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Restraint Stress Modulation of the Murine Humoral Response to Influenza A Virus

Dissertation

Presented in Partial Fulfillment of the Requirements of the Degree Doctor of Philosophy in the Graduate School of The Ohio State University by

Xiaohong Chu, B.D.S. (Bachelor of Dental Surgery)

The Ohio State University

1996

Dissertation Committee
J. F. Sheridan
P. T. Marucha
J. H. Hughes
J. D. Walters

Approved by
J. F. Sheridan
Adviser
Department of Oral Biology
ABSTRACT

Restraint stress (RST) induced significant alterations in murine humoral immune response to influenza A viral infection. The numbers of influenza-specific antibody-secreting cells (ASCs) were enumerated in these studies to evaluate humoral responses. The numbers of IgM and IgG ASCs were significantly suppressed in lung, spleen and mediastinal lymph nodes (MLN), while their numbers were significantly enhanced in superficial cervical lymph nodes (SCV) in RST mice. In addition, the IgG subtype (IgG1 and IgG2a) responses were altered by RST. In MLN, both IgG1 and IgG2a ASC numbers were significantly decreased by stress, while in SCV, both IgG1 and IgG2a ASC numbers were significantly increased by stress. These studies implied that RST may modulate humoral responses in mucosal and nonmucosal lymph nodes differently while T helper 1 (Th1) and T helper 2 (Th2) functions may not be differentially modulated.

Furthermore, as T helper and cytotoxic T cell functions are modulated by glucocorticoids (GC) and catecholamines (CAT) in RST, the roles of GC and CAT in RST-induced modulation on ASC responses were investigated. The results showed that treatment with glucocorticoid receptor antagonist, RU486, partially restored the mononuclear cell numbers and the IgM and IgG ASC numbers in MLN, but treatment with β2-adrenoceptor antagonist, nadolol, did not restore the mononuclear cell numbers nor the IgM and IgG ASC
numbers in SCV. This study indicated that GC played a role in RST-induced alteration of ASC responses while CAT was not involved, or did not act through the β₂-adrenoceptor, in the modulation. These findings suggested that RST modulated ASC responses through GC-dependent and GC-nondependent mechanisms. Other neuroendocrine mediators (such as ACTH and CRF) may be involved in the modulation of ASC responses.
To my parents, my husband John, my daughter Michelle and my sisters
ACKNOWLEDGMENTS

I express my sincere appreciation to my advisor, Dr. Sheridan, for his guidance and support throughout my research. Thanks go to the other members of my advisory committee, Drs. Phillip Marucha, John Hughes and John Walters for their suggestions and comments. Gratitude is expressed to Dr. Ruth Nordlander for the use of her dissecting microscope.

Special thanks go to Dr. Alley for accepting me into the Oral Biology program, for his faith in me and for his help throughout the years. I would like to express my gratitude to Ms. Cathy Dobbs and Dr. Ningguo Feng for their excellent input and suggestions.

I would like to thank my parents, my sisters, and my husband for their continued unconditional love and support through the years. Without your help, I could not have achieved my goals.
VITA

July 4, 1964 ......................................................... Born in Beijing, China

1987 ......................................................................... B.D.S. School of Stomatology,
Beijing Medical University.
Beijing, China.

1987-1990 ................................................................. General Practice Dentist,
Beijing Friendship Hospital
Beijing, China.

1991-Present ............................................................. Graduate Teaching Associate,
Department of Oral Biology,
College of Dentistry,
The Ohio State University,
Columbus, Ohio.

FIELDS OF STUDY

Major Field: Oral Biology
Studies in immunology
J. F. Sheridan, Ph.D., Advisor
# TABLE OF CONTENTS

**PAGE**

ABSTRACT ........................................................................................................ ii

DEDICATION .................................................................................................... iv

ACKNOWLEDGMENTS .................................................................................... v

VITA ...................................................................................................................... vi

TABLE OF CONTENTS ................................................................................... vii

LIST OF TABLES ............................................................................................... ix

LIST OF FIGURES ............................................................................................. x

INTRODUCTION ................................................................................................ 1

**CHAPTER**

I. ENUMERATION OF INFLUENZA-SPECIFIC ANTIBODY SECRETING CELLS BY THE ENZYME-LINKED IMMUNOSPOT (ELISPOT) ASSAY .......................................................... 26

   INTRODUCTION ................................................................................ 26
   MATERIALS AND METHODS ........................................................ 29
   RESULTS .............................................................................................. 32
   DISCUSSION ........................................................................................66

II. RESTRAINT STRESS MODULATION ON MURINE ANTIBODY RESPONSES TO INFLUENZA A INFECTION ................................................................. 71

   INTRODUCTION ................................................................................ 71
   MATERIALS AND METHODS ........................................................ 74
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. SPECIFICITY OF SECONDARY ANTIBODY (GOAT ANTI-MOUSE IgM HRP)</td>
<td>41</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ELISPOT ASSAY PROCEDURES</td>
<td>38</td>
</tr>
<tr>
<td>2. DETERMINATION OF SECONDARY ANTIBODY WORKING DILUTION</td>
<td>40</td>
</tr>
<tr>
<td>3. DETERMINATION OF OPTIMUM CELL CONCENTRATION FOR ELISPOT ASSAY (MLN HIGH CONCENTRATION)</td>
<td>43</td>
</tr>
<tr>
<td>4. DETERMINATION OF OPTIMUM CELL CONCENTRATION FOR ELISPOT ASSAY (SPLEEN HIGH CONCENTRATION)</td>
<td>45</td>
</tr>
<tr>
<td>5. DETERMINATION OF OPTIMUM CELL CONCENTRATION FOR ELISPOT ASSAY (MLN LOW CONCENTRATION)</td>
<td>47</td>
</tr>
<tr>
<td>6. DETERMINATION OF OPTIMUM CELL CONCENTRATION FOR ELISPOT ASSAY (SPLEEN LOW CONCENTRATION)</td>
<td>49</td>
</tr>
<tr>
<td>7. DETERMINATION OF OPTIMUM CELL CONCENTRATION FOR ELISPOT ASSAY (MLN IgG1/G2a)</td>
<td>51</td>
</tr>
<tr>
<td>8. INFLUENZA-SPECIFIC ASC NUMBERS IN NORMAL NONINFECTED MICE SCV AND MLN</td>
<td>53</td>
</tr>
<tr>
<td>9. INFLUENZA-SPECIFIC ASC NUMBERS IN NORMAL NONINFECTED MICE LUNG AND SPLEEN</td>
<td>55</td>
</tr>
<tr>
<td>10. SCV IgM AND IgG ASC RESPONSES DURING PRIMARY INFLUENZA INFECTION IN C57Bl/6 MICE</td>
<td>57</td>
</tr>
<tr>
<td>11. MLN IgM AND IgG ASC RESPONSES DURING PRIMARY INFLUENZA INFECTION IN C57Bl/6 MICE</td>
<td>59</td>
</tr>
<tr>
<td>12. SPLEEN IgM AND IgG ASC RESPONSES DURING PRIMARY INFLUENZA INFECTION IN C57Bl/6 MICE</td>
<td>61</td>
</tr>
</tbody>
</table>
13. LUNG IgM AND IgG ASC RESPONSES DURING PRIMARY INFLUENZA INFECTION IN C57BI/6 MICE ........................................ 63
14. MLN AND LUNG IgA ASC RESPONSES DURING PRIMARY INFLUENZA INFECTION IN C57BI/6 MICE ........................................ 65
15. RST EFFECT ON CELLULARITY IN INFLUENZA INFECTED CF57BI/6 MICE ....................................................................................... 81
16. RST EFFECT ON IgM AND IgG ASC RESPONSES IN SCV .......... 83
17. RST EFFECT ON IgM AND IgG ASC RESPONSES IN MLN ...... 85
18. RST EFFECT ON IgM AND IgG ASC RESPONSES IN SPLEEN... 87
19. RST EFFECT ON IgM AND IgG ASC RESPONSES IN LUN ...... 89
20. RST EFFECT ON IgG1 AND IgG2A ASC RESPONSES IN SCV.... 91
21. RST EFFECT ON IgG1 AND IgG2A ASC RESPONSES IN MLN.... 93
22. RU486 EFFECT ON LYMPHANOPATHY OF MLN ................. 95
23. RU486 EFFECT ON IgM ASC RESPONSES OF RST MICE .......... 97
24. RU486 EFFECT ON IgG ASC RESPONSES OF RST MICE .......... 99
25. RU486 EFFECT ON THE SIZE OF IgM ASC IN RST ............... 101
26. RU486 EFFECT ON THE SIZE OF IgG ASC IN RST ............... 103
27. NADOLOL EFFECT ON LYMPHANOPATHY OF MLN .......... 105
28. NADOLOL EFFECT ON IgM ASC RESPONSES OF RST MICE .... 107
29. NADOLOL EFFECT ON IgG ASC RESPONSES OF RST MICE .... 109
**Introduction**

**Influenza**

Influenza is a viral respiratory infection which causes morbidity in patients of all age groups, and mortality in young children, elderly individuals, and immunocompromised patients. The symptoms of influenza infection are similar to those caused by other respiratory viruses (e.g., pharyngitis, croup, tracheobronchitis and bronchiolitis), but its epidemic nature and mortality from complications are important characteristics.

There are three types of influenza viruses, A, B and C, distinguished on the basis of the identity of the major internal protein antigens, the nucleoprotein (NP) and the matrix (M1) proteins. Influenza A, B and C are all human pathogens, whereas they also infect animals. The large gene pool of influenza A virus in human, animals and aquatic birds results in the continuous changing of antigenicity and fast evolution of influenza A virus. Continued evolution of the influenza A virus causes local sporadic, regional epidemics and occasional pandemics of diseases in humans and animals. Although the gene pools of influenza B and C are also present in dogs and swine in addition to human (1, 2), their gene pools are smaller than influenza A, therefore, the structure and antigenicity of influenza B and C change less
frequently. Influenza B may also cause human epidemics, but influenza C virus only causes mild infection in human (3).

The reason that the pandemics happened in 1957, 1968 and 1977 was the changes in viral antigenicity of influenza A viruses (4). Since the genes of influenza A virus are segmented, coinfection of a cell with two influenza viruses may result in progeny virions containing various assortments of genes from both parents. Thus gene reassortment and point mutations during viral replication are the major reasons for changes in antigenicity and generation of new influenza A viruses.

**Influenza A virus structure**

Influenza A viruses are members of the *Orthomyxoviridae* family. They have special affinities for mucopolysaccharides and glycoproteins (in particular, for sialic acid-containing receptors on cell surfaces).

Influenza A viruses are pleomorphic, usually 80-120nm in diameter, but filamentous forms are commonly seen. Viral particles consist of a host-derived lipid bilayer envelope in which the virus-encoded glycoproteins are anchored as spikes, an inner shell of matrix protein lining the inside of the envelope, and an nucleocapsids core formed by viral RNA and nucleocapsid proteins (NP) at the center. Viral RNA polymerases are attached to one end of each nucleocapsid (5).

**Influenza A viral proteins**

**Envelope proteins**
Glycoproteins

Two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), comprise the spikes protruding from the envelope surface. HA molecules are evenly distributed and NA spikes are in patches. HA mediates the initial attachment of a viral particle to its cellular receptor, a sialic acid molecule on the host cell surface, as well as fusion of viral envelope to the host membrane. NA has an enzymatic function, namely, the removal of a sialic acid residue from any glycoconjugate possessing the terminal sugar, thus permitting the escape of progeny virions from host cell, separating virus particles from inhibitory mucopolysaccharides in the respiratory tract and facilitating virus spread (6).

Direct amino acid sequencing and X-ray crystallography have provided the information about functional domains and antigenic regions of HA (7, 8). At the amino terminus, a signal peptide, which directs the nascent HA molecule into the endoplasmic reticulum, is followed by the HA1 and HA2 peptide chains. At the carboxyl terminus, the hydrophobic transmembrane domain is next to the cytoplasmic domain.

The removal of the arginine residue between the HA1 and HA2 chains by intracellular trypsin-like enzymes (9, 10) activates the HA molecule, as the noncovalently linked HA1 and HA2 undergo conformational change and allow virus entry into the host cell. After the viruses attach to receptors and are endocytosed into host cell, the virus vesicles fuse with endosome and its pH is lowered. The decreased pH causes conformational change in the HA molecule which brings the HA2 amino terminus closer to the vesicle membrane, which in turn fuses the virus envelope with the host membrane and delivers the nucleocapsid into cytoplasm. Then the viral genes move into the nucleus and start transcription and replication.
The cleavage of HA into HA1 and HA2 is considered the final stage of virus maturation. HA of mammalian influenza viruses are probably cleaved by extracellular protease of the respiratory tract in the host.

Each HA monomer forms an elongated stalk capped by a large and a small globular region at each end. The HA spike is a trimmer of the HA molecule. The hydrophobic forces between the stalks hold the three molecules together. The attachment sites for cellular receptors are located at the top surfaces of the large globule and they are highly conserved. The antigenic sites also appear on the large globule and are free to mutate extensively, contributing to the great antigenic variability of influenza A viruses, without affecting the overall framework of the HA trimmer. Since HA is the major target of the host immune response, selection for amino acid substitutions driven by immune pressure starts from substitution of bases in gene replication. About one base substitution in the HA gene occurs in each replication cycle (3).

The NA spike on the viral envelope is composed of tetrameres of NA molecules. A slender stalk topped by a box-like head region. The catalytic site is situated at the top of the head of each subunit (11). Antigenic changes are confined to the amino acid chains that do not contribute to the structural framework of the head.

HA and NA molecules of each type of influenza virus have their variations. For example, there are 14 HA subtypes and 9 NA subtypes in influenza A viruses, according to their differences in amino acid sequences. For example, H2-H14 subtypes differ from H1 by at least 30% in their amino acid sequence and they are serologically not cross-reactive. Strains in one subtype are partially serologically cross-reactive (12).
The influenza A viruses are classified using a nomenclature defined by host, origin, strain and year of isolation, and HA and NA subtypes given in parentheses, e.g. influenza A/human/PR8/34/(H1N1).

**Nonglycoproteins**

Matrix proteins include M1 and M2. M1 is the major structure protein that covers the nucleocapsid core and lines the lipid envelope. It is the bridge between the nucleocapsid and carboxyl terminus of envelope proteins, probably involved in initiating progeny virion assembly. M2 is membrane-associated and present in virions in very small amounts. It is essential for virion maturation and entry, since it is a proton channel to control the pH of the Golgi during HA synthesis and allows acidification of the virus vesicle during virus uncoating. There is other evidence supporting this concept. Specific mutations in M2 confer resistance to the antiviral drug, amantadine (13), and antibodies to M2 protein restrict virus growth (14).

**Nucleocapsid proteins**

RNA dependent RNA polymerases (P) are less abundant than nucleoproteins (NP) in the nucleocapsid. They attach to the end of nucleocapsid and biochemical evidence showed that P proteins are clustered at the 3' end of template, as if poised to initiate RNA synthesis (15). Three influenza A viral polymerases, PB1, PB2 and PA, play a role in viral transcription. PB1 and PA also function in viral replication process. All the nucleocapsid proteins contain specific nuclear transport sequences that direct them to the nucleus. After viral uncoating, the nucleocapsid migrate into host cell nucleus and polymerase complex begin mRNA transcription.
NP protein triggers the switch of RNA synthesis from transcription to replication. NP gene analysis indicates that NP may be a determinant of host range, since their gene sequences fall into five groups according to their host range (16, 17, 18).

**Nonstructural proteins**

The functions of influenza virus nonstructural proteins (NS1 and NS2) are unknown. They are not assembled into the virus but accumulate in host cell nuclei and may have a function related to virus-specific RNA synthesis (19).

**Influenza A virus genome**

The single-strand RNA molecule of influenza A virus encoded 10 genes in 8 segments with negative polarity. Each segment is encapsidated by NP proteins separately. The segments are numbered 1 to 8 in order of decreasing molecular weight. PB2, PB1 and PA are encoded by segments 1 to 3, respectively. Segments 4 and 6 encode for envelope glycoproteins HA and NA, respectively. Segment 5 is the gene for NP protein. Each of the two smallest segments yield at least 2 different kinds of transcript by differential splicing during transcription. M1 is the product of complete transcription of segment 7, whereas M2 is a truncated transcript of segment 7 using a different reading frame. NS1 occupies the continuous RNA template of segment 8 and NS2 is encoded by a spliced mRNA sequence (12).
**Influenza A virus transcription and replication**

The RNA of influenza A virus has negative polarity. A positive polarity mRNA is needed for gene expression. Since viral RNA polymerases need a capped and methylated primer synthesized by host RNA polymerase II to initiate transcription, an virus-encoded endonuclease cleaves a capped 10 to 13-base-long oligonucleotide donor mRNA from host transcript (12). The resulting primer has a G residue methylated in the 7 position and an hydroxyl group at 3' end. Viral RNA polymerase PB2 recognizes and binds to 5' cap, whereas PB1 catalyzes the extension of the primer using the viral RNA as template. The three P proteins work together as a noncovalently linked complex and move along the template (20, 21, 22). Transcription stops 15 to 22 nucleotides before reaching the 5' end of viral RNA template. The transcription of NP and NS1 RNA segments occurs early in infection and the mRNAs specifying surface proteins (e.g., HA, NA, and M1) becoming more prominent later (23). Therefore, the abundance of the viral peptides is controlled at the transcriptional level.

The proteins that transcribe influenza virus mRNA segments also carry out their replication, except PB2 protein (24). NP proteins are involved in regulating these alternative uses of the same complement of proteins. Viral genome replication is switched on by the abundance of NP proteins. Assembly of the new nucleocapsids deplete the available pool of NP proteins, decreasing their concentration below the needed level and switching on the transcriptional mode that will replace them. This is a facile way to achieve a balance between transcription and replication.
After the nucleocapsid is assembled within the nucleus, it moves toward the surface of cell. Since the viral envelope proteins are expressed on host cell membrane and virus uses a piece of host membrane as its envelope, the viral envelope is assembled at the cell surface. In the budding process, M1 protein bridges the nucleocapsid and carboxyl terminus of glycoproteins that have inserted into the nascent virus envelope patches.

**Influenza A virus genetic variability and epidemiology**

The RNA polymerases of influenza A virus do not have proofreading function, therefore approximately 1 in $10^4$ bases may be incorrectly inserted, substituted or deleted in replication process, which results in amino acid sequence changes and alteration of antigenicity (4, 25). These point mutations are the reason for minor antigenicity change (antigen drift). Each round of RNA virus replication results in a mixed population with many variants. The accumulation of mutations may cause some of the mutants to have the potential of becoming dominant under the right selective conditions.

Gene reassortment may occur if two or more influenza viruses coinfect the same host cell, because the replicas of parent genomes are equally selected into a new virus. Large amounts of genetic materials from each parental strain being introduced into this new virus results in major changes in antigenicity (antigen shift). Although RNA recombination among negative-stranded viruses is uncommon, insertion of cellular mRNA sequences into influenza HA gene with acquisition of virulence has been demonstrated, offering another possible mechanism of antigen shift (26).
The continuing evolution may occur in each of the eight gene segments, but it is most prominent in the surface glycoproteins HA and NA. The genes coding for surface proteins are subject to strong selection pressure by neutralizing antibodies of host immune systems. Host antibodies directed against HA block attachment of virus, preventing infection (27). In contrast, antibodies against NA block its enzymatic activity and prevent progeny virus release and spread of infection (28).

Reassortant viruses with new genes for surface proteins have a selective advantage over the parent viruses because they are able to escape the host immune responses. The emerged new viruses, with changes in HA and NA determinants, do not bind, or bind with low avidity, to the antibodies that were induced in previous infection of other subtypes of influenza viruses. Thus, if the new viruses are sufficiently infectious, they can escape immune responses completely or partially, resulting in pandemic or epidemic influenza infections, respectively.

Aquatic wild birds are considered the primordial reservoir of all influenza A viruses for avian and mammalian species. The gene pool of influenza A viruses in aquatic birds provides all the genetic diversity required for the emergence of pandemic influenza viruses for humans, lower animals, and birds (3). Cross-species infections have been shown to occur between swine and humans (29). Because influenza A viruses have gene pools in human, animals and aquatic birds, the possibility of coinfection and gene reassortment of influenza A is higher than that of influenza B and C, which have smaller gene pools. Thus, influenza A viruses can cause pandemic infection, whereas influenza B and C may only cause epidemic or sporadic infections.
The serologic analysis of individuals who survived influenza epidemics, and direct viral isolation and gene sequencing, have helped to elucidate the evolution of human influenza A virus subtypes. Around 1900, H3N8 emerged and replaced H2N2, which prevailed earlier. In 1918, "Spanish influenza" H1N1 pandemic occurred (30). H1N1 circulated in humans for many years until H2N2 reemerged and replaced H1N1 in the 1957 "Asian influenza" epidemic. H3N2 "Hong Kong" virus pandemic happened in 1968, followed by a 1977 pandemic, in which the H1N1 "Russian" virus, genetically identical to an influenza A virus which circulated in the human population in 1950, reappeared, causing widespread epidemics with low mortality. Since that time, H1N1 and H3N2 have been co-circulating in the human population (3).

It is postulated that southern Asia is an influenza epicenter, since influenza A viruses of all species may circulate in ducks and pigs in the tropical and subtropical region all year around. Close contact between human and animals provide the opportunity for interspecies transmission and genetic exchange among influenza viruses. Subsequent recombinations may yield novel viruses against which there is little protective antibody in the population or which may be more virulent than previous strains (31).

**Pathogenesis of influenza viral infection**

Influenza viruses can be transmitted between individuals through respiratory droplets or direct contact of contaminated surfaces. The symptoms of influenza A infection are similar to those caused by other respiratory viruses.
The viruses attach to and then replicate in host cells. The viral replication and antiviral immune responses result in damages to or even desquamation of the epithelial cells of upper respiratory tract and pulmonary tissues. Desquamation and inflammation of larynx, trachea and bronchi lead to the clinical symptoms (32). If immune responses limit the viral replication and infection from spreading, then the epithelial cells regenerate and the pulmonary function recovers.

In immunocompromised individual, the elderly and young children, the initial upper respiratory infection may spread and cause influenza pneumonia. There are three patterns of human lower tract involvement: influenza pneumonia followed by bacterial superinfection, concurrent influenza and bacterial pneumonia, and rapidly progressive “pure” viral pneumonia. The first two types are more common than the third type, but the third type has the highest mortality rate. The gross appearance of the lung in influenza pneumonia is nonspecific, as is the case for all viral pneumonia. In fatal cases, the lungs are heavy, edematous, and hemorrhagic, often with increased firmness, and often with obvious tracheobronchitis on gross examination (33).

Although influenza viruses are not natural pathogens for mice, the virus strains which are adapted in lab mice may cause upper and lower respiratory tract infection. The less-virulent strain remain confined to the upper respiratory tract, whereas more virulent strains spread down the trachea to produce bronchial and bronchiolar epithelial necrosis. Inhalation of the viruses in fine droplets (34) or intranasal administration of virus to an anesthetized mouse (35) leads to pneumonia, while intranasal administration to awake mice produces
tracheitis (36). The influenza pneumonia often results in severe damage to lung air exchange and subsequent death.

**Anti-influenza immunity**

The immune responses to the viral challenge are both viral non-specific and viral specific. The initial events following infection is influenza non-specific, and involve complement, natural killer (NK) cells, resident and infiltrating macrophages (Mφ) and interferons (IFN). Antigen specific cell-mediated immunity includes the interactions between antigen presenting cells (APC) and CD8+ cytotoxic T lymphocytes (CTL), aided by CD4+ T helper (Th) cell-produced cytokines. An antigen specific humoral immune response is mediated by influenza specific antibodies secreted by plasma cells, which is also aided by Th cytokines (3, 37).

**Influenza virus non-specific immunity**

Influenza non-specific immunity, or natural immunity, includes fever, Mφ, NK, interferon and complement responses (38). Their function is to limit viral spread and initiate recovery.

Pretreatment of mice with *P. acnes* (*Corynebacterium parvum*) before a lethal virus challenge results in lower lung viral titers and lower mortality (39). This protective effect correlates with increased lung interferon levels, Mφ content, and NK cell activity in the absence of any changes in lymphocyte function. Another experiment showed that survival of athymic mice following a sublethal infection with influenza virus is dependent on either the
nonspecific immune response or IgM production, but neither mechanism is sufficient for complete recovery (40).

Fever plays a role in host defense mechanism, since the height of fever is correlated to the subsequent rate of decline of nasal virus titer, and the suppression of fever in ferrets by nonpharmacologic means results in delayed clearance of virus (41, 42).

Influenza viruses undergo abortive replication in Mφ (43, 44). The infected Mφs act as antigen presenting cells and also produce α- and β-interferon (45, 46). In addition, they may mediate lysis of infected cells independent of antibody (47). Pulmonary type I (α and β) interferon rises rapidly during murine influenza infection and correlates directly with the degree of viral replication. Alveolar Mφ and lymphocytes recovered early from infected lungs are the major sources of interferon (45). NK cells limit the viral spread by lysis of virus-infected cells and by production of type I interferon (48).

Complement may be activated by virus and virus-infected cells by the classical or alternative pathways (49). Although complement binding in the absence of antibody may neutralize virus or result in lysis of viral particles, the major protective role of complement is to mediate antibody-dependent lysis of infected cells (50).

**Influenza Virus Specific Immune Responses**

Murine influenza infection happens in both the upper and lower respiratory tract. Viral replication occurs initially in the epithelial cells and later extends to the alveolar macrophages and alveolar cells (51). Cytopathic effect of influenza virus infection leads to cell death and desquamation. Virus titer increases rapidly in lungs and reaches a maximum only a few days after intranasal infection. The peak levels of interferon in lung lavage fluid are
reached afterwards, followed by the appearance of virus neutralizing antibodies in the serum and lung lavage fluid (52). Influenza specific CD8+ CTLs appear prior to the appearance of virus-specific antibody secreting cells (ASCs) in lung. Also, the time CTLs are detected coincides with a precipitous decline in pulmonary virus titer (53).

Both cellular and humoral immune responses are important in the host defense to influenza infection. Antibodies to the HA have been shown to be critical for virus neutralization and for protection against subsequent infection (54, 55), whereas cytotoxic T cells play a role in the clearance of the virus in an ongoing infection (40). Both of these responses appear to be T helper (Th) cell dependent (56, 57).

Cell-mediated immunity (CMI) to influenza virus

There is a general agreement that T lymphocytes play a role both in recovery from lethal pulmonary influenza virus infection of mice (58, 59, 60, 61, 62, 63, 64) and in the pathogenesis of viral pneumonia in mice (58, 59, 60). CD8+ CTLs are responsible for the clearance of infected cells from pneumonic lungs (65, 66) and for the termination of infection in the absence of the CD4+ T helper cells (67), although compensatory mechanisms are able to clear virus slowly without CD8+ cells (68).

Dendritic cells are the major antigen presenting cells (APC) in mediastinal lymph nodes (MLN) that drain the lungs and are responsible for the initiation of the immune response (69). Pulmonary dendritic cells are able to present antigen associated with MHC-II to T cells after they migrate into MLN (70, 71, 72), but their capacity to present antigen is actively downregulated by adjacent macrophages via secreted products (e.g. IFNα) in the
lung. This may be viewed as a protective mechanism to limit T-cell mediated damage to host epithelial tissues in the lung and airways (73).

Lung consolidation is related to virus replication and CTL response. The action of CTL induces pathological damage in the lung but it is not the only cause of such damage. A vigorous CMI response to an influenza virus infection can be mounted in the lung with minimal pathology occurring, a deciding factor probably being the level of virus replication in the lung (74).

CD8+ T cells are primed in the regional draining lymph nodes and then migrate back to the site of virus growth in the respiratory epithelium (67), where they lyse virus-infected cells. In mediastinal lymph nodes (MLN), CTL precursors are exposed to Th cell cytokines and to viral antigens on antigen presenting cells, after which the influenza specific CTL clones expand and differentiate into mature CTLs. These CTLs move out of the MLN and travel via vascular system to the infected lung. CTL precursor activation may also occur in spleen, which acquires virus or viral fragments from blood. The development of the CTL response in the lymphoid tissue and movement of the cells to lung span an interval of 7-10 days following infection, which parallels the period of viral clearance from the lung. The CTL memory cells in circulation have a half-life for about 2-3 years after infection (66).

Primed CD8+ memory CTLs also provide some protection (e.g., reduce lung virus titer and death rate) against heterologous influenza A viruses since the response is often to a conserved viral antigen (e.g. nucleoprotein molecule, matrix protein) (75, 76). MHC-I restricted CD8+ CTL killing of infected cells is not the sole mechanism of viral clearance, as mice depleted of CD8+ T cells or MHC-I molecules clear viruses by the activation of CD4+
CTL or other mechanisms (e.g., CD4+ T helper cells promoting local B cell response or helping γ/δ CTLs). This redundancy in CMI is beneficial for the host (68).

Th cells recognize fragments of internal antigens of influenza virus presented by MHC-II molecules and do not discriminate between different subtypes of influenza viruses within a type (37). Depletion of Th cells results in decreased IgG production, lower CTL function and delayed virus clearance (77, 78). Therefore, Th is essential for CMI and humoral responses to influenza virus.

**Humoral immune response to influenza virus**

Influenza virus-specific B cells in the lung and upper respiratory tract draining lymph nodes, as well as in spleen, are also activated by Th cytokines and APCs. They proliferate and differentiate into effectors or precursors of ASC / plasma cells, which subsequently migrate from the lymphoid tissues to lung through circulation system (79). IgM and IgG often appear in serum after primary infection, while IgA forms the major response in nasal secretions (80, 81).

Anti-M, NP, NA and HA antibodies develop frequently after infection, but only anti-HA antibodies are able to neutralize viruses and interfere with their attachment to the host cells. Evidence showed that physiological amounts of IgG antibodies to the HA molecule protected mice from influenza infection in the absence of an active host response, while passive transfer of large amounts of either anti-M or anti-NP antibodies provided no protection, and anti-NA antibody provided limited protection (55). Therefore, it is generally accepted that antibodies to the HA molecule play a primary role in antibody-mediated
protection and antibodies to the NA molecule play a secondary role in reducing virus yield from cells and the spread of virus (82).

Anti-HA antibodies provide subtype specific protection, that is, only the reinfection of viruses with the same HA antigenicity (homotypic infection) can be prevented (54). There is no cross-protection to a different subtype, since the anti-HA antibodies are specific for that subtype of HA molecule. Heterotypic immune serum passively transferred in mice is less protective than homotypic serum (54). Epidemiological observations in humans also indicate that infection with one subtype does not confer immunity to the other subtypes (83).

In addition to neutralizing viruses, antibodies may modify infection by restricting viral spread by lysis of virus-infected cells. This effect is mediated by the lytic action of complement or by antibody-dependent cell-mediated cytotoxicity (ADCC).

During infection, serum antibody helps in reducing virus shedding and in tracheal epithelium regeneration (84). Therefore, while serum antibodies may not be essential for recovery, if present, they help the process.

Experiments using intranasal administration of anti-IgA have proved that the level of nasal secretory IgA is also related to the prevention of reinfection. Abrogation IgG and IgM in nasal secretion did not affect virus shedding and infection spreading, while abrogating IgA did (85). Anti-HA IgA and IgG in nasal washings and serum derived IgG in the lower part of lung are the major defense against reinfection (38).
Stress and Immunity

Homeostasis denotes the relatively stable conditions of the internal environment that result from compensating regulatory responses performed by homeostatic control systems (86). The hypothalamus is crucial to homeostatic regulation and is a principal site for regulating the behavior essential to the survival of an individual. The integration achieved by the hypothalamus often includes correlation of neural and endocrine functions.

Stressors interrupt the homeostasis of the body and induce a series of responses in nervous and endocrine systems. The anatomical and physiological pathways of stress modulation of the neuroendocrine system have been investigated extensively. It has been found that responses to stressors are initiated by the hypothalamus and translated into action by the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (87, 88, 89, 90). Activation of the HPA axis and SNS leads to the release of glucocorticoids (GC) from adrenal cortex and the release of catecholamines (CAT) from sympathetic nerve terminals and adrenal medulla.

Various aspects of immune function are modulated by stress. GC and CAT are considered to be among the major immunomodulatory factors of the neuroendocrine system. Sympathetic innervation of both primary and secondary organs of the immune system has been demonstrated (91, 92, 93, 94). β-adrenergic receptors have been found on T and B lymphocytes (95, 96, 97), macrophages (98, 99), neutrophils (100), and natural killer (NK) cells (101). Direct SNS contact of terminal nerve process with T lymphocytes in lymphoid organs has been shown by Felten's group (102). In addition, adrenergic innervation of the
vasculature and lymphatic ducts have been demonstrated and may play a role in lymphocyte trafficking (103, 104, 105).

GC receptors have been shown on mononuclear cells (106, 107). In addition, glucocorticoid response elements (GREs), which bind to the GC-receptor complex, have been found in some cytokine genes. Furthermore, GC modulation of cytokine gene expression has been shown by some studies. GC inhibits lymphocyte IL-2 gene expression at the transcriptional level and macrophages IL-1 gene expression at both the transcriptional and posttranscriptional levels (108, 109, 110, 111, 112). GCs are known to modulate the expression of adhesion molecules, thus play a role in changing the pattern of recirculation or trafficking of lymphocytes (113, 114, 115, 116, 117).

The HPA axis products, other than GC, also have immunomodulatory capacity. Activation of hypothalamus results in the release of corticotropin-releasing factor (CRF), which induces the secretion of adrenocorticotropic hormone (ACTH), opioid peptides and other proopiomelanocortin (POMC) gene products from the pituitary gland. ACTH, in turn, stimulates the production and secretion of GC from the adrenal cortex. ACTH receptor expression has been found on mononuclear cells (118, 119, 120). In addition, ACTH has been shown to inhibit antibody production (121) and enhance B cell proliferation (122). Also, ACTH inhibits T cell IFN-γ production (123), IFN-γ-induced macrophage activation and MHC-II expression (124, 125). CRF receptors have been demonstrated on macrophages (126, 127) and CRF alteration of NK activity has been shown (128, 129, 130).

The interaction between immune and neuroendocrine systems is bidirectional. Cytokine (e.g., IL-1, IL-6, TNF and IFN) receptors have been found in the central nervous
system (131) and activated lymphocytes produce factors analogous to many of the pituitary hormones (e.g., ACTH and CRF) (132). It has been suggested that the immune system may act as a sensory afferent organ for the CNS, providing it with a feedback in response to change within the immune apparatus corresponding to antigen challenge (133).

The immune process has generally been considered to be a self-regulatory process controlled by a number of mechanisms (e.g., cytokines, cell-cell contact, receptor regulation). However, an additional mechanism for immune regulation exists, namely modulation by the CNS through the HPA axis and SNS. To investigate modulation of the immune system, stress is often used as a stimulus to activate the neuroendocrine system. The studies in our lab are mainly focused on the modulation of the immune response by two major classes of neuroendocrine mediators, namely, GC and CAT.

**CAT and GC receptors and signal transduction pathways**

β-adrenergic receptor is linked via the Gs protein to adenylate cyclase. Activated adenylate cyclase converts ATP to cAMP, which then activates cAMP-dependent protein kinase A, which in turn phosphorylates various proteins in the cytosol and cell membrane (134). These proteins can regulate many aspects of lymphocyte function and lead to the CAT-specific effects.

GC receptors, like other steroid receptors, are located in the cytoplasm. The hormone penetrates freely into the cell, where it binds to the receptor to form a nonactive complex. Through an unknown process, the complex is activated in the cytosol (which is characterized by having high affinity for DNA), then the complex moves into nucleus and binds to
regulatory elements (GREs) of certain genes. As a result, transcription of specific genes is regulated. The hormone thereby increases or decreases the levels of mRNAs and usually of the proteins that the genes encode. Those proteins, which may be enzymes, secretory products, regulators of various functions including transcription of other genes, are the primary effectors of GC actions (135).

The structure of the GC receptor is composed of a steroid binding domain, a DNA binding domain and two Hsp90 molecules. Its molecular weight is about 300KD. The Hsp90 molecules are associated with the exposure and coverage of the DNA-binding domain. During GC receptor complex activation, the Hsp90 molecules dissociate from the complex (135).

**GC and CAT modulation on immune responses**

GCs exert regulatory influences in either a positive or negative way on virtually every stage of the inflammatory and immune response in animals and men (136, 137, 138). GCs deplete the circulating lymphocytes from lymph nodes, spleen and thymus by redistributing them into other body compartments (e.g., bone marrow) (114, 139, 140, 141, 142). The mechanisms of the trafficking regulation is not clear, but possibly results from modulation of adhesion molecule expression on lymphocytes and on endothelial cells.

GCs impair monocyte random movement, chemotaxis and bactericidal activity after only brief periods of contact with the hormone (143). Also, IL-2 and IFN-γ gene transcription (111, 144), IL-3 transcription (145), IL-1β translation and posttranslation regulation (109) are suppressed by GC.
It appears that T cells are more susceptible to GC immunosuppressive effect than B cells (139, 142, 146). And B cell traffic is not as significantly affected by GC as T cell traffic (147). The same in vitro concentration range of corticosteroids which suppress certain T cell functions is often essential for maximal Ig production. Specifically, it has been demonstrated that Ig production is significantly enhanced in the presence of in vitro GC within physiologic range (148, 149, 150). Some in vivo studies in animals and man also showed enhanced Ig production by GC (151, 152). The enhancing effect is exerted predominantly on the early activation process and not later on the differentiation of B cells to plasma cells (153).

The appearance of α-adrenoceptors on lymphocytes has not been proved by ligand binding techniques and thus their existence remains controversial. On the other hand, β-adrenoceptors on lymphocytes are coupled to adenyl cyclase (134). Agents that inhibit adenylate cyclase or activate guanylate cyclase and/or phospholipase C are usually stimulatory (154). It is generally true that hormones that activate adenylate cyclase inhibit T lymphocyte function (155), but an elevation of cAMP early in an immune response can activate B cells.

Simultaneous stimulation of T cells with mitogen and β-adrenergic receptor agonist isoproterenol results in diminished cellular proliferation which is associated with a marked synergistic rise in the intracellular cAMP (156). Stimulation of T cells with β-adrenergic agonist results in a minor increase of cAMP level. Mitogen alone has little effect on cAMP synthesis, since the secondary messenger linked to the T cell receptor and to mitogen responses is the phosphatidylinositol system. Activation of this system results in inositol triphosphate (IP₃) and diacylglycerol (DAG) production by the action of phospholipase C (PLC). IP₃ stimulates the release of calcium from intracellular stores and DAG activates
protein kinase C (PKC), initiating a cascade of events which ultimately results in cell proliferation. The interactions between the cAMP and phosphatidylinositol pathways result in the decrease T cell proliferation.

Experimentally induced increases in intracellular cAMP levels stimulate in vitro antibody production of B cells isolated from spleen (157). In addition, β2-adrenergic agonist induces an increase in both the amount of antibody produced and the number of antigen-specific antibody producing cells (158). It has also been reported that increasing cAMP early in the culture period increased antibody synthesis, but sustained elevation of cAMP beyond 24hr inhibited antibody synthesis (159, 160, 161). Elevation of cAMP in G0 phase B cells is essential for them to enter G1 phase, but elevated cAMP inhibits B cells from entering the S phase (162). It is suggested that after antigenic contact a cell surface alteration may lead to a decrease in the intracellular level of cyclic AMP, which could be a signal for DNA activation, cell differentiation and proliferation. This decrease might be prevented by exogenous cAMP (163). Chamber's group (134) suggested that CAT regulation of the immune response includes cAMP-dependent and cAMP-independent mechanisms. Given that an antibody response depends on T cell function, CAT modulation of an antibody response is more complicated than that of T or B cells alone.

Studies in which adrenergic agonist-induced lymphocytes egress from lymph nodes (164) and spleen (165) were inhibited by the administration of adrenergic antagonists without altering blood flow rates, suggested that the control of leukocyte emigration is extremely complex, depending on the release of multiple neuroactive substances which affect not only
the blood and lymph flow rates, but probably also the expression of leukocyte adhesion molecules (166, 167).

In addition, GC modulates lymphocytes response to CAT. Clinical treatment of bronchial asthma with corticosteroids and β-adrenergic drugs have demonstrated that GC can amplify CAT responsiveness by stimulating cAMP accumulation in lymphocytes and by enhancing cellular enzyme responsiveness to the modulation effect of cAMP (168, 169, 170).

The mechanism of GC enhancing cAMP level is unclear. Furthermore, GC enhanced adrenergic receptor expression has been shown by several studies (171, 172). Elevated GC level may amplify lymphocyte sensitivity to β-adrenergic stimulation by increasing the receptor density (173).

The mechanisms of RST-induced suppression of CTL cytolytic function was investigated in our lab using GC and CAT receptor antagonists RU486 and nadolol, respectively. It was found that treatment with RU486 reversed the stress-induced diminution of cellularity in local lymph nodes, while treatment with nadolol and RU486 completely reversed the stress-induced suppression of CTL activation. These findings suggested that both GC- and CAT-mediated mechanisms are operative in the suppression of anti-viral cellular immunity (174).

**Purpose of this study**

RST was shown to modulate serum antibody responses to influenza virus in mice. It delayed the serum antibody class switching but did not affect the magnitude of antibody responses (175). In addition, RST also suppressed IL-2 production in spleen and MLN (176).
We hypothesize that Th1 function is suppressed but Th2 function is unchanged or enhanced by RST. Since class switching to IgG2 depends on Th1 IFN-γ production and class switching to IgG1 relies on Th2 IL-4 production, we speculate that IgG2 response is suppressed while IgG1 response is unchanged or enhanced by RST.

In addition, since IL-2 is a B cell growth factor and its production in MLN and spleen are decreased in RST, we hypothesize that IgM and IgG responses in MLN and spleen are suppressed. Also, as a corollary hypothesis we propose that antibody production in lymphoid tissues other than MLN and spleen, which have suppressed IL-2 production, could be enhanced by RST, since the magnitude of serum antibody production is unchanged by RST. To test these hypotheses, the humoral responses in different lymphoid tissues will be examined and RST modulation on the responses will be investigated.

Furthermore, since GC and CAT has been shown to modulate antibody responses in *vitro* and *in vivo*, and CD4+ and CD8+ T cell function are affected by GC and CAT in RST, we hypothesize that GC and CAT may also be involved in regulating anti-viral humoral immunity. To test this hypothesis, GC and CAT receptor antagonists (RU486 and nadolol, respectively) will be used in RST mice to examine if they are able to block the RST effect on antibody responses.
Chapter I
Enumeration of Influenza-Specific Antibody Secreting Cells
by the Enzyme-Linked Immunospot (ELISPOT) Assay

Introduction

The ELISPOT assay was initially developed by Sedgwick and Holt in 1983 to measure antigen-specific IgE secreting cells (177, 178, 179). The assay was based on the enzyme-linked immunosorbent assay (ELISA) and was called the ELISA plaque assay. In the meantime, several other groups developed similar assays, Czerkinsky's group named it the enzyme-linked immunosorbent spot (ELISPOT) assay (180), and Moller et al. described it as filter immunosorbent plaque assay (FIPA) (181).

The basic principle behind the ELISPOT assay is similar to the ELISA. Antigen is bound to a solid support (i.e., plastic or nitrocellulose) in a well. A blocking solution (e.g., bovine serum albumin) is added to the wells to bind the remaining protein-binding sites on the solid surface. Cells are added to wells and allowed to incubate. During the incubation period, antibody is secreted from antibody-secreting cells (ASC) and binds to neighboring antigen. The cells are washed from the plate leaving the antibody bound to the antigen in discrete areas. An enzyme conjugated anti-immunoglobulin antibody is added to the wells (e.g., horseradish conjugated goat anti-mouse IgG). A substrate solution is added and an insoluble
product is produced in discrete regions in which antibody is bound to antigen. The spot
produced by the insoluble product represent an antibody-secreting cell (ASC) (Fig. 1). To
determine the isotype of an ASC, antibodies specific for different heavy chains can be used.

The ELISPOT assay has been modified by many labs to measure different parameters.
It is most commonly used to enumerate antibody secreting cells (182) and cytokine producing
cells (183). By using different enzyme-substrate systems, the number of ASCs with different
isotypes can be counted in one well (184). The specificity of the assay and the optimum length
of cell incubation time have been examined by many groups (180, 185). The assay was
found to have very high antigen specificity. The optimum incubation time for cell culture is
4 hours and longer times only make the spot blur without increasing the spot numbers. The
result of an ELISPOT assay determines the number of antigen-specific ASCs, which is
different from an ELISA result which determines the amount of antibody produced. Usually
there are differences in the appearance of spots and the spot size depends on the antibody
affinity and the amount of antibody produced by the ASC (180, 181, 186).

Several different apparatuses were tried in our lab to get clear spots and reproducible
results. The system used by Merchant’s group (187) was tried with modification. But using
nitrocellulose as solid phase produced nonspecific binding of antibodies and the apparatus
(Minifold®, Schleicher & Schuell, Inc. Keene, NH 03431) could not hold the cell culture
medium long enough to have the antibodies bind to antigen before the medium leaked through
the nitrocellulose film. Then we modified the system developed by Orr et al. (188), which
used flat bottom high-binding 96-well polystyrene plates (Costar) and overcame the
nonspecific binding and leakage problems.
Influenza A viruses introduced intranasally induce both cellular and humoral immune responses. A previous serum study showed that, during primary infection, serum IgM and IgG titers peaked around day 10 post infection, while the IgA titer peaked at day 14. Both IgG and IgA titers had a plateau after reaching peaks, but the IgM titer decreased quickly afterwards (175). Serum antibodies are secreted by plasma cells from different lymphoid tissues in the body, therefore the serum antibody response reflects a systemic humoral response. Since each lymphoid tissue has its unique antigen draining region and unique microenvironment for lymphocyte activation and differentiation, the pattern of antibody responses in each lymphoid tissue could be different from that in serum. Therefore, to investigate the cellular events of stress modulation of the antibody response to influenza virus, the ELISPOT assay was developed to examine the humoral immune responses in lung and lymphoid tissues. We first studied the kinetics, isotypes and magnitude of the influenza-specific antibody response in the lungs, mediastinal lymph nodes (MLN), superficial cervical lymph nodes (SCV) and spleen. Subsequently, neuroendocrine modulation of the antibody response was examined in restraint stressed mice (which will be discussed in chapter II).

We hypothesized that in response to an influenza viral infection each lymphoid tissue has its own kinetics, isotype pattern and magnitude of ASC responses.

**Materials and methods**

**Animal**

Virus-antibody-free C57Bl/6 male mice, 4-6 weeks old, were purchased from Charles River Laboratory (Wilmington, MA). Mice were housed five per cage and provided food and
water ad libitum. The 12 hour light and dark cycle began at 6am. The mice were allowed to acclimate for one week before the experiments started.

**Virus**

Influenza A/Puerto Rico/8/34 (PR8) (H1N1) virus was obtained from the American Type Culture Collection (Rockville, MD) and cultured in the allantoic cavity of 10-day-old fertilized chicken eggs. Infectious allantoic fluid was collected, clarified by low-speed centrifugation, and stored at -70°C. The virus titer was determined to be 800 hemagglutinating units (HAU) per milliliter using human 'O' erythrocytes.

**Infection of mice**

Mice were anesthetized with an intraperitoneal injection (40 µl/10 g body weight) of a mixture of 0.4 mg/ml xylazine (Rompun®, Haver-Lockhart, Shawnee, KS) and 7.8 mg/ml Ketamine (Ketaset®, Bristol Laboratories, Syracuse, NY). All animals were intranasally infected with 20 HAU of PR8 virus in a volume of 50 µl using an Eppendorf pipette. Pre-infection serum samples were routinely screened for antibody to influenza virus to assure that all mice were seronegative prior to experimentation.

**Cell preparation**

Animals were sacrificed by cervical dislocation several days post infection (p. i.). Single cell suspensions were prepared by grinding the tissues in 500 µl Hanks' balanced salt solution (HBSS, Gibco. Grand Island, NY) using glass microhomogenizers (Radnoti Glass,
Monrovia, CA). Cells from mediastinal and superficial cervical lymph nodes were washed twice in HBSS and resuspended at a density of $1 \times 10^7$/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco). Spleen cells were washed in HBSS twice and incubated in 0.17M NH₄Cl on ice for 5 minutes to lyse red blood cells, then washed in HBSS twice again before they were resuspended in DMEM with 10% FBS. Lungs were finely minced with sharp instruments and incubated in collagenase (type I, Worthington Biochemical Corporation. 972.4 unit / 2.6 ml HBSS / lung) at 4°C for 90 minutes, followed by HBSS wash and red blood cell lysis as above.

**Examination of antibody specificity using ELISA assay**

Horseradish peroxidase conjugated goat anti-mouse IgM (55568 Cappel, West Chester, Pennsylvania), IgG (55566 Cappel, West Chester, Pennsylvania), IgG1 (1070-05 Southern Biotechnology Associates Inc., Birmingham, Alabama), IgG2a (1080-05 Southern Biotechnology Associates Inc., Birmingham, Alabama) and IgA (1040-05 Southern Biotechnology Associates Inc., Birmingham, Alabama) were purchased to use in ELISPOT assay. The working dilution for each secondary antibody was first determined by ELISA assay as was the specificity of these antibodies. Briefly, polystyrene plates (25805-96 Corning Glass Works, Corning, New York) were coated with 50μl of mouse myeloma protein (MOPC 104E IgM, MOPC 21 IgG1, UPC 10 IgG2a, 8402.05 IgG and 8402.01 IgA. Bionetics Laboratory Products. Litton Bionetics Inc., Kinsington, MD) in bicarbonate coating buffer (pH 9.6) at 4°C for overnight and washed 3 times with 0.05% Tween in phosphate-buffered
saline (PBST), then blocked with 50µl 10% goat serum (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) for 2 hours (h) at 37°C and washed with PBST 3 times. Fifty microliters of secondary antibody dilutions were added to the plate and incubated for 2h at 37°C, followed by 3 times PBST wash. Finally, 50µl ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) A-1888, Sigma, St. Louis, MO) substrate was added and optical density was determined using an EL-310 ELISA reader (Bio Tek, Burlington, VT) at 405nm. The working dilution of each secondary antibody was determined by comparing the curve of each dilution on titration graph. Then the specificity of each secondary antibody was examined by ELISA coating the plates with mouse myeloma proteins of different isotypes. The crossreaction of a secondary antibody at the working dilution was checked against myeloma proteins of opposite specificities.

**Detection of anti-influenza antibody secreting cell (ASC) numbers by ELISPOT assay**

ASCs were detected by using an ELISPOT assay modified from the method described by Orr *et al.* (188). Briefly, polystyrene plates (96-well, flat-bottom, high binding; Costar, Cambridge, MA) were precoated with 100µl of PR8 virus (2HAU in bicarbonate buffer, pH 9.6) for 1h at 37°C, washed 3 times with PBST, blocked with 100µl 10% goat serum in PBS for 1h at 37°C, and washed 3 times with PBST. The plates were incubated with 100µl of the isolated cells in 6 duplicates (1 x 10^6 to 2.5 x 10^5 cell / well) in DMEM with 10% FBS for 4h at 37°C in 10% CO2, followed by 3 times washes with PBST. Then 100µl of horseradish peroxidase conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a or IgA diluted 1:200 in PBS with 10% goat serum was added and incubated overnight at 4°C. After 3 washes with PBST,
100μl TMB substrate (3,3',5,5'-tetramethylbenzidine, Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) was added to each well. Twenty to 30 minutes later, specific ASCs were observed as dark blue spots under a dissecting microscope. The specific ASC number of each sample was calculated from the spot numbers of the 6 wells and expressed as the number of ASCs per $2.5 \times 10^5$, $5 \times 10^5$ or $1 \times 10^6$ mononuclear cells.

**Statistical analysis**

The mean number of ASCs ± the standard deviation (SD) was calculated for each group. The statistical significance of the differences in the mean number of ASCs among samples of different days post infection was assessed by Kruskal-Wallis test and Mann-Whitney U test on SYSTAT (Systat Inc., 1990-1992).

**Results**

**Determination of secondary antibody working dilution.** An ELISA assay was used to determine the working dilution for each secondary antibody. Figure 2 shows a representative titration curve of goat anti-mouse IgM HRP. Mouse IgM myeloma protein (1mg/ml) of MOPC 104E cell line (Bionetics Laboratory Products, Kensington, MD) was serially diluted and used as coating antigen. Goat anti-mouse IgM HRP was diluted and incubated with the antigen. Optical density values are plotted against coating antigen dilutions in the titration curves. The titration curves demonstrated that antibodies at 1:100 and 1:200 dilutions possessed similar binding patterns, and the antibody sensitivity decreased as the antibody was diluted further. Therefore, 1:200 was determined as working dilution for goat anti-mouse
IgM HRP. All the other secondary antibodies were tested the same way and their working dilutions were also 1:200.

Examine the specificity of secondary antibodies. In order to enumerate the isotype specific ASCs in ELISPOT assay, the specificity of each secondary antibody was tested. Table 1 presents the O.D. values of an ELISA assay which examined the cross reaction of goat anti-mouse IgM HRP with mouse IgG1, IgG2a and IgA proteins. The O.D. values of goat anti-mouse IgM HRP reacting with mouse IgM was around 1.0, while the O.D. values of other groups were much lower (0.076 to 0.353). Therefore, at working dilution, the cross reactions of goat anti-mouse IgM HRP with mouse immunoglobulin proteins other than IgM were not significant.

Determine the optimum cell concentrations for ELISPOT assay. Preliminary experiments were performed to standardize the ELISPOT assay for enumeration of influenza virus-specific ASCs. Seven days p.i., mononuclear cells were obtained from secondary lymphoid tissues (spleen and MLN) and used in the ELISPOT assay. Results showed that the number of influenza virus-specific IgM, IgG, IgG1 and IgG2a ASCs per well increased as the number of mononuclear cells in each well was increased from $2.5 \times 10^5$ to $1 \times 10^6$, while at higher cell concentrations, the ASC number did not increase proportionally (Figs. 3, 4, 5, 6, 7). This may have been due to the fact that the surface area of the well was not large enough to have more ASCs to contact the bottom and develop into spots, or the amount of medium (100μl) was not sufficient to support the growth of higher number of cells.
Alternatively, the cell crowding that occurred at high density may have had a negative effect on the ASCs. Therefore, cell suspension concentrations of $2.5 \times 10^6$ / ml to $1 \times 10^7$ / ml were selected for all further experiments.

**Determination of the cut-off for ELISPOT assay.** Influenza-specific ASCs were infrequently ($<10 / 10^6$ cells) detected in lung, SCV, MLN and spleen cell suspensions from normal, noninfected mice (Figs. 8 and 9). ASCs were rarely ($<10 / 10^6$ cells) detected when cell suspensions from influenza virus-infected mice were assayed on plates coated with bicarbonate buffer only (data not shown). In addition, the wells without any cells produced less than 10 spots (data not shown). Thus, we considered any well with less than 10 spots / well as a negative response.

**Kinetics of IgM and IgG ASCs in SCV, MLN and spleen during a primary influenza infection.** Mice were infected intranasally with 20HAU PR8 virus. Isotype specific ASCs to influenza virus were enumerated in SCV, MLN and spleen at different days p. i., using the ELISPOT assay. The kinetic study was repeated three times and a representative result is shown in Figures 10, 11, 12. The numbers of IgM and IgG ASCs peaked on day 7 p. i. in all the tissues, and they declined sharply on day 10 post infection. On day 7 p. i., the numbers of IgM and IgG ASCs were significantly greater (P<0.05) than those on day 3. MLN had the highest IgM and IgG ASC numbers at all sites which may indicate that B cell response is the strongest in MLN. Also, MLN had higher numbers of IgG ASCs than IgM ASCs, while spleen had more IgM ASCs than IgG ASCs. These results are consistent with previous
studies (76, 194). The superficial cervical lymph nodes had similar ASC numbers of both isotypes.

**Kinetics of IgM and IgG ASCs in lung homogenate during primary infection.** The kinetics of IgM and IgG ASC responses were detected in the lung using ELISPOT assay on different days post intranasal infection. Figure 13 is the result of a representative experiment. Influenza-specific IgM and IgG ASCs appeared and peaked on day 7 p.i., then IgM ASCs remained high while the IgG ASCs decreased afterwards and disappeared by day 14 p.i.

On day 7, the numbers of IgM and IgG ASCs in the lung were similar and they were not as high as those in the lymphoid tissues. These results suggest that the kinetic of lung IgG ASC is similar to the IgG kinetics of lymphoid tissues, but IgM ASCs persist in lung for a longer period of time. This finding is consistent with Jones and Ada's (194) result which showed persistent high number of IgM ASCs in lung for about 3 weeks post intranasal infection. It is probably because that IgM is more efficient than IgG in neutralizing viruses at inflammation site.

**Kinetics of IgA ASCs in MLN and lung during primary influenza infection.** Figure 14 presents a representative study of the kinetics of IgA ASCs in MLN and lung. IgA ASCs appeared in MLN on day 7, peaked on day 10 and reached plateau phase on day 14. The day 10 and day 14 MLN IgA ASC numbers were significantly (P<0.05) greater than that of day 3. In lung, IgA ASCs appeared on day 10, reached peak on day 14 and its magnitude was similar to that of MLN. Lung IgA ASCs on day 10 and day 14 were significantly (P<0.05)
higher than that on day 3. These results indicate that IgA ASCs persist in MLN and lung for a period of time probably because of their engagement in virus neutralization. In addition, the IgA ASCs peaked later than IgM and IgG ASCs, which indicates the transition of IgM response to IgA response due to antibody class switching.
Figure 1. ELISPOT assay procedures. See materials and methods for details.
ELISPOT Assay

Add antigen (PR8 virus, 1 HAU/well) to plate.

1 hr 37°C

Add blocking solution (10% goat serum) to block the remaining protein binding sites on the plate.

1 hr 37°C

Add ASCs and incubate. Secreted antibody will bind to antigen.

4 hr 37°C

Wash cells from plate. Add goat anti-mouse Ig-HRP.

Figure 1
Figure 2. Determination of secondary antibody working dilution. ELISA plate was coated with mouse myeloma protein (MOPC 104E, IgM λ1, Bionetics Laboratory Products, 1mg/ml). Serial dilutions of secondary antibody (goat anti-mouse IgM-HRP) were incubated in the wells. O.D. reading was plotted against coating antigen dilutions. Antibody dilution at 1:200 was determined to be the working concentration.
Goat Anti-mouse IgM HRP Titration

Figure 2
Table 1. Specificity of secondary antibody (goat anti-mouse IgM HRP). O. D. readings of ELISA assay examine cross reaction of the antibody (at working concentration 1:200) to the other isotype immunoglobulin proteins.

<table>
<thead>
<tr>
<th>Antigen Concentration</th>
<th>Optical Density</th>
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<tbody>
<tr>
<td></td>
<td>2µg / ml</td>
</tr>
<tr>
<td>Mouse IgM Myeloma Protein MOPC 104E</td>
<td>0.907</td>
</tr>
<tr>
<td>Mouse IgA Myeloma Protein 8402.01</td>
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</tr>
<tr>
<td>Mouse IgG1 Myeloma Protein MOPC 21</td>
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</tr>
<tr>
<td>Mouse IgG2a Myeloma Protein UPC 10</td>
<td>0.110</td>
</tr>
</tbody>
</table>
Figure 3. Determination of optimum cell concentrations for ELISpot assay. The results showed that cell concentration higher than 1,000,000 cell/well did not provide proportionally higher number of IgM and IgG spots or ASCs with day 7 infected mediastinal lymph node cells.
Day 7 P. I. MLN ASC Responses
IgM and IgG

![Bar Chart]

Figure 3
Figure 4  Determination of optimum cell concentrations for ELISPOT assay. The results showed that cell concentration higher than 1,000,000 cell / well did not provide proportionally higher number of IgM and IgG spots or ASCs with day 7 infected spleen cells.
Figure 4

Day 7 P. I. Spleen ASC
IgM and IgG

Figure 4
Figure 5. Determination of optimum cell concentrations for ELISPOT assay. The results showed the linearity relationship between mononuclear cell numbers and IgM and IgG ASC numbers when cell concentration was 250,000 / well to 1,000,000 / well. The mononuclear cells were collected from day 7 post infection mediastinal lymph nodes.
Day 7 P. I. MLN ASC Responses
IgM and IgG

Figure 5
Figure 6. Determination of optimum cell concentrations for ELISPOT assay. The results showed the linearity relationship between mononuclear cell number and IgM and IgG ASC numbers when cell concentration was 250,000 / well to 1,000,000 / well. The mononuclear cells were collected from day 7 post infection spleen.
Day 7 P. I. Spleen ASC Responses
IgM and IgG

Figure 6
Figure 7. Determination of optimum cell concentrations for ELISPOT assay. The results showed the linearity relationship between mononuclear cell numbers and IgG1 and IgG2a ASC numbers when cell concentration was 250,000 / well to 1,000,000 / well. The mononuclear cells were collected from day 7 post infection mediastinal lymph nodes.
Day 7 P. I. MLN ASC Responses
IgG1 and IgG2a

![Bar chart showing ASC responses to different cell concentrations for IgG1 and IgG2a.](image)

Figure 7
Figure 8. Influenza-specific IgM and IgG ASC numbers in normal noninfected mice. ASCs of IgM and IgG in SCV and MLN were lower than 10 in each well. Each data point was the average of 6 duplications of a pooled sample from 5 mice.
Normal Noninfected Mice ASC
IgM and IgG

Figure 8
Figure 9. Influenza-specific IgM and IgG ASC numbers in normal noninfected mice. ASCs of IgM and IgG in spleen and lung were lower than 10 in each well. Each data point was the average of 6 duplications of a pooled sample from 5 mice.
Figure 9

Normal Noninfected Mice ASC

IgM and IgG
Figure 10. SCV IgM and IgG ASC responses during primary influenza infection in C57Bl/6 mice. Results expressed as ASC number per 250,000 mononuclear cells. N is the number of animals used for an experiment. Each result is the average of 3-6 duplicates of a pooled sample. Day 3 and day 7 (N=10), day 10 and day 14 (N=8). Significant elevation above day 3 control (p<0.05) indicated by asterisk.
Kinetics of Superficial Cervical Lymph Node IgM and IgG ASC Responses

Figure 10
Figure 11. MLN IgM and IgG ASC responses during primary influenza infection in C57Bl/6 mice. Results expressed as ASC number per 500,000 mononuclear cells. N is the number of animals used for an experiment. Each result is the average of 3-6 duplicates of a pooled sample. Day 3 and day 7 (N=10), day 10 and day 14 (N=8). Significant elevation above day 3 control (p<0.05) indicated by asterisk.
Kinetics of Mediastinal Lymph Node IgM and IgG ASC Responses

Figure 11
Figure 12. Spleen IgM and IgG ASC responses during primary influenza infection in C57Bl/6 mice. Results expressed as ASC number per 250,000 mononuclear cells. N is the number of animals used for an experiment. Each result is the average of 3-6 duplicates of a pooled sample. Day 3 and day 7 (N=10), day 10 and day 14 (N=8). Significant elevation above day 3 control (p<0.05) indicated by asterisk.
Figure 12

Kinetics of Spleen ASC Responses
IgM and IgG
Figure 13. Lung IgM and IgG ASC responses during primary influenza infection in C57Bl/6 mice. Results expressed as ASC number per 1,000,000 mononuclear cells. N is the number of animals used for an experiment. Each result is the average of 3-6 duplicates of a pooled sample. Day 5 (N=5), day 7 (N=4), day 10 (N=5) and day 14 (N=2). Significant elevation above day 5 control (p<0.05) indicated by asterisk.
Kinetics of Lung ASC Responses
IgM and IgG

Days Post Infection

ASC / 1,000,000 mononuclear cells

5 7 10 14

Figure 13
Figure 14. Lung and MLN IgA ASC responses during primary influenza infection in C57Bl/6 mice. Results expressed as ASC number per 1,000,000 mononuclear cells. N is the number of animals used for an experiment. Each result is the average of 3-6 duplicates of a pooled sample. Day 5 (N=5), day 7 (N=4), day 10 (N=5) and day 14 (N=2). Significant elevation above day 5 control (p<0.05) indicated by asterisk.
Kinetics of IgA ASC Responses of MLN and Lung

Figure 14
Discussion

The ELISPOT assay provided reproducible results for enumeration of influenza-specific antibody-secreting cells in the lung and secondary lymphoid tissues. Detection of influenza-specific antibody in the lung is often impossible, since the antibodies are bound to viruses or virus-infected cells and the amount of free antibodies is low. With the ELISPOT assay, antibody-secreting cells or plasma cells can be detected without the interference of viral antigens. Therefore, the ELISPOT assay is a simple, specific, sensitive and reliable method for evaluation of the number of antigen-specific plasma cells.

The influenza virus-induced inflammatory response in mice is characterized by severe pulmonary mononuclear cell infiltration, edema and consolidation. Usually the mononuclear cell infiltration starts 2 days after intranasal influenza infection and peaks around day 7 post infection. By day 10 post infection, the cell infiltration in lungs is diminished and inflammatory response is resolving. At 30 days post infection, the lungs appear normal (189). The mononuclear cells consist of macrophages, null, T and B lymphocytes, while macrophage and T cells provide the major increments (190). Some early studies of lung immunoglobulin-bearing cells showed conflicting results concerning the kinetics of each subtype (191, 192). There have been several studies of lung influenza-specific antibody secreting cells in the past two decades using ELISPOT assay and hemolysis plaque assay (193, 194, 195). Their results indicated that the lung ASCs appear approximately one week after infection, which overlapped the time of mononuclear cell infiltration, and supported the notion that antibody responses develop after cell-mediated responses appear (53).
In the present study the ASC responses in SCV, MLN, spleen and lung were investigated. The kinetics of MLN, spleen and lung are consistent with previous studies by other groups (53, 193, 194, 195). However, this is the first study that investigated the kinetics of SCV IgM and IgG ASCs in a primary influenza infection. The results indicate that the kinetics of IgM and IgG ASCs in lung, draining lymph nodes and spleen are similar, while the number of ASC differs in each tissue. This finding does not support the working hypothesis that each lymphoid tissue has its unique kinetics of ASC responses.

The ASCs detected in MLN, SCV and spleen are plasma cells (terminally differentiated B cells), which are activated in the lymph nodes or spleen cells. Influenza viruses introduced intranasally replicate in the epithelial cells of respiratory tract. Viral antigens appear to be endocytosed and processed by Mφ and dendritic cells which reside in mucous membrane and pulmonary interstitial tissues. Then the antigen presenting cells (APCs) migrate through afferent lymphatics into the lymph nodes that drain the respiratory tract, such as MLN and SCV. In the lymph nodes, the antigen fragments are presented to influenza-specific T cells which interact with antigen-MHC II complex on APCs through T cell receptor (TCR)-CD3 complex. The binding signal is transduced into the T cells via inositol phospholipid system resulting in the T cells transition from G0 to G1 stage, the production of IL-2 and the expression of IL-2 receptors on the cell membrane. IL-2 acting in an autocrine fashion causes the influenza-specific T cell clonal expansion and production of other cytokines, such as IL-4, IL-5 and IFN-γ, which are required for B cell activation and differentiation (196).
Cytokines produced by the T cells also affects B cells in the lymph node. The influenza-specific B cells acquire viral antigen in respiratory tract and migrate into lymph nodes, where they contact T helper (Th) cells and are stimulated by T cell cytokines. Antigen binding through membrane immunoglobulin (mIg) induces signal transduction via inositol phospholipid system, T-B contact induces elevation of intracellular cAMP level, and cytokine binding activates protein kinase activity in B cells (197). These signals induce B cell activation, proliferation and differentiation. The cellular events in the spleen may be similar to those in the lymph nodes, except that spleen acquires influenza viral antigens from the blood instead of from the pulmonary antigen presenting cells, as the viruses replicate in respiratory tract resulting in the spillover of virus or viral antigens into blood. Therefore, the ASCs detected in MLN, SCV and spleen are plasma cells, the terminally differentiated B cells, which are activated in the lymph nodes or spleen cells.

The kinetics of SCV, MLN and spleen anti-influenza virus IgM and IgG ASCs in the present study showed that the ASCs accumulate in the tissues for a short period of time and ASC numbers decline rapidly afterwards. These observations were comparable with the results previously reported by Reiss (53) and Jones (194), which showed sharp peaks of ASC numbers in MLN and spleen, although their magnitude and peak response days were different from the present study. The differences could be due to a number of factors including the differences in the virulence of the strains of virus and/or the infection dose. This could be supported by the observation made by Jones and Ada, who showed that using a lower dose of influenza virus causes delayed onset and lower magnitude of antibody responses in mice (194).
The sharp peaks of ASCs in lymph nodes and spleen may be the result of lymphocyte migration and/or lower magnitude of B cell proliferation and differentiation late in infection. ASCs may migrate from lymphoid tissues into inflammation site later in infection and secrete antibodies there. On the other hand, it has been shown that lung viral titer decreases before antibody appears in serum (53), therefore, B cell proliferation and high ASC number may last for a few days and then the ASC number declines in the lymphoid tissues as a result of less antigen stimulation.

Previous studies showed that serum IgG titer remained high for a few weeks after influenza infection (175). However, in this study the IgG ASC number was low at the site inflammation and in the secondary lymphoid tissues 10 days after infection. Therefore, ASCs and serum antibody titers had different patterns of kinetics, that is, the serum antibody titers were increasing while the ASC numbers were decreasing and the serum antibody level remained stable for at least 3 weeks post infection (175). This difference could be explained by the fact that each ASC produces more antibodies later in infection, therefore even though the ASC number is decreasing, the total amount of antibody produced is increasing. An alternative explanation may be that since the half-life of serum antibody is about 3 weeks long and the serum antibody titer is the result of accumulating antibody production over a period of time, the titer could be high late in infection although the number of ASC is low.

Serum antibody responses usually show a transition from IgM response to IgG and IgA responses, but this study showed that IgM and IgG responses peaked at the same time, while IgM to IgA transition exist as described in other publications (198). Previously Feng et al. (175) showed that IgM and IgG peaked at the same time in the serum and IgA peaked
few days later. Other groups measuring influenza specific antibody-secreting cells in lung and spleen showed similar overlapping patterns of IgM and IgG responses (194, 195). This maybe the result of an infection with virus which induced strong T cell responses and early production of antibody class switching cytokines (such as IL-4 and IL-5), which resulted in the early appearance of IgG secreting cells.

The migration of plasma cells throughout the body is controversial. Some investigators considered plasma cells to be "non-circulating" cells that stay in lymphoid tissues to secrete antibodies into lymph and blood (199). This notion could be supported by the fact that there are no plasma cells in normal blood smears (200). Others indicate that plasma cells or their precursors present in circulation and migrate into inflammation site. For instance, Jones and Ada (194) detected influenza-specific IgM-secreting cells in the blood 5 days post intranasal infection, while Kaltreider et al. (79) showed that lymphocytes from MLN are able to migrate into the lung of infected recipient. Therefore, the ASCs in lungs may be the B cells that are activated in lymphoid tissues, migrate into lung and terminally differentiate into plasma cells in the inflammatory environment in the lung.

In conclusion, the result of this study showed that ASC secreting IgM and IgG existed in secondary lymphoid tissues for a short period of time (3-4 days) following a primary infection, while they last longer in lung (7-9 days). Furthermore, IgM and IgG ASC responses peaked in SCV, MLN, spleen and lung on day 7 post infection, while IgA ASC responses peaked on day 10 in MLN and on day 14 in lung.
Chapter II
Restraint Stress Modulation of Murine Antibody Responses to Influenza A Viral Infection

Introduction

Restraint stress (RST) activates the hypothalamic-pituitary-adrenal axis and the autonomic nervous system, resulting in the release of glucocorticoid and catecholamines. These hormones modulate various aspects of the murine anti-viral responses. As previously reported, RST suppresses both local lymph node and systemic splenic IL-2 responses to influenza virus (176), as well as decreases cytolytic functions of cytotoxic T cells (CTL) in a herpes simplex virus infection (174, 201, 202). In addition, RST modulates serum antibody responses to influenza infection (175, 176).

Influenza A viruses introduced via the bronchial route induce both the cellular and humoral immune responses, resulting in bronchitis and pneumonia in mice. Viral clearance and recovery from lethal pulmonary influenza virus infection mainly depends on the generation of a cellular immune response, while the prevention of reinfection relies on the production of strain specific antibodies in serum and nasal secretion by plasma cells (38, 203). T helper cells play key roles in CTL and B cell responses, as cytokine production by T helper cells result in the activation, proliferation and differentiation, as well as function of CTL and
B cells. For instance, IL-2 is essential for the development of functional CTLs from pre-CTLs, also it acts on B cells as both a growth factor and a stimulus for antibody synthesis; in addition, IFN-γ, IL-4 and IL-5 are B cell growth and class switching factors (199, 205).

Since T helper cell function is critical in the anti-influenza responses and RST suppresses IL-2 production, which indicates decreased T helper 1 (Th1) cell function, we postulated that RST may alter T helper 2 (Th2) cell cytokine production (e.g. IL-4, IL-5 and IL-10) resulting in the modulation of B cell antibody production and antibody class switching. The results of a serum antibody study showed that the antibody production was not affected but class switching was delayed (175). We speculated that the reasons were: 1) the alteration of T helper cell trafficking which results in decreased IL-2 production in local lymph nodes and spleen but normal support to B cell activation and maturation in other lymphoid tissues which result in unaltered serum antibody production (176); 2) the differential resistance of Th1 and Th2 helper cells to stress-induced changes which results in decreased Th1 cytokine production but unchanged Th2 cytokine production (176); 3) the regulation of the susceptibility of B cell response to stress by antigen availability, which indicated that antibody response is more sensitive to stress in early stage of infection while viral antigen is less abundant (175).

Serum antibodies are secreted by plasma cells from different lymphoid tissues in the body, particularly the spleen, therefore the serum antibody response reflects systemic humoral immunity. Since each lymphoid tissue has its unique antigen draining region and unique microenvironment for lymphocyte activation and differentiation, the pattern of antibody responses in each lymphoid tissue could be different from that in serum. The results of a
recent study by Fazekas and colleagues (193) supports this notion, which indicates that influenza viruses introduced via respiratory tracts induce IgM response in lung, mediastinal lymph node (MLN) and spleen, and IgG and IgA responses only in lung and MLN, but predominantly IgG2a response in the serum.

Therefore, to investigate the cellular events of RST modulation on anti-influenza humoral response, we studied the antibody responses by using the lymphocytes from individual lymphoid tissues instead of measuring circulating antibody level. An enzyme-linked immunospot (ELISPOT) assay was developed to detect the individual anti-influenza IgM, IgG (G1 and G2a) and IgA antibody secreting cells (ASC) in lymphoid tissues and analyzed the differences of ASC numbers between stressed and non-stressed groups.

Given that glucocorticoid enhances Th2 cytokine (IL-4) production and suppresses Th1 cytokine (IL-2) production (205), and restraint stress elevates serum glucocorticoid level, we hypothesized that Th1 function is suppressed in stressed mice whereas Th2 function is either increased or unchanged. Therefore, it was expected that there would be increased or unchanged IgG1 ASCs and decreased IgG2a ASCs in stressed mice. In addition, since IL-2 affects B cell growth and differentiation, suppressed Th1 function would also result in decreased ASC numbers of IgM, IgG and IgA.

It is known that GC and CAT play roles in RST-induced modulation of T cell responses (174), in addition, GC and β2-adrenoceptors are expressed on B cells (95, 97). It was hypothesized that GC and CAT may be the mediators modulating humoral responses. To test this hypothesis, RU486 and nadolol were used in stressed mice to block the effect of GC and CAT, respectively.
The results of this study showed that restraint stress significantly suppressed the numbers of influenza-specific IgM and IgG secreting cells in MLN, lung and spleen while increasing their numbers in superficial cervical lymph nodes, the numbers of both IgG1 and IgG2a ASCs were suppressed in MLN and enhanced in SCV by RST. These findings suggest that restraint stress may modulate antibody responses in mucosal and nonmucosal lymph nodes differently, while Th1 and Th2 responses were not differentially regulated. Furthermore, RU486 treatment partially restored the IgM and IgG ASC responses in MLN, whereas nadolol did not affect the stress-induced suppression of ASC. These results suggested that GC plays a role in stress-induced modulation of humoral responses but that CAT may not be involved, or may not act through β2-adrenoceptors.

Materials and methods

Animals

As in chapter I.

Restraint Protocol

Restraint mice were kept in well-ventilated 50 ml conical polypropylene tubes for 16 hours each day (from 5pm to 9 am) within their cages. They could move back and forth but could not turn around or reach their food and water while in the tubes. Mice were given free access to food and water upon release from the tubes.

Restraint stress started one day prior to infection and continued from one day post infection to the day of study.

Virus
Infection of mice

As in chapter I.

Drugs

The β2-adrenergic receptor antagonist nadolol (Corgard; Bristol-Myers Squibb, Princeton, NJ) was administered in the form of a sustained-release pellet which was implanted subcutaneously using a trochar. The pellet contained 0.5mg of nadolol and was designed to release nadolol at a continuous rate over 21 days (Innovative Research, Toledo, OH). A pellet containing α-lactose was implanted subcutaneously in vehicle-treated mice. Pellets were implanted 1 day prior to RST.

The glucocorticoid receptor antagonist RU486 (Roussel-UCLAF, Romainville, France) was administered daily by subcutaneous injection at a dose of 25mg / kg (0.1 ml of 5mg/ml RU486 in PEG 400). Control group was treated with daily subcutaneous injection with 0.1 ml of PEG 400. Daily injection started 1 day prior to RST and continued to 6 days post-infection. Each day, injection was performed 1 hour before RST.

Cell preparation

As in chapter I.

Detection of anti-influenza antibody secreting cell (ASC) numbers by ELISPOT assay

As in chapter I.

Statistical analysis
The mean number of ASCs ± the standard deviation (SD) was calculated for each group. The statistical significance of the difference in the mean numbers of ASCs between the stress and nonstress groups was assessed by Kruskal-Wallis test and Mann-Whitney U test on SYSTAT (Systat Inc. 1990-1992).

**Results**

**Restraint stress decreased mononuclear cell numbers in lung, SCV, MLN and spleen.** We have reported previously that RST decreased mononuclear cell numbers in the lung and MLN. In this study we further examined the spleen and SCV mononuclear cell numbers, and we found that they were also significantly decreased by RST. Each infection may provide different cell yield, but the difference between the RST and control groups was consistently significant. An example of the results is shown in Figure 15. The numbers of mononuclear cells in SCV, MLN, spleen and lung were decreased 2 to 5 fold in RST group.

**Effects of restraint stress on IgM and IgG ASCs.** The kinetic study in chapter I showed that the peak of IgM and IgG ASC responses in all the tissues examined was on day 7, we therefore used 7 day infected mice to compare the ASCs of the stressed and non-stressed control groups in order to examine the effect of restraint stress on the numbers of IgM and IgG ASC in different tissues. Repeated experiments demonstrated that the numbers of IgM and IgG ASCs were significantly suppressed in mediastinal lymph nodes, spleen and lung while they were significantly increased in superficial cervical lymph nodes by restraint stress. One example of the results is shown in Figures 16, 17, 18, 19. In Figure 16, both IgM and
IgG ASCs were significantly (P<0.05) increased by RST in SCV. In Figures 17, 18 and 19, IgM and IgG ASC responses were significantly (P<0.05) decreased by RST in MLN, spleen and lung.

**Effect of restraint stress on IgG1 and IgG2a ASCs.** To investigate the Th1 and Th2 responses in anti-influenza infection and determine if stress modulated them, the IgG1 and IgG2a ASCs were examined in mediastinal lymph nodes and superficial cervical lymph nodes on day 7 p.i., as day 7 represents the peak IgG ASC accumulation at the sites (Figs. 20 and 21). In MLN, IgG2a was the dominant subtype of IgG response in the infected mice; both IgG1 and IgG2a ASC numbers were significantly (P<0.05) decreased by stress. In SCV, both subtypes had weak responses in infected mice and stress significantly (P<0.05) increased IgG1 and IgG2a ASC numbers, while IgG1 was the dominant subtype in the RST group.

**RU486 partially blocked the RST-induced suppression of ASC responses in MLN.** GC has been shown to modulate lymphocyte trafficking and to inhibit cytolytic function of CTLs. Therefore, the role of GC in RST-induced suppression of ASC responses in MLN was examined. This experiment was repeated four times, the results of two experiments are shown in Figures 22, 23, 24, 25 and 26. PEG was used as solvent of RU486 and was injected in the mice of control group. In Fig. 22, RS/PEG group showed 2-3 fold lower cell numbers than Infected/PEG (INF/PEG) group, while the number of mononuclear cells in RS and RU486 treated (RS/RU) group was 1-2 fold higher than that of RS/PEG group. These results are consistent with our previous work (174) which indicated that RU486 restore lymph
nodes cellularity in stressed mice and GC play a role in lymphocyte trafficking. Figs. 23 and 24 show that RU486 treatment partially restored the IgM and IgG ASC responses in RST mice. In Fig. 23, stressed and PEG injected (RS/PEG) groups had significantly (P<0.05) lower IgM ASCs than INF/PEG control groups. RS/RU groups had significantly (P<0.05) higher numbers of IgM ASC than RS/PEG, although the ASC response was weak compared to the INF/PEG control group. Also, RU486 seemed to decrease IgM ASC number in INF/RU control group (P<0.05 comparing to INF/PEG group) of experiment 1, but it did not hold up in experiment 2. In Fig. 24, the RS/PEG group ASC number was significantly lower than INF/PEG group, so RST suppression of IgG ASC number was obvious (P<0.05). In addition, RS/RU group showed significantly (P<0.05) higher numbers of IgG ASCs than RS/PEG control group. Therefore, RU486 partially blocked the RST-induced suppression of IgG ASC response in MLN.

We have found that RST not only decreases ASC numbers in MLN but also decreases the ASC spot size. It was indicated by Moller et al. (181) that spot size probably relates to antibody production, that is bigger spots mean more antibodies than smaller ones. Other groups (180, 186, 206) suggested that the affinity of antibodies secreted from ASC may affect spot size. To investigate if the ASC spot size change was related to the effect of GC, we also examined the numbers of big spots (macrospot) and small spots (microspot). The results are shown in Figs. 25 and 26. In Fig. 25, RU486 decreased IgM macrospot number while affecting total spot number in INF/RU group, RST mainly decreased ASC macrospot in RS/PEG group and RU486 increased ASC macrospot number without significantly changing the microspot number in RS/RU group. In Fig. 26, RST and RU486 effect on IgG ASCs in
RS/PEG and RS/RU groups were similar to those on IgM ASCs. The only difference for IgG ASC was that RU486 decreased IgG macrospot number without changing the total IgG ASC number in INF/RU group.

Nadolol did not block RST-induced suppression of ASC responses in MLN. Catecholamines have been shown to affect B cell responses in vitro and in vivo (150, 158, 207, 208, 209, 210, 211), as well as CTL cytolytic function (174). Therefore, the role of catecholamines acting at β2-adrenoceptor in RST-induced suppression of ASC response in MLN was investigated. The results of a representative experiment are shown in Figs. 27, 28 and 29. Fig. 27 shows that the number of mononuclear cells was decreased in RS/VEH group and was not restored by nadolol in RS/NAD group. Figs. 28 and 29 show that the IgM and IgG ASCs were significantly (P<0.05) decreased by RST (RS/VEH group) and nadolol did not restore (P>0.05) the decreased ASC numbers in RS/NAD group.
Figure 15. RST suppressed the cellularity of SCV, MLN, spleen and lung in influenza infected C57Bl/6 mice. Experiment 1 includes 3 groups: the noninfected control (CON) group, the infected (INF) control group, and the infected and stressed (RST) group. Experiment 2 includes 2 groups: INF and RST. Each result is the mononuclear cell count of a pooled sample from several mice. N is the number of animals used in a experiment. In experiment 1, CON N=10, INF N=9 and RST N=16. In experiment 2, INF N=8 and RST N=7.
Mononuclear Cell Number
Day 7 Post Infection

Experiment 1

SCV | MLN | Spleen | Lung
---|---|---|---
CON | INF | RST

Experiment 2

SCV | MLN | Spleen
---|---|---
INF | INF | RST

Figure 15
Figure 16. RST significantly changed IgM and IgG ASCs in SCV. ASC numbers are the average of the results of 4 to 6 duplicates from a pooled sample. N is the number of animals used in a group. INF group, N=8, RST group, N=7. In Fig. 16, RST significantly (P<0.05) increased the IgM and IgG ASC numbers in SCV.
Superficial Cervical Lymph Node
IgM and IgG ASCs in RST Mice

Figure 16
Figure 17. RST significantly changed IgM and IgG ASCs in MLN. ASC numbers are the average of the results of 4 to 6 duplicates from a pooled sample. N is the number of animals used in a group. INF group, N=8; RST group, N=7. In Fig. 17, RST significantly (P<0.05) suppressed the IgM and IgG ASC numbers in MLN.
Mediastinal Lymph Nodes IgM and IgG ASCs in RST Mice

Figure 17
Figure 18. RST significantly changed IgM and IgG ASCs in spleen. ASC numbers are the average of the results of 4 to 6 duplicates from a pooled sample. N is the number of animals used in a group. INF group, N=8; RST group, N=7. In Fig. 18, the spleen IgM ASC numbers were significantly suppressed by RST, while IgG ASC numbers were at baseline and RST effect could not be determined.
Spleen IgM and IgG ASCs in RST mice

Figure 18
Figure 19. Lung IgM and IgG ASCs were significantly suppressed by RST. ASC numbers are the average of the results of 4 to 6 duplicates from a pooled sample. N is the number of animals used in a group. INF group, N=5, RST group, N=2. P<0.05 for both IgM and IgG ASCs.
Lung IgM and IgG ASCs in RST Mice

Figure 19
Figure 20. RST significantly (P<0.05) increases the IgG1 and IgG2a ASC responses in SCV, while IgG1 ASC response is dominant in RST mice. ASC numbers are the average of the results of 4 to 6 duplicates from a pooled sample. N is the number of animals used in a group. INF group, N=8; RST group, N=7.
Superficial Cervical Lymph Node IgG1 and IgG2a ASCs in RST Mice

![Graph showing the distribution of ASCs and mononuclear cells in RST mice.](image)

Figure 20
Figure 21. RST significantly ($P<0.05$) suppresses the IgG1 and IgG2a ASC responses in MLN, while IgG2a ASC response is dominant in RST mice. ASC numbers are the average of the results of 4 to 6 duplicates from a pooled sample. $N$ is the number of animals used in a group. INF group, $N=8$; RST group, $N=7$. 
Mediastinal Lymph Nodes IgG1 and IgG2a ASCs in RST Mice

Figure 21
Figure 22. RU486 restored lymphadenopathy of MLN in RST mice. INF/RU and RS/RU groups were treated with RU486 dissolved in PEG. INF/PEG and RS/PEG groups were treated with PEG as control. Each result is the mononuclear cell count from a pooled sample of several mice. N is the number of animals used in a group. In experiment 1, each group N=5 except RS/RU group N=6. In experiment 2, INF/PEG N=3, INF/RU N=5, RS/PEG N=5 and RS/RU N=4.
RU486 Restores Lymphadenopathy of MLN in RST Mice

Experiment 1

Experiment 2

Figure 22

95
Figure 23. RU486 partially restored the IgM ASC responses in MLN of RST mice. Experiment 1 and 2 were done with the mice that were used in Fig. 22. In Fig. 23, IgM ASCs of RS/PEG group were significantly (P<0.05) lower than those of INF/PEG group. IgM ASCs of RS/RU group were significantly (P<0.05) higher than RS/PEG group. In addition, experiment 1 showed that RU486 alone may suppress IgM ASC response in infected mice as P<0.05 comparing INF/RU group with INF/PEG group.
RU486 Treatment of RST Mice
MLN IgM ASC Responses

Experiment 1

Experiment 2

Figure 23
Figure 24. RU486 partially restored the IgG ASC responses in MLN of RST mice. Experiment 1 and 2 were done with the mice that were used in Fig. 22. In Fig. 24, IgG ASCs of RS/PEG group were significantly (P<0.05) lower than those of INF/PEG group. IgG ASCs of RS/RU group were significantly (P<0.05) higher than RS/PEG group. In addition, RU486 alone did not suppress IgG ASC response in infected mice as P>0.05 for most dilutions, when comparing INF/RU group with INF/PEG group.
RU486 Treatment of RST Mice
MLN IgG ASC Responses

Experiment 1

Experiment 2

Figure 24
Figure 25. Effect of GC on IgM ASC spot size in RST. In experiment 1 of Fig. 22, the ASCs were also counted as macrospot or microspot according to their spot sizes. RST significantly suppressed the total IgM ASC number in RS/PEG group while macrospot number decreased more than microspot number. RU486 partially restored both macro- and microspot number in RS/RU group. In addition, RU486 alone decreased macrospot number dramatically without changing microspot number.
Effect of RST on IgM Production by ASCs

Figure 25
Figure 26. Effect of GC on IgG ASC spot size in RST. In experiment 1 of Fig. 22, the ASCs were also counted as macrospot or microspot according to their spot sizes. RST significantly suppressed the total IgG ASC number in RS/PEG group while macrospot number decreased more than microspot number. RU486 partially restored both macro- and microspot number in RS/RU group. In addition, RU486 alone decreased macrospot number dramatically without changing total spot number.
Effect of RST on IgG Production by ASCs

![Bar chart showing ASCs production in different treatment groups.]

**Figure 26**
Figure 27. Nadolol did not restore the MLN lymphadenopathy in RST mice. Nadolol pellets were implanted in INF/NAD and RS/NAD group while vehicle pellets were implanted in INF/VEH and RS/VEH group. N is the number of mice used in a group. INF/VEH N=4, INF/NAD N=4, RS/VEH N=8 and RS/NAD N=8. Each result is the mononuclear cell count of a pooled sample from several mice. Mononuclear cell number in RS/VEH group was 1/3 lower than that in INF/VEH group, nadolol did not increase the cell count of RST mice.
Nadolol Does Not Restore MLN Lymphadenopathy in RST Mice

![Graph showing million mononuclear cells per mouse across different treatment groups: INF/VEH, INF/NAD, RS/VEH, RS/NAD.]

Figure 27
Figure 28. Nadolol did not restore IgM ASC response in RST mice. Experiment was done with the mice used in Fig. 27. IgM ASC in RS/VEH group was significantly (P<0.05) lower than that of INF/VEH group. IgM ASC number of RS/NAD group was not significantly different from that of RS/VEH group. RST significantly suppressed IgM ASCs in MLN, but nadolol did not restore the ASC response.
Nadolol Treatment of RST Mice
MLN IgM ASC Responses

Figure 28
Figure 29. Nadolol did not restore IgG ASC response in RST mice. Experiment was done with the mice used in Fig. 27. IgG ASC in RS/VEH group was significantly (P<0.05) lower than that of INF/VEH group. IgG ASC number of RS/NAD group was not significantly different from that of RS/VEH group. RST significantly suppressed IgG ASCs in MLN, but nadolol did not restore the ASC response.
Nadolol Treatment of RST Mice
MLN IgG ASC Responses

Figure 29
**Discussion**

The results of this study demonstrated that stress decreased the mononuclear cell numbers in spleen, inflammation site (lung) and regional lymph nodes (SCV and MLN). RU486 partially restored the MLN cellularity of stressed mice but nadolol did not. These findings are consistent with previous work of Dobbs (174) and Hermann (212) which using glucocorticoid receptor antagonist RU486 proved that the suppression of lymphadenopathy and regulation of lymphocyte trafficking are associated with the elevated glucocorticoid activity in stressed mice.

The number of virus-specific ASCs in each organ is related to B cell activation, proliferation and trafficking. RST may affect every aspect of this process. Zwilling *et al.* (125) showed that RST suppresses MHC-II expression on peritoneal Mφ which may result in suppression of their antigen presentation function. This may be true of pulmonary macrophages and dendritic cells which undertake antigen processing and antigen presentation in an influenza infection. In addition, Sonnenfeld *et al.* (213) found that shock-induced suppression of MHC-II expression is related to catecholamines production. Therefore, RST-induced suppression of antigen presentation may result in fewer B cells being activated by the viral antigens.

On the other hand, since B cell activation and proliferation, as well as antibody class switching are influenced by T helper cell cytokines, an alternative reason for decreased ASCs at these sites may be the diminished T cell cytokine production and less help for B cell proliferation and class switching. Recent studies in our lab showed that both Th1 and Th2
cytokine responses are down-regulated in MLN, lung and spleen by restraint stress and the regulation is at the transcriptional level (214, 215). Therefore, the decrease of IgM ASC number may be the result of IL-2 production suppression, while IgG ASC number diminution may result from decreased production of the class switching factors.

As to B cell trafficking, it is highly possible that the influenza-specific ASCs moved from MLN, spleen and lung to bone marrow, SCV or other sites during stress. GC has been shown to regulate lymphocyte trafficking and adhesion molecule expression (114, 117, 142, 216, 217). Adhesion molecule expression on mucosal and non-mucosal lymph nodes are different (218, 219, 220). The homing receptors on lymphocytes and their ligands, addressin, on vascular endothelium for mucosal lymph nodes are VLA-4 and VCAM-1, respectively, and for nonmucosal lymph nodes are L-selectin (also designated LECAM-1, MEL-14 and LAM-1) and MECA-79 ligand, respectively (218). SCV drains mucosal surfaces and MLN drains the deep portion of the lung which is considered a nonmucosal site. We postulated that the expression of lymphocyte adhesion molecules and endothelial addressins in the mucosal and nonmucosal lymph nodes may be differentially modulated by RST, resulting in the increased ASC movement to a mucosal site, such as SCV.

Increased ASCs in SCV could also be explained as the result of differential innervation of sympathetic nervous system in different lymphoid organs (102). Catecholamines released from sympathetic nerve endings are known to regulate lymphocyte activation and trafficking (134, 156, 221, 222, 223). Stress-elevated serum glucocorticoid and tissue catecholamine may interact with each other (171, 172, 173, 224, 225, 226) and regulate the immune response and lymphocyte trafficking. Therefore, SCV and MLN may represent different
microenvironments in modulating B cell response as a result of differential SNS innervation.

We speculate that glucocorticoid and catecholamine interaction results in enhanced antibody response in SCV but decreased antibody response in MLN.

Th1 and Th2 cells interact with each other and regulate cytokine production of each other. IL-4 produced by Th2 cells helps B cells switch from IgM ASCs to IgG1 ASCs, whereas IFN-γ produced by Th1 cells induces B cells switch from IgM ASCs to IgG2a ASCs (227). In the present study, RST reduced the numbers of both IgG1 and IgG2a secreting cell in MLN and increased those in SCV suggesting that Th1 and Th2 functions were not differentially regulated by RST as we hypothesized. The result not only supported previous work which demonstrated stress-induced suppression of IL-2 production in MLN and spleen (176), but also confirmed recent findings that both Th1 and Th2 cytokine gene expression were suppressed in MLN, lung and spleen (215).

It has been shown in Daynes' study that IL-2 production is decreased and IL-4 production is increased by GC. In addition, Moynihan et al. (228) have shown that pheromones emitted by foot-shock-stressed mice decreased IL-2 production but increased IL-4 production in recipient mice. Therefore, the results of present study indicate that factors in addition to GC are involved in the modulation of Th cell function. Evidence has been shown that ACTH and CRF modulate T helper cell function (122, 229, 230). The production of multiple neuroendocrine mediators resulting from the RST-induced activation of HPA axis and SNS may cause the suppression of both Th1 and Th2 functions.

In conclusion, we investigated the effect of restraint stress on the number of influenza specific antibody secreting cells at inflammation site, regional lymph nodes and spleen, and
we found that the IgM and IgG ASC numbers were suppressed in lung, MLN and spleen but were increased in SCV, probably because that mucosal and non-mucosal sites are affected differently by restraint stress. Also, Th1 and Th2 may not be differentially modulated in RST, as a result of multi-mediator interaction. Furthermore, GC plays a role in the stress-induced modulation of ASC response while CAT is not involved, or may not act through β2-adrenoceptors.
Human influenza is predominantly an upper respiratory tract infection with some central airway involvement. Typically, symptoms include nasal discharge, cough, fever, headache, myalgia, malaise and depression but occasionally neurological and gastrointestinal effects are also seen. However, it may be lethal in elderly people, young children and immune compromised patients when complications (such as viral pneumonitis, secondary bacterial pneumonitis or heart failure) develop. Therefore, prevention of infection is critical for reducing influenza-related morbidity and mortality.

The serum antibody level is elevated after natural influenza infection and is related to prevention of homotypic reinfection. Epidemiologic studies have shown a clear relationship between the level of serum antibodies to the current virus and resistance to natural infection; the severity of the clinical symptoms of induced infection in humans is inversely related to influenza-specific antibody levels. The memory of humoral immune response may last for over 20 years as evidence has shown that older people who had experienced H1N1 subtype infection were spared the pandemic infection in 1977 when H1N1 subtype reappear. Therefore, humoral immunity to influenza virus is essential for preventing reinfection. Currently, both inactivated and live influenza vaccines are used to immunize susceptible
people and they have been shown to be effective in preventing the occurrence of complications and mortality. However, the responses of elderly people to vaccination are often not satisfactory. One reason is that their immune system undergo senescence and respond poorly. On the other hand, a portion of the elderly people are living under psychological stress while taking care of a chronically ill spouse or living in isolation.

There has been compelling evidence that the immune system is not only self-regulatory, but also under control of the central nervous system. The immune system, nervous system and endocrine system interact with one another to modulate the body's function under different conditions. Stress has been shown to be associated with the susceptibility to diseases. For example, stress is related to the susceptibility to respiratory viral infection (231) and the increased pulmonary metastases of mammary tumor (232). The mechanisms of stress-induced immunomodulation have been studied intensively. It is known that the signals of physical and psychological stress are sent to the hypothalamus, which is the center of body's homeostatic regulatory system, resulting in the activation of the HPA axis and the sympathetic nervous system. Some of the neuroendocrine products released from the HPA axis and SNS have been shown to be involved in the immunomodulation (such as GC, CAT, ACTH, CRF, endorphin).

Most of the studies that investigate neuroendocrine modulation of the immune response were performed in vitro, this reductionist approach makes the experimental system more controllable and the results easier to interpret. However, the in vivo response could be different and the results of in vitro studies may not be applicable. Therefore, animal models have been shown to be important in investigating neuroendocrine modulation of immune
responses (174, 175, 176). A murine influenza infection model and a restraint stress paradigm have been set up to investigate RST-induced modulation of cellular and humoral responses. It has been shown that cytolytic function of CTL cells and cytokine production of T helper cells are modulated by glucocorticoids and catecholamines in RST (174, 214). Additionally, while the IgM, IgG and IgA antibody titers are not significantly altered, the peaks of their responses are reached several days later in RST group than in nonstressed group (175).

It appears that even though the serum antibody response is slower in RST mice, RST would not affect long term immune memory and prevention of reinfection, as RST does not affect the ultimate antibody titer. However, the influenza-specific antibodies are also involved in neutralizing viruses and limiting infection spreading during infection. Studies which showed that antibodies neutralize viruses and help the regeneration of epithelial cells in respiratory tract indicated that serum antibody is not essential but is beneficial for recovery from infection (84). On the other hand, the vaccines for immunization contain the antigens that are predicted to be prevalent in the future, but the prediction could be mistaken and the appearance of a different strain is possible. Under this condition, the delay of several days in reaching the peak of antibody response may result in infection spreading and complications developing in immunocompromised patients.

A recent study of Kiecolt-Glaser et. al showed that chronic stress suppressed the increase in serum antibody titer after influenza vaccination in elderly people (233). The immunological responses to influenza virus vaccination is poorer in caregivers who take care of their spouses with Alzheimer disease than in matched noncaregiving control subjects. This finding indicated that the magnitude of serum antibody response in humans is affected by
chronic stress, although it is not in agreement with the result of mice restraint stress model which used an infection rather than a vaccination. However, the difference could be due to that restraint stress is a model of acute-chronic stress, which could activate neuroendocrine system differently from a chronic stress, or that species differences exist in response to vaccination and stress.

Therefore, to investigate the mechanisms of neuroendocrine modulation of humoral response and to explore a possible method for improving the humoral response in elderly people, this study was set up to examine the cellular mechanisms of neuroendocrine modulation of the murine humoral response to influenza viral infection in a restraint stress model.

In this study, the ELISPOT assay was used to examine the humoral responses by quantitating the number of influenza-specific antibody-secreting cells. The results were different from a previous serum antibody titer study which showed total antibody production (175). The advantage of the ELISPOT assay is to evaluate humoral responses in each lymphoid tissue without the interference of viral antigen and without in vitro stimulation. In addition, the results of the ELISPOT assay give insight of the local humoral response at cellular level, while the results of serum ELISA study reflect systemic humoral immunity.

We hypothesized that the lung, MLN, SCV and spleen would have different kinetics, magnitude and isotypes of ASC responses, since the availability of influenza viral antigen and the microenvironment in each tissue is different. The results showed that the peak responses of IgM and IgG ASC in all the sites were on day 7 p. l., although the magnitude of the ASC response was different among them. MLN had the highest IgM and IgG ASC numbers and
the response in spleen and lung were weak. This finding could be explained by the fact that MLN is the respiratory tract draining lymph nodes, and B cell activation, proliferation and differentiation are more rigorous as more antigens are drained into them compared to the spleen. In addition, since ASC in the lungs are B cells activated in lymphoid tissues which migrated into the lung, the similarity of the IgM and IgG kinetics in all the tissues tested may suggest that the ASCs migrate out of lymphoid tissues soon after proliferation.

The sharp peak of IgM and IgG ASC responses in MLN, SCV and spleen is an unexpected result. The decline of the ASC numbers could be the result of lymphocyte migration into other tissues or decreased B cell proliferation and differentiation as a result of less antigen stimulation and less T cell cytokine production late in infection. In addition, as many B cells in germinal center of lymphoid tissues undergo apoptosis after activation and proliferation, apoptosis could be a reason for the decline of ASC numbers. The survival from apoptosis is achieved by signaling through the B cell surface CD40 molecules which interact with CD40 ligand on T helper cells, and through B cell surface Ig which interact with follicular dendritic cells (FDC) acting as antigen presenting cells (234). The surviving cells become plasma cells or memory cells. Because the ASCs detected after day 7 p. I. could be the cells that went through apoptosis, any factors that affect T-B interaction or FDC antigen presentation may alter B cell survival and change ASC numbers in lymphoid tissues.

The second part of this study was the effect of RST on ASC responses. The similarity of the kinetics of ASC responses in the various tissues made it easy to examine the neuroendocrine modulation of the ASC responses. At the peak of IgM and IgG ASC responses, RST groups and nonstressed groups were compared for IgM and IgG ASC
responses. The results showed that MLN, spleen and lung had suppressed ASC responses while the SCV had an enhanced ASC response in RST groups. This result does not support the hypothesis that IgM and IgG ASC responses were both suppressed by RST. It was unexpected that the SCV ASC responses would be enhanced by RST. There has been evidence that lymphocytes migrate to the bone marrow during stress as elevated GC level modulates lymphocyte trafficking (142). However, to our knowledge, this is the first study which showed that lymphocytes migrate to mucosal lymph nodes, or that humoral responses are enhanced, in mucosal lymph nodes during stress.

It is known that mucosal and nonmucosal lymph nodes express different addressins for lymphocyte homing (218, 219, 220) and that GC affects adhesion molecule expression (117, 142, 216, 217). Therefore, it is possible that the expression of adhesion molecules for mucosal and nonmucosal lymph nodes are differentially regulated by neuroendocrine mediators resulting in increased migration of T or/and B cells to mucosal lymph nodes. An alternative explanation is that the differences in SNS innervation of MLN and SCV may result in the differences of the microenvironments for B cell activation when SNS is activated by RST. Another possible reason is that RST may modulate the Th1 and Th2 interaction in SCV resulting in the enhanced IgM and IgG ASC responses. The upper respiratory tract is challenged by environmental stimulus constantly and its draining lymph nodes, such as SCV, are engaged in humoral responses by secreting IgA. Therefore, the Th2 response is dominant in SCV while Th1 response is normally downregulated. During stress, the downregulation of Th1 may be modulated and the expression of Th1 cytokines is increased resulting in the enhanced IgM and IgG ASC responses. The mechanisms of enhanced SCV IgM and IgG
ASC responses need to be further investigated from the apoptosis aspect. The effect of RST on the modulation of germinal center B cell apoptosis and the differential modulation of apoptosis in SCV and MLN could be the answer.

The study of the mechanisms of the RST-induced modulation of humoral responses showed that RU486 (a GC receptor antagonist) treatment partially restore the RST-induced suppression of ASC responses in MLN while treatment of nadolol (a $\beta_2$-adrenoceptor antagonist) did not restore the suppression. These findings indicate that GC is one of the mediators that modulate humoral responses while CAT is not involved in the modulation of humoral responses or does not act through $\beta_2$-adrenoceptor. This conclusion may be of clinical importance as RU486 could be used in stressed-patients to increase their humoral responses and to reduce their susceptibility to influenza viral infection. The results of this mechanism study leave the question open as to the other factors that involve in the modulation of humoral response. ACTH, CRF and endorphin have been shown to modulate antibody responses and their role in the RST-induced modulation of humoral responses is worth of future investigation.
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125


