney, J.E. (2000). Influenza vaccination is less effective for stimulating a granzyme B response in older adults, In: International Congress Series (Oxford, UK), in press.

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