INTERVENTIONS FOR PRIMARY PREVENTION OF POST-BRONCHIOLITIS ALLERGIC SENSITIZATION IN GUINEA PIGS

by

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ABSTRACT

Asthma is a serious health problem that affects over 2.4 million Canadians. Asthma and allergy are closely linked and 80% of asthmatic children are allergic to at least one inhaled antigen. Among several conditions of diverse etiology that are included in asthma, we studied post-bronchiolitis asthma and allergy in children, in which an episode of acute bronchiolitis precedes the onset of asthma. By focusing on the Th1/Th2 paradigm concept, in which shifting the Th1/Th2 balance towards Th2 is characteristic of allergic responses, we proposed two types of interventions for preventing the post-bronchiolitis allergic sensitization and asthma in a guinea pig model of respiratory syncytial virus (RSV) infection and ovalbumin (OA) sensitization. These interventions, which included inoculation of animals with relatively "low doses" of RSV or administration of CpG-ODN (synthetic oligodeoxynucleotides rich in cytosine and guanine) before experimental RSV infection plus OA exposures, were applied to shift the Th1/Th2 towards Th1 responses. We compared 4 groups of animals (n=10-12/group), including controls and animals that had the intervention. We compared airway inflammatory cells (eosinophils and T cells) by point counting of the Hansel stained and anti-CD3 immunostained formalin-fixed paraffin embedded lung sections. Using ELISA, we also measured the titers of serum OA-specific IgG1 antibodies, which increase during allergic sensitization and reflect Th2 responses. We also compared lung IFNγ/IL-5 gene expression ratios as indices of Th1/Th2 balance, by semi-quantitative RT-PCR method. The results showed that low dose RSV had no effect on any of the indices of allergy and asthma examined. However, CpG-ODN intervention decreased the percentage of inflammatory cells and the titers of IgG1 antibodies, and increased the lung IFNγ/IL-5 gene expression ratios. We also tested the effect of CpG-ODN on uninfected OA-sensitized guinea pigs and the results showed that CpG-ODN did not affect OA sensitization. In conclusion, the "low dose" RSV intervention had no effect on post-bronchiolitis allergic sensitization of guinea pigs but the CpG-ODN administration provided partial protection against combination of RSV infection and OA sensitization.
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CHAPTER 1: ASTHMA

1.1. GENERAL INTRODUCTION

Asthma is a serious health problem in Canada and affects both children and adults. According to the 1998-99 National Population Health Survey (NPHS), over 2.4 million Canadians (10.7% of children and 7.5% of adults) suffer from this chronic condition [1]. The mortality and morbidity associated with asthma affects the life of asthmatic patients and their families as well as the community and is estimated to cost the Canadian health care system $0.8 to $1 billion annually [2].

1.2. DEFINITION

Instead of being a single entity, asthma includes several conditions of diverse etiology and pathogenesis that share similar clinical features [3]. Due to this diversity, asthma is difficult to define and instead it is characterized by its hallmarks of reversible airflow obstruction, non-specific bronchial hyperreactivity, chronic airway inflammation and airway remodeling [4,5]. Each of the hallmarks is described below.

1.2.1. Reversible Airflow Obstruction

Decreased airflow rates during expiration that are often associated with an elevated functional residual capacity are characteristic for airflow obstruction [6]. Using lung spirometry, airflow obstruction is diagnosed by measuring the forced expiratory volume in 1 second (FEV₁) and the forced vital capacity (FVC) [6]. The decreased ratio of FEV₁ to FVC (<75%) is indicative of airflow obstruction [7]. However, in contrast to chronic obstructive pulmonary diseases (COPD), airflow obstruction in asthma is episodic and has a pharmacological and spontaneous reversibility [6]. The reversibility of the obstruction in patients with obstructive lung disease could be measured by changes in FEV₁ before and after administration of an inhaled bronchodilator. In adults, an increase of at least 12% and 200 mL in FEV₁ or 15% and 200 mL in FVC defines reversibility [7]. In children, reversibility has been defined by 10% increase in FEV₁ [8]. In situations for which spirometry is not diagnostic, bronchial provocation testing will be needed to confirm non-specific bronchial hyperreactivity in the airways of asthmatics [6].
1.2.2 Non-Specific Bronchial Hyperreactivity

Bronchial hyperreactivity refers to the airways of asthmatic patients having a lower threshold (increased sensitivity) and greater narrowing (increased maximal response) in response to exposure to nonspecific bronchoconstrictors such as cold air, exercise, and a number of inhaled substances (e.g., methacoline) [9]. Increasing amounts of a bronchoconstrictive agent can be given to induce the response in patients. A test is considered positive when a \( \geq 20\% \) drop in FEV\(_1\) is observed after inhaling a nebulized solution of methacoline at a dose of 16mg/mL or less [7].

1.2.3. Airway Inflammation

The inflammatory process in airways of asthmatics has been recognized for more than 100 years. The chronic airway inflammation in asthma affects the proximal and distal conducting airways but relatively spares the parenchyma [11]. The airway inflammation is characterized by infiltration of inflammatory cells [11]. The inflammatory cells are infiltrated in all compartments of the airway and include T lymphocytes, mast cells, basophils, macrophages, and eosinophils and in some forms of asthma, neutrophils [11].

1.2.4. Airway Remodeling

Airway inflammation plays a central role in asthma, but it does not explain the chronicity and progression of this disease [10]. For example, the use of corticosteroids in the therapy of asthma controls symptoms but does not cure the disease [11]. However, other structural changes in all compartments of the airway that result in airway wall thickening, have attracted the notice of researchers interested in asthma. These changes are called “airway remodeling” and are characterized by epithelial goblet cell hyperplasia and metaplasia, increased accumulation of extracellular matrix in subepithelial connective tissue and adventitia of the airway wall, smooth muscle hypertrophy and hyperplasia, increased vascularity, and proliferation of nerves [12].

1.3. ETIOLOGY OF ASTHMA

The etiology of asthma involves the interaction between genetic factors and environmental exposures. However, being a “complex genetic disorder”, classical Mendelian patterns of inheritance do not apply to asthma [13]. Environmental exposures are also believed to play important roles in the development of asthma [14].
It is thought that genetic factors (e.g. atopy) predispose individuals to environmental allergic reactions. The environmental factors that lead to allergic responses include animal dander, dust mite, cockroach allergens and workplace contaminants. In addition there are other factors that increase the possibility of developing asthma in people exposed to allergens and examples of these contributing factors are viral respiratory infections, air pollution, and exposure to cigarette smoke during pregnancy and childhood. [1]

In contrast to factors mentioned above, breast-feeding and avoiding the exposure of infants and young children to allergens and cigarette smoke may decrease the risk of asthma [1].

1.3.1. Atopy and Asthma

The term “atopy” refers to a state of IgE-mediated allergic response to environmental allergens [15]. Despite there being no standardized definition of atopy, it can be assessed in several ways, including measurement of total serum IgE and allergen-specific IgE, positive skin-test reaction to intradermally-injected allergens, or association with other conditions such as infantile eczema, allergic rhinitis and food allergies [15]. Asthma and atopy are closely linked, and if the age of patients is considered, > 80% of asthmatic children are allergic to one or more inhaled allergens [16].

1.3.2. Bronchiolitis

Association of bronchiolitis in infancy with recurrent wheezing and asthma during childhood has been reported frequently [18-21] and will be described in the next chapter. Acute bronchiolitis is a common, predominantly viral illness that affects lower respiratory tract of children less than 24 months old. The disease occurs sporadically and has peak incidence during the winter and early spring. Respiratory syncytial virus (RSV) accounts for the majority of cases and almost all of the remainder are produced by parainfluenza 3 virus, *Mycoplasma*, some adenoviruses, and occasionally other viruses [17].

1.3.2.1 Clinical Features

A history of exposure to older children or adults with minor respiratory diseases within the week before the onset of bronchiolitis is usual. First the infant develops upper respiratory infection symptoms with or without loss of appetite and fever and after several days, paroxysmal wheezy cough and dyspnea occurs. Respiratory distress results in tachypnea and cyanotic
appearance as well as diminished food intake, which in turn causes dehydration, irritability, and lethargy. Increased respiratory effort leads to nasal flaring and intercostal and subcostal retractions. The liver and spleen may be palpable due to their depression by overinflated lungs. If the bronchiolitis is not severe enough to cause a complete airway obstruction, widespread fine crackles and wheezes are heard. Conjunctivitis or otitis may accompany the illness. The course of bronchiolitis in previously healthy infants is typically 3 to 10 days. Overall, 1-2% of children diagnosed with bronchiolitis require hospitalization, and of these hospitalized patients, 3% to 7% develop respiratory failure, and 1% die. Complications such as atelectasis, apnea, and respiratory failure are more common in children who have significant cardiopulmonary disease or immunodeficiency [17,22].

1.3.2.2 Diagnosis

Virus may be demonstrated in nasopharyngeal secretions by culture, antigen detection (e.g., by immunofluorescence assay (IFA) or enzyme immunoassay (EIA)) or by amplification of virus-specific nucleic acid by use of polymerase chain reaction (PCR) [23]. Chest x-ray findings are nonspecific and may reveal diffuse hyperinflation of the lungs or scattered areas of consolidation, which represent alveolar inflammation or atelectasis [22].

1.3.2.3 Treatment

The treatment for bronchiolitis is mainly supportive and includes administration of oral fluids, antipyretics, and oxygen. Bronchodilators sometimes are beneficial and this is usually shown by a trial administration. Indications for hospitalization in otherwise healthy children are tachypnea, marked retractions, listlessness, and poor oral intake. In addition, children with immunodeficiency or underlying cardiopulmonary disease should be admitted to the hospital. In case of apnea, rising $P_{a}CO_2$ value, listlessness and chest retractions intubation and mechanical ventilation are required. Ventilated patients receive corticosteroids, theophylline, and furosemide [17,22].

Ribavirin, a broad-spectrum antiviral agent is administered for early treatment of RSV bronchiolitis in high-risk infants (e.g., infants with congenital heart disease, bronchopulmonary dysplasia, lung and chest wall anomalies, and immunodeficiency) and also less than 6-week-old infants or those who are severely affected ($P_{a}O_2 < 65$ mm Hg, rising $P_{a}CO_2$). Improved oxygenation, improved clinical scores, and lower levels of mediators of inflammation have been observed after administration of ribavirin [24].
However, use of ribavirin does not reduce mortality or duration of hospitalization in infants with RSV bronchiolitis [19]. Furthermore, the use of ribavirin is restricted because of its expense and potential teratogenic effects on hospital staff [19].

1.3.2.4 Prophylaxis

Passive immunization with the immunoglobulin containing high titers of anti-RSV antibody or monoclonal antibody against F protein of RSV (palivizumab) has been reported to result in less severe illness and lower risk of hospitalization for RSV infection [24]. Given practical considerations of cost and logistic of administration, prophylaxis for RSV is only recommended in high-risk infants who have an underlying condition such as bronchopulmonary dysplasia or a history of premature birth [22].

1.3.2.5 Immunization

The unfortunate experience during the original trials of a formalin-inactivated vaccine, in which after subsequent RSV infection 80% of vaccine recipients compared with 5% of controls were hospitalized and two vaccine recipients died [25], has delayed the vaccine development. Furthermore, particular problems such as existence of several antigenic strains for RSV, immaturity of immune system during early life, presence of maternal antibodies during the first months of infancy, and limitations of available animal models, the development of a vaccine has been delayed [23]. Despite these problems, research on live attenuated vaccines, genetically engineered viral vaccines, live viral vectors, and subunit vaccines is proceeding [23].

1.4. IMBALANCE BETWEEN TH1 AND TH2 CELLS AND THE ORIGIN OF ASTHMA

CD4+ T lymphocytes, also known as T helper (Th) cells can be classified into subsets, based on biosecretory profiles of cytokines [26]. Of these subsets, Th1 and Th2 cells have opposing roles and regulate immune responses to intracellular and extracellular pathogens, respectively [27]. Murine Th1 cells are involved in cellular immunity by producing interferon-γ (IFNγ), interleukin-2 (IL-2), and tumor necrosis factor-β (TNFβ), thereby promoting the development of CD8+ cytotoxic T lymphocytes and macrophages, while Th2 cells by producing IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 are involved in humoral immunity, which is associated with allergy and parasitic infestations [27,28]. Although production of some cytokines such as IL-2, IL-6, IL-10, and IL-13 in humans is not limited to a single subset of CD4+ T cells, the
overall Th1/Th2 pattern is similar to the mouse [27]. A variety of factors determine the commitment to a Th1 or Th2 response including the local cytokine milieu, the dose of antigen, the antigen-presenting cell (APC), and the delivery of the costimulatory signals from the APC to T cells [28]. Briefly, T cells are regulated by antigen-specific signals and costimulatory signals mediated by CD28 on T cells and B7-1 (CD80) and B7-2 (CD86) on APCs add an additional level of regulation on T cells [29]. Several in vitro and in vivo studies have reported that B7/CD28 signals induce the Th2 responses [29]. A period of antigenic stimulation is required to make isolation of CD4⁺ T cells capable of a Th1 or Th2 response [30]. This stimulation, which can also occur during active or resolved infections, categorizes the T-cells into primed memory T cells [30]. IL-12 produced by APCs has a key role in commitment of naïve CD4⁺ T cell precursors to Th1 cells [28]. IL-12 by activating signal transducer and activator of transcription 4 (Stat4) results in Th1 responses [28]. Th1 cells in turn produce IFNγ, which upregulates the IL-12 receptor and inhibits the growth of Th2 cells [28]. On the other hand Stat6 mediates development of Th2 responses by IL-4 [28]. IL-4 also downregulates IL-12 receptors making the commitment into Th2 cells easy [28].

Taken together, an increase in the differentiation and function of one of these CD4⁺ subsets decreases the differentiation and function of the opposing one, indicating existence of a Th1/Th2 balance in the body that can be skewed in different conditions. It is thought that a Th1 and Th2 balance shifted toward the Th2 response, contributes to the etiology of atopic diseases such as asthma [30]. It is believed that during normal pregnancy the immune response shifts towards Th2 response, to protect rejection of the fetus by immunologically different maternal tissues [31]. In newborns the Th1/Th2 balance is still skewed toward Th2 cells and some investigators have suggested that exposure to stimuli which shift the Th1/Th2 balance toward Th1 during early infancy can reduce the risk of allergy and asthma in childhood [30].

Although several interventions have been proposed for priming Th1 host response, the efficacy of these interventions in preventing the onset of asthma and allergy in children is controversial [32,33]. Despite this controversy, two types of suggested interventions that we have applied in our experiments are described below:

1.4.1 Th1 Priming with Different Doses of Antigens

Studies on the effect of tuberculosis [34] and Leishmaniasis [35] on the T cell cytokine profile show that Th1/Th2 responses are determined by the infectious dose during first exposure. In particular, regardless of whether the pathogen is bacterial or parasitic, exposures to
comparatively low doses of initial pathogen stimulate a long-lasting, protective Th1 response, while initial high infectious doses stimulate a harmful mixed Th1+Th2 response. These findings support the possibility of developing interventions that can preferentially stimulate a Th1 response.

1.4.2 Th1 priming with CpG oligodeoxynucleotides (ODNS)

CpG-ODNs are synthetic oligodeoxynucleotide agents characterized by unmethylated cytosine-guanine (CG) dinucleotides in particular motifs [36]. These CpG motifs have potent immunostimulatory effects and are found abundantly in bacterial DNA [36]. Unmethylated CpG motifs do not exist in vertebrate DNA and the vertebrate immune system recognizes the CpG motifs by Toll-like receptor 9 (TLR9) [37]. CpG motifs can activate a number of transcription factors such as nuclear factor κB (NF-κB) and activator protein-1 (AP-1) [37]. This activation induces macrophages and dendritic cells to secrete cytokines such as IL-12 and IL-18 and natural killer cells to secrete IFNγ [38]. In murine models of asthma, application of CpG-ODN has resulted in suppression of allergen-induced asthma associated with a shift of Th1/Th2 balance toward Th1 responses [39-41].

1.5 SUMMARY

In summary asthma and allergy are closely linked, with >80% of asthmatic children being allergic to one or more inhaled allergens. There is epidemiological and experimental evidence that viral respiratory tract infections such as RSV acute bronchiolitis may contribute to allergic sensitization. It is believed that asthma and allergy originate from shift of Th1/Th2 balance toward Th2 responses. Accordingly, several interventions have been proposed to shift the Th1/Th2 balance away from Th2 responses, thereby protecting against allergy and asthma. Two of these interventions that are related to this thesis are described in this chapter including priming Th1 responses with low doses of antigens and priming Th1 responses with CpG-ODNs.
CHAPTER 2: RESPIRATORY SYNCYTIAL VIRUS

2.1. HISTORICAL BACKGROUND

Respiratory syncytial virus (RSV) was first isolated in 1956 from chimpanzees that had common cold symptoms and the new virus was called the "Chimpanzee coryza agent" [42]. The following year, the same virus was isolated from infants with respiratory disease [43] and the name "respiratory syncytial virus" was given to the virus because of its ability to fuse infected cells into syncytia in some tissue culture cell lines. Today RSV is regarded as "the most important viral agent of serious respiratory tract disease in the pediatric population worldwide" [44]. Up to 90% of bronchiolitis cases and about 50% of pneumonia during infancy occurs as a result of RSV infection [45]. Almost all children are infected with RSV during the first two years of life; 2.5% are hospitalized, and 0.1% die [45]. More than 45 years of research has brought neither a good treatment nor any vaccine [46].

2.2. CLASSIFICATION OF RSV

RSV is categorized in the genus Pneumovirus within the family Paramyxoviridae. Viruses in the Paramyxoviridae family have a single-stranded, negative-polarity RNA genome. The genome is located in a helical nucleocapsid that is RNAse-resistant and also contains the viral polymerase. The genome is transcribed in a stop-restart mode, in which the polymerase produces subgenomic messenger RNAs (mRNAs). The viral replicative cycle takes place in the cytoplasm of the host. The virus is enveloped by the plasma membrane of the infected cell and enters the host cell by fusion to the surface [44].

2.3 RSV STRUCTURE

The RSV virion is about 150-300 nm in diameter and consists of a nucleocapsid within a lipid envelope [44]. The genome is a single-stranded, non-segmented RNA with approximately 15,200 nucleotides that encode 11 proteins, three of which (G, F, and SH) are transmembrane surface glycoproteins [46]. The diameter of these surface glycoproteins is 11-20 nm and they are spaced at intervals of 6-10 nm in the envelope [44]. The G (Glycoprotein) protein is involved in the viral attachment to the host cell surface and the F (Fusion) protein is required for fusion of the viral envelope or infected cell membranes with the uninfected cell membranes [46]. The two...
major antigenic groups of RSV (A and B) are mainly classified according to their G protein variations, but the F protein is highly conserved between the strains [24]. The function of SH (small hydrophobic) protein is not known and its presence is not essential for maintaining viral viability [46]. The nucleocapsid consists of N (nucleocapsid), P (phosphoprotein) M2-1 proteins and L proteins [46]. L protein is the RNA polymerase and the other nucleocapsid proteins are also involved in transcriptional activity [46]. The M2-2 protein, which is low in infected cells but increases during infection, is involved in regulation of transcription [44]. However, the M2-2 protein is not essential for growth and replication of the virus. The M (matrix) protein organizes assembly of envelope proteins and nucleocapsid proteins and is vital for viral viability [44]. RSV has also nonstructural proteins (NS1 and NS2), which enhance virus growth but they are not essential proteins for viability [44]. Nonstructural proteins may be antagonists for interferon-α and β [44,46].

2.4 RSV REPLICATIVE CYCLE

The RSV can survive on inanimate objects for hours and transmission can occur through self-inoculation of secretions from hands [47]. In addition, large-particle aerosols are able to transmit the virus to the nasal mucosa or conjunctivae [47].

Cell surface attachment is the first step in the viral infection process, which occurs via the G protein. The cellular receptor(s) for G protein has not been identified. Once attached, the fusion of viral envelope to the host cell membrane occurs and then the nucleocapsid is released into the cytoplasm. In the cytoplasm, mRNA transcription and genomic RNA replication take place. Viral proteins and genome are then assembled and the mature virions either leave the cell by budding or produce RSV cell-to-cell fusion and syncytia formation [46].

2.5 HOST RESPONSE TO RSV INFECTION

It is not well understood why RSV infection is more severe in some infants. One reason could be the genetic variations and indeed recently an association has been found for the RSV bronchiolitis and an IL-8 genetic polymorphism [48]. The host immune response has a key role in producing the lower respiratory tract disease caused by RSV infection [49]. The importance of the host response to RSV infection was discovered after the disastrous formalin-killed RSV vaccine trial, in which subsequent natural infection caused more severe disease [25].
2.5.1 Innate Immunity to RSV

Epithelial cells and alveolar macrophages mediate the innate immune response to RSV. For example, epithelial cells produce opsonins and collectins. Surfactant A, one of the collectins produced by epithelial cells, enhances the opsonisation of RSV by monocytes and macrophages and thereby is involved in RSV clearance [50].

RSV infection also induces epithelial cells to secrete cytokines such as IL-1α [51], Tumor necrosis factor-alpha (TNF-α) [51], IL-8 [52], and RANTES (regulation upon activation normal T cell-expressed and secreted) [53]. IL-1α upregulates the expression of intracellular adhesion molecule (ICAM)-1 [51] in RSV-infected cells, and thereby may be involved in neutrophil and eosinophil trafficking in the airways after RSV infection. TNF-α is a potent chemotactic factor and a neutrophil activator and IL-8 is the most important factor for neutrophil chemotaxis [54]. In addition, RANTES is an effective chemoattractant for T cells and eosinophils [54]. Taken together, these mediators are chemotactic for neutrophils, T helper cells and eosinophils and may take part in the pathogenesis of RSV-induced wheezing.

Alveolar macrophages also release inflammatory cytokines such as TNF-α, IL-8, and IL-6 (a lymphocyte activator) when infected with RSV [55]. It has been reported that the innate immune response to the viral F protein is mediated by Toll-like receptor (TLR) 4 and CD14 receptor on monocytes [56].

2.5.2 Humoral Immunity to RSV

All full-term newborns have maternal RSV-neutralizing antibodies in their sera [57,58]. Although mainly transferred transplacentally, antibodies can be delivered to the newborn by breast-feeding. [59]. The titer of maternal antibodies declines during the first few months after birth and if neutralizing antibodies are detectable after 7 months of age, they are produced by infection [57]. Observations that prophylaxis with RSV-specific IgG reduces hospitalization from subsequent RSV infection [60] and breastfeeding reduces the likelihood of severe bronchiolitis [61] provide evidence that antibodies have neutralizing effects against RSV. In contrast, more severe disease and wheezing after RSV infection are associated with higher levels of RSV-specific IgE [62].
2.5.3 Cellular Immunity to RSV

Some observations suggest that cell-mediated immune responses start within 10 days of RSV infection [63,64]. Individuals who have cellular immunodeficiency have longer courses of RSV-induced disease, indicating a critical role for cell-mediated responses [65,66]. Both protective and disease-enhancing roles have been observed for CD8\(^+\) and CD4\(^+\) T lymphocytes in RSV-infected mice, including reduction in viral shedding but with increased pulmonary damage [67].

As mentioned in Chapter 1.4, CD4\(^+\) T cells are classified into two functionally distinct Th1 and Th2 subsets. Viruses are intracellular pathogens and Th1 responses are involved in cell-mediated responses against intracellular pathogens, therefore in general it is believed that a normal host response to viruses is mediated by Th1 cells. Live RSV infection in mice stimulated a Th1 response, whereas immunization with formalin-inactivated virus resulted in a Th2 (allergy-associated) response [68]. It is suggested that formalin-inactivated vaccine stimulated a Th2-type response and thereby resulted in the more severe disease following subsequent RSV infection in humans [69]. Studies on presence and relevance of Th1 versus Th2 responses to RSV infection of humans have reported contradictory results [70,71,72].

2.6 RSV PATHOLOGY

Since lung specimens are not obtained very often from very young children, there is little known about RSV pathology in mild human cases. However, in severe cases of RSV bronchiolitis some histological changes have been observed in bronchioles including destruction of epithelial cells, peribronchiolar inflammation, edema of submucosal and adventitial tissues, and profuse mucus production. Bronchiolar obstruction leads to either collapse of the airways or air trapping. In the pneumonia cases, alveoli are involved and alveolar walls are infiltrated with mononuclear cells [44].

2.7. RSV AND ASTHMA

There is considerable evidence that RSV bronchiolitis might play a key role in development of recurrent wheezing and asthma [18-21]. Although the pathogenesis of post-bronchiolitis asthma is not well understood, several hypotheses are proposed to explain the mechanism behind this development including:
1. A ‘hit and run driver’ mechanism for the virus, in which despite viral clearance from the lungs, a chronic sequela remains. Evidence for this mechanism is provided by a rat model of parainfluenza type I (Sendai) virus lung infection, in which bronchial hyperresponsiveness and mast cell hyperplasia last for at least three months, without evidence of concomitant viral persistence [73].

2. Enhancement of allergen sensitization by the viral infections. Increased airway responsiveness and lung inflammation after allergen sensitization in RSV-infected mice [74] and also increased levels OA-specific IgG1 antibody in RSV-infected, OA-sensitized guinea pigs [75] provide supporting evidence for this mechanism.

3. Persistence of viral infection. This potential mechanism is supported by a study on guinea pigs that reported association of chronic intrapulmonary RSV persistence with bronchial hyperresponsiveness and airway inflammation [76].

In contrast to those who consider a causal link between viral infection and asthma, some investigators regard a protective role for viruses against development of allergy and asthma [77,78,79]. Proponents of this concept point to the Th1-type response after a viral exposure in early life [77]. Their hypothesis is further supported by studies that show a lower possibility of asthma development at 6 to 13 years of age in children who receive more exposures to viruses during infancy, in daycare settings or from their older siblings [80]. Indeed, the rise in asthma and allergy in western countries could be explained by the “hygiene hypothesis”. According to this theory, higher levels of hygienic conditions and thereby less pathogen exposure in infants is associated with an increased risk of asthma development in children. This hypothesis can be explained by considering a Th1 response after viral infections that brings protection to asthma and allergy [81]. However, as mentioned in Section 2.5.3, it is controversial whether RSV stimulates a predominant Th1 or Th2 response in humans, such that the applicability of hygiene hypothesis in the context of acute bronchiolitis and asthma remains unclear.

2.8 SUMMARY

RSV is the most common cause of acute bronchiolitis in infants. There is considerable evidence suggesting causal link between respiratory viral infections, especially RSV infections, and allergy and asthma, but the mechanism behind this link is poorly understood. On the other hand, some investigators consider a protective role for respiratory viral infections against allergy and asthma, but whether the host response to RSV bronchiolitis is predominantly Th1 or Th2
mediated is controversial. In our opinion, some of this controversy could be explained by considering the doses of infectious virus to which the infant was first exposed. This postulation provided a basis for one of the interventions we applied for primary prevention of post-bronchiolitis allergic sensitization in this thesis.
CHAPTER 3: ANIMAL MODEL FOR POST-BRONCHIOLITIS ASTHMA

3.1. ANIMAL MODELS FOR RSV BRONCHIOLITIS

The tragic trial of formalin-inactivated (FI) RSV vaccine, in which vaccinees developed more severe disease after subsequent natural RSV infection, was a stimulus for finding a relevant small animal model for RSV infection [82]. So far many different animal models have been proposed and in this chapter some advantages and limitations of these models are reviewed.

Not only is the genetic relatedness of chimpanzees to humans more than any other laboratory animal, chimpanzees also become ill after being infected with human RSV [42]. However, chimpanzees are not readily available and their purchase and maintenance is expensive. In addition terminal experiments on chimpanzees are not allowed [82].

Monkeys are genetically closer to human than many other species and terminal experiments on them are not forbidden. Moreover, some strains of monkeys show signs of rhinorrhea after human RSV infection. However, expense, lack of inbreeding, and inadequately available species-specific immunologic reagents are limitations for using these animals in RSV research [82].

The calf is another example of a large animal model for RSV infection and calves can become infected with bovine RSV, a pathogen that is related to the human virus. The disease caused by bovine RSV in calves is similar to the one caused by human RSV in humans in many ways. For example, it is limited to respiratory tract, results in an epidemic disease with a peak during winter months, induces an incomplete immunity, and high levels of maternal antibody attenuate the disease. Moreover, the severity of disease is highest among neonates. However, expense, lack of inbred animals, and inadequately available species-specific reagents are limitations for using calves as an animal model. In addition, human and bovine RSVs are different in terms of amino acid homology, propagation in vitro and titering, which adds to the disadvantages for modeling human RSV infection [82].

Research on small animal models has the benefits of easy handling and low cost of purchase and maintenance. Several small animals have been used as models for RSV infection including cotton rats, mice, guinea pigs, ferrets, and hamsters.

The mouse and the cotton rat have the problem of not developing acute lower respiratory tract disease or an impressive bronchiolar inflammation [83,84]. For example, more than $10^7$ plaque-forming units (pfu) of RSV is required for producing bronchial inflammation in mice.
[83], while intranasal inoculation of only 500 pfu is sufficient to develop infection in 100% of human volunteers [85]. In contrast to humans who are susceptible to RSV bronchiolitis during their infancy, cotton rats of all ages [84] and mice only after maturity [83] develop pulmonary infection. The advantage of using cotton rats over mice is their 100-fold greater permissiveness to RSV with 10-fold higher titers of serum antibody [83,86]. Moreover, like humans, the cotton rat develops vaccine-enhanced disease [82]. However, lack of species-specific reagents and the absence of congenic, transgenic and knock out strains are limitations of using cotton rats over mice [82].

Virus permissiveness in guinea pigs is more than cotton rats and mice (10^3-10^4 pfu in guinea-pigs compared to >10^7 pfu in mice) with evidence of virus replicating in the lungs [87]. Moreover, not only have RSV genome and/or protein have been detected in the guinea pig lung for at least 100 days [76,88,89] after experimental inoculation but also the replicating virus was isolated from guinea pig lungs 60 days after inoculation [90], providing evidence for persistence of intact, infectious virus in vivo. Limitations for using this model are shortage of inbred strains as well as lack of species-specific reagents [82,91].

Although both guinea pigs [92] and ferrets [82] possess the advantage of developing pulmonary infection only before maturity and thereby modeling the more susceptibility of infants than adults to RSV lung infection, the knowledge on ferret models is not as vast as guinea pigs, hence using ferrets do not offer any advantage over guinea pigs.

In Syrian hamsters RSV replicates in both nose and lungs, but the permissiveness is similar to mouse [82]. Overall, the knowledge about RSV infection in hamsters is not enough to encourage using them over other proposed models.

### 3.2. GUINEA PIG AS A MODEL FOR ASTHMA

The Cam-Hartley guinea pig has been used as a model of allergic sensitization and challenge to aerosolized OA [93]. The allergic response in this animal model has been associated with non-specific bronchial hyperresponsiveness to acetylcholine challenge, airway eosinophil infiltrates and OA-specific IgG1 production (the main class of antibody involved in allergic responses of guinea pigs [94]) [75]. In addition, inoculation of juvenile Cam-Hartley guinea pigs with human RSV results in long-term persistence of the virus, airway hyperresponsiveness to acetylcholine challenge, airway eosinophil infiltrates and RSV-specific IgG1 production [76,90]. RSV infection in guinea pigs has also been shown to enhance OA-specific IgG1 production [75],
similar to the results reported in mice, in which RSV infection intensified antigen-specific IgE production [95,96]. Moreover, RSV infection results in bronchial hyperresponsiveness, allergic airway inflammation and skewing of the lung Th1/Th2 cytokine ratios toward Th2 responses in strain 2 and strain 13 guinea pigs, which are inbred for delayed hypersensitivity and allergic reactions, respectively [97].

3.3. OTHER ADVANTAGES OF USING GUINEA PIGS

Lung tissue in guinea pigs is abundant in comparison to other small animal species and allows the investigator to use the lungs of the same animal for different purposes such as molecular biology, histology and tissue culture work [91,97]. Having three major lobes on the right side and two major lobes on the left side, the gross anatomy of guinea pig lung is roughly similar to the human lung [91]. Moreover, the well-defined terminal bronchioles with subtending alveolar ducts in guinea pigs provide another advantage for them to model human lung diseases [91].

3.4 LIMITATIONS OF USING GUINEA PIG IN MOLECULAR BIOLOGY

Tissue handling for mRNA measurement purposes in guinea pigs requires extensive precautions because these animals have very high levels of endogenous ribonuclease. In addition, guinea pig gene and protein sequences are less characterized than those of mouse and rat. However, the number of these sequences is growing rapidly for guinea pigs and there is also evidence of cross-reaction of some human reagents with guinea pig specimens [91].

3.5 SUMMARY

In summary, no perfect animal model is available for RSV infection. Being used as models for both bronchiolitis and allergic sensitization, guinea pigs offer benefits of relatively higher RSV permissiveness for developing experimental acute bronchiolitis. In addition, guinea pigs produce IgG1 antibodies in response to RSV and OA and this capability is relevant to modeling post-bronchiolitis wheezing and asthma in this species. More general issues such as easy handling, relatively low purchase and maintenance cost, similarity of guinea pig lung to human lung, and availability of abundant lung tissue from a single animal are added benefits of using guinea pigs to model human RSV lung infection.
CHAPTER 4: WORKING HYPOTHESIS, SPECIFIC AIMS AND STRATEGY

The working hypothesis of this thesis was based primarily on the general hypothesis that interventions designed to optimize Th1/Th2 balance away from Th2 (allergic) responses will inhibit RSV-enhanced allergic sensitization to aeroallergen.

4.1 GOAL

The goal was to determine whether “low dose” RSV infection (a RSV-specific intervention) or CpG-ODN administration (a general intervention) would inhibit the development of RSV-enhanced allergic sensitization of guinea pigs to aerosolized OA, and inhibit allergic airway inflammation and production of OA-specific IgG1 antibodies.

4.2 WORKING HYPOTHESIS

Maintaining Th1/Th2 balance away from Th2 responses by low dose RSV infection or CpG-ODN administration of guinea pigs, prior to exposure to OA-containing aerosols, will decrease the high dose RSV-enhanced OA-specific IgG1 antibody production and decrease allergic airway inflammation.

4.3 SPECIFIC AIMS

1. To determine the effects of low dose RSV infection or CpG-ODN administration on production of RSV-enhanced OA-specific IgG1 antibodies after subsequent exposure to high dose RSV plus OA-containing aerosols;
2. To determine the effects of low dose RSV infection or CpG-ODN administration on subsequent high dose RSV plus OA-induced allergic airway inflammation;
3. To determine the effects of low dose RSV infection or CpG-ODN administration on lung IFNγ/IL-5 ratios from subsequent high dose RSV plus OA-exposures.
4.4 STRATEGY

4.4.1 Low Dose RSV

The amount of low dose RSV for use as an intervention has been determined in a previous work in our lab [98]. Briefly, 18 juvenile Cam Hartley guinea pigs were divided into three groups (n=6/group) as follows:

“High dose” RSV infection (1.3 \times 10^4 pfu)
“Low dose” RSV infection (1.3 \times 10^2 pfu)
“Sham inoculated”, uninfected controls

Lung homogenates from three animals of each group were prepared for RSV genome detection by RT-PCR, and cytokine measurement by semi-quantitative RT-PCR during acute bronchiolitis (one week post-inoculation). After three weeks of inoculation sera from remaining animals underwent ELISA for RSV-specific IgG1 measurement. Table 4.1 summarizes the results of this experiment, in which animals that received “low dose” RSV had evidence of intrapulmonary virus, without skewing of lung Th1/Th2 toward Th2, and without production of detectable levels of RSV-specific IgG1. High dose RSV infection also resulted in evidence of intrapulmonary virus, but showed skewing of Th1/Th2 balance toward Th2, and production of detectable RSV-specific IgG1. The result of this study indicated that \sim 10^2 pfu of RSV is acceptable for use as a “low dose” viral exposure.

4.4.2 CpG-ODN Dose and Route of Administration

The optimal dose and route of CpG-ODN administration was not known in guinea pigs. A preliminary experiment reported in the next chapter was designed to examine the effects of different CpG-ODN dosing regimen on lung Th1/Th2 balance in guinea pigs.

4.4.3 OA-Specific IgG1 Titers

OA-specific IgG1 was measured according to the ELISA method described by Dakhama et al. [75].

4.4.4 Allergic Airway Inflammation

Infiltrates in small airways of asthmatics are rich in eosinophils and T cells [11,99]. The percentage of eosinophils and T cells in small airways of guinea pigs were measured as indices
of airway inflammation by using Image Pro PLUS image analysis system (Media Cybernetic, inc; Silver Spring, MD, USA).

4.4.5 Lung IFNγ/IL-5 Ratios

Lung specimens were homogenized and the ratios of IFN γ/IL-5 messenger RNA were measured with a semi-quantitative RT-PCR method developed in this thesis.

4.5 SUMMARY

A Cam Hartley guinea pig model was used to test the working hypothesis that manifestations of high dose RSV-enhanced OA sensitization such as OA-specific IgG1 antibody production and airway inflammation would be decreased by maintaining Th1/Th2 balance away from Th2 responses. "Low dose" RSV inoculation and CpG-ODN administration were the interventions that we used for producing a Th1/Th2 balance away from the Th2 responses. A quantitative histology method and ELISA were used for assessment of the airway inflammation and OA-specific IgG1 antibody measurement, respectively. Levels of gene expression of selected Th1 and Th2 cytokines were measured in lung homogenates using a semi-quantitative RT-PCR method.
Table 4.1. Results of low dose RSV infection on IFNγ/IL-5 ratio and RSV-specific IgG1 [98]

<table>
<thead>
<tr>
<th>Group</th>
<th>RSV RT-PCR (+) (Day 7) (n=3/group)</th>
<th>IFNγ/IL-5 (Day 7) (mean ± SD) (n=3/group)</th>
<th>RSV-specific IgG1 titer (Day 21) (n=3/group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected controls</td>
<td>0/3</td>
<td>1.20 ± 0.12</td>
<td>&lt;1:5; &lt;1:5; 1:5</td>
</tr>
<tr>
<td>Low dose RSV (1.3 x 10^3 pfu)</td>
<td>3/3</td>
<td>1.14 ± 0.14</td>
<td>&lt;1:5; &lt;1:5; 1:5</td>
</tr>
<tr>
<td>High dose RSV (1.3 x 10^4 pfu)</td>
<td>3/3</td>
<td>0.65 ± 0.11*</td>
<td>1:500; 1:500; 1:250**</td>
</tr>
</tbody>
</table>

* p<0.04 compared to uninfected controls and low dose RSV groups

** p<0.001 compared to uninfected controls and low dose RSV groups
CHAPTER 5: PRELIMINARY STUDIES

In order to test the working hypothesis mentioned in Chapter 4, we first did some preliminary studies to determine the optimal dose and route of CpG-ODN administration to guinea pigs and establish a semi-quantitative RT-PCR method for selected Th1 and Th2 cytokines.

5.1 CpG-ODN DOSING STUDY OF GUINEA PIGS TO DETERMINE HOW DOSE AND ROUTE OF ADMINISTRATION AFFECTS PULMONARY TH1/TH2 BALANCE

This preliminary experiment was done to determine the optimal dose and route of CpG-ODN (CpG-ODN 2007; Coley Pharmaceutical Group, Inc., Wellesley, MA) administration in guinea pigs. The CpG-ODN contained 22 nucleotides with three CpG motifs (TCGTCGTTGTCGTTTTGTCGTT). The doses and routes of CpG-ODN administration as well as the duration of immunotherapy were recommended by Dr. Heather Davis, Science Officer for Infectious Disease, Coley Pharmaceutical Group, Inc.

5.1.1 Experimental Design

Five groups of female juvenile (n=4/group), 1-month old Cam Hartley guinea pigs were studied as follows:

Group 1: No CpG-ODN
Group 2: 30 μg CpG-ODN (biweekly x 4 weeks), subcutaneous
Group 3: 100 μg CpG-ODN (biweekly x 4 weeks), subcutaneous
Group 4: 30 μg CpG-ODN (biweekly x 4 weeks), intranasal
Group 5: 100 μg CpG-ODN (biweekly x 4 weeks), intranasal

5.1.2 Specimens

Animals were killed 1-week after last CpG-ODN dose by intraperitoneal injection of an overdose of barbiturate (Euthanyl Forte, 1:1 diluted in saline; MTC Pharmaceuticals, Cambridge, ON). Lungs were harvested and flash frozen and kept in −70°C for subsequent mechanical

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1 Work described in section 5.1 has been published: Tayyari F, Ashfaq MK, Manson HE, Hegele RG. Comparison of dose and route of administration of CpG oligonucleotides (CpG ODN) on pulmonary Th1/Th2 cytokine balance in guinea pigs. Am J Respir Crit Care Med 2003;167:A724 (Abstract).
homogenization by Tissue-Tearor® (BIOSPEC Products, Inc; Racine, WI) and extraction of total RNA by use of RNeasy Midi-Kits (Qiagen, Corp; Hilden, Germany). During RNA extraction on-column DNase digestion (Qiagen, Corp) was applied to remove any contaminating DNA. Levels of guinea pig IFNγ (Th1 cytokine), IL-5 (Th2 cytokine) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene) were measured using semi-quantitative RT-PCR method, which is further described in the next section.

5.1.3 Data Analysis

After normalization of IFNγ and IL-5 optical density (OD) to corresponding GAPDH OD, the ratio of IFNγ/IL-5 was calculated for each animal. The normalized cytokine values and ratios underwent logarithmic transformation, which better approximated a normal distribution for the data. One-way analysis of variance (ANOVA) was used to compare mean levels of IFNγ/GAPDH, IL-5/GAPDH, and IFNγ/IL-5 among the 5 groups of animals (SPSS v.11, Statistical Product and Services Solutions; Chicago IL) and a post hoc two-tailed t-test was used to examine the differences between pairs of groups. P value < 0.05 was set as level of statistical significance.

5.1.4 Results

All animals tolerated CpG-ODN administration very well and no animal death or side effect was observed from the CpG-ODN treatment. Since the value of lung IFNγ in one animal from Group 1 was equal to zero and could not be logarithmically transformed, the animal was excluded from data analysis. As illustrated in Figure 5.1, animals in Group 1 (no CpG-ODN treatment) had significantly lower IFNγ gene expression than animals given CpG-ODN (p < 0.05). Figure 5.2 shows that there was a trend toward higher IL-5 gene expression in the guinea pig lung after CpG-ODN treatment except for Group 5 (high dose, intranasal route). Group 5 had significantly lower levels of IL-5 gene expression than other CpG-ODN-treated groups. Figure 5.3 illustrates the IFNγ/IL-5 ratios in groups of animals. All CpG-ODN groups showed significant Th1 shift in pulmonary Th1/Th2 balance in comparison to untreated controls. The extent of Th1 shift was influenced by dose and route of administration of CpG-ODN, in that Group 5 (high dose, intranasal route) had significantly greater Th1 shift in Th1/Th2 balance than Group 2 (low dose, subcutaneous route).
5.1.5 Summary

As the goal of CpG-ODN treatment of guinea pigs is to maximize increase in Th1 and minimize any possible increase in Th2 in the lungs, in this thesis, we used the protocol of 100 μg CpG-ODN, delivered intranasal, biweekly over 4 weeks as the intervention.

5.2 CYTOKINE GENE EXPRESSION MEASUREMENT

This preliminary study was done for establishing a sensitive method for measurement of cytokine gene expression in guinea pig lung specimens. The methods used for RNA extraction and reverse transcription are described in the next chapter. In this section, we describe how the primers were designed, how the sensitivity of our method was measured, and how we provided evidence that our PCR products were true amplicons of the relevant cytokines.

5.2.1 Primer Designing for PCR

Using http://www.ncbi.nlm.nih.gov web site guinea pig sequences for IL-5 and β-actin and IFNγ messenger RNA (or complementary DNA) were obtained and aligned with human genomic DNA for determining different exons. In the case of IFNγ the sequence was also aligned with mouse and rat genomic DNA. Primers for guinea pig β-actin, IL-5, and IFNγ were designed using Primer3 software available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. The primers were selected from different exons. Table 5.1 shows the sequence of selected primers. At the time of our preliminary experiments the sequences for guinea pig GAPDH or β-actin were not available and therefore we were using mouse and rat primer sequences for GAPDH. However, before starting our major experiment the guinea pig β-actin sequence became available and we switched to it as our housekeeping gene.

5.2.2 Polymerase Chain Reaction (PCR) Optimization

Details of our PCR method are provided in Chapter 6. The concentration of primers and Mg²⁺ were determined according to finding a single band without smears on PCR product gel images. The cycles of amplification were optimized such that the numbers of amplicons were in the exponential phase (Figures 5.4 to 5.6).
5.2.3 PCR Sensitivity Measurement

After gel electrophoresis (described in Chapter 6), the PCR products were purified from the gel using QIAquick Gel Extraction Kit (Qiagen, Corp). According to the manufacturer’s instruction, the band was cut from the gel with a clean scalpel and put in a tube. After weighing, Buffer QG was added to the tube (3 μL of the Buffer for each μg of gel). The gel was dissolved by a 10 min-incubation at 50°C and isopropanol (1 μL for each μg of gel) was added to the mixture. The mixture underwent centrifugation on QIAquick column for 1 min and the column underwent washing with 0.5 mL of Buffer QG and centrifugation for 1 min. The next step was washing with 0.75 mL of Buffer PE and centrifugation for another 1 min. After an extra 1 min centrifugation the column was placed in a new tube and eluted in 50 μL of Buffer EB (10 mM Tris.Cl, pH 8.5). The optical density (OD) of the elution was measured and then the concentration of DNA was calculated by Equation 2 (Appendix). The copy equivalents were measured by means of Equation 3 (Appendix) and known amounts of copy equivalents underwent PCR and gel electrophoresis for sensitivity determination. Distilled water was used instead of copy equivalents as negative control. The sensitivity of the method was measured according to detectable bands such that detecting 10 copy equivalents would provide an acceptable sensitivity. Since 1-10 copy equivalents of our cytokines or housekeeping gene were detectable (Fig. 5.7), we considered the methods sensitive enough for our major experiments.

5.2.4 Sequencing the PCR Products

PCR products were extracted from gels (as described in Section 5.2.3) and the purified products were sent to NAPS Unit DNA Sequencing (NAPS; University of British Columbia, Vancouver, Canada). The results of sequencing are shown in table 5.2. “BLAST 2 sequences” program located in the http://www.ncbi.nlm.nih.gov/blast/bl2eq/bl2.html website was used to test the alignment of the reported sequences with the relevant guinea pig nucleotide sequences. The result of this alignment showed that our PCR products were 99-100% matched with the relevant cytokines.

5.2.5 Summary

In summary, we designed primers for measuring selected Th1 and Th2 cytokines and βactin by semi-quantitative PCR methods. Our methods were sensitive enough to detect 1-10
copy equivalents of selected Th1 and Th2 cytokines and β-actin and PCR product sequencing confirmed our amplicons being true copies of relevant cytokines.
Fig. 5.1 Natural logarithm of IFNγ/IL-5 ratios in different groups of our preliminary experiment. Group 1: No CpG-ODN, Group 2: 30 µg CpG-ODN (biweekly x 4 weeks), subcutaneous, Group 3: 100 µg CpG-ODN (biweekly x 4 weeks), subcutaneous, Group 4: 30 µg CpG-ODN (biweekly x 4 weeks), intranasal, Group 5: 100 µg CpG-ODN (biweekly x 4 weeks), intranasal. Note the significantly increased level of IFNγ/GAPDH in CpG-ODN treated groups. The *p* values were as follows: Group 1 versus Group 2: *p* < 0.04; Group 1 versus Group 3: *p* < 0.03; Group 1 versus Group 4: *p* < 0.02; Group 1 versus Group 5: *p* < 0.03; all other group comparisons: *p* > 0.05. (Group 1: n=3; Groups 2-5: n=4)
Fig. 5.2 Natural logarithm of lung IL-5/GAPDH ratios in different groups of our preliminary experiment. Group 1: No CpG-ODN, Group 2: 30 μg CpG-ODN (biweekly x 4 weeks), subcutaneous, Group 3: 100 μg CpG-ODN (biweekly x 4 weeks), subcutaneous, Group 4: 30 μg CpG-ODN (biweekly x 4 weeks), intranasal, Group 5: 100 μg CpG-ODN (biweekly x 4 weeks), intranasal. Note the trend toward higher IL-5 gene expression in the CpG-ODN pre-treated animals except for the Group 5. Group 5 had significantly lower levels of IL-5 gene expression than other CpG-ODN pre-treated groups. The p values were as follows: Group 2 versus Group 5: p < 0.001; Group 3 versus Group 5: p < 0.001; Group 4 versus Group 5: p < 0.02; All other group comparisons: p > 0.05. (Group 1: n=3; Groups 2-5: n=4)
Fig. 5.3 Natural logarithm of lung IFNγ/IL-5 ratios in different groups of our preliminary experiment. Group 1: No CpG-ODN, Group 2: 30 μg CpG-ODN (biweekly x 4 weeks), subcutaneous, Group 3: 100 μg CpG-ODN (biweekly x 4 weeks), subcutaneous, Group 4: 30 μg CpG-ODN (biweekly x 4 weeks), intranasal, Group 5: 100 μg CpG-ODN (biweekly x 4 weeks), intranasal. Note the significantly higher IFNγ/IL-5 ratio in the CpG-ODN pre-treated animals. Moreover, the ratio of IFNγ/IL-5 in Group 5 was significantly higher than Group 2. The p values are as follows: Group 1 versus Group 2: p < 0.03; Group 1 versus Group 3: p < 0.02; Group 1 versus Group 4: p < 0.02; Group 1 versus Group 5: p < 0.01; Group 2 versus Group 5: p < 0.002; All other group comparisons: p > 0.05. (Group 1: n=3; Groups 2-5: n=4)
<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequences</th>
<th>Product Size (base pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Antisense 5’ GGGGTGTGAAAGTCTCGAA 3’</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>Sense 5’ ACTGGGACGACATGGAGAAG 3’</td>
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<tr>
<td>IFNγ</td>
<td>Antisense 5’ AGGAGACGATTTGGCTCTGA 3’</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>Sense 5’ GAAGTTCTTTGGACCTGATCG 3’</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>Antisense 5’ CCAGAGCTTCCCCCTTGTA 3’</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td>Sense 5’ TGTCTGTGCTGTGCCATCC 3’</td>
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</tbody>
</table>
Fig. 5.4 Cycles of amplification for β-actin. The cDNA used in PCR reaction was 1:10 diluted. 30 cycles of amplification was chosen to obtain the amplicons in exponential phase. Lane 1 is an empty lane. PCR products loaded in lanes 2-7 were produced from the same amount of reagents and cDNA. Negative controls (distilled water instead of cDNA) were loaded in different gels (not shown). (sm = size markers)
Fig. 5.5 Cycles of amplification for IFNγ. 40 cycles of amplification was chosen for obtaining the amplicons in exponential phase. PCR products loaded in lanes 1-5 were produced from the same amount of reagents and cDNA. Negative controls (distilled water instead of cDNA) were loaded in different gels (not shown). (sm = size marker)
Fig. 5.6 Cycles of amplification for IL-5. 40 cycles of amplification was chosen for obtaining the amplicons in exponential phase. The lane between sm (size marker) and 45 cycles is empty. Negative controls (distilled water instead of cDNA) were loaded in different gels (not shown).
Fig 5.7 Sensitivity of PCR methods (copy equivalents used are shown above the bands). One to ten copy equivalents provided detectable bands after PCR and gel electrophoresis.
Table 5.2 Sequences of our PCR Products Reported by NAPS.

<table>
<thead>
<tr>
<th>Cytokine or Housekeeping gene</th>
<th>Primer used</th>
<th>Sequence reported by NAPS</th>
</tr>
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<tr>
<td><strong>IL-5</strong></td>
<td>Sense</td>
<td>GCAGCAGAGTGCACTCTGAGGCGCTGGNAANAGAGACCCCTGACCTCTGCTTT</td>
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<tr>
<td></td>
<td></td>
<td>CTACTCATCGAAGCTCTGCTTTAAGCGAAGACTCTGAGGATTTCTTGTCC</td>
</tr>
<tr>
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<td>TGACATGAAATCACCACATTTGCATAGTAGACANTCTCTCAAGGANNTGA</td>
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<td></td>
<td></td>
<td>CACANTGACNCAACTCGNCTACNNNGCTACNNNGAGGGAAGCTCNGNANNNN*</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>GNTGTCTNGTNGTNTNAATTACCTTGGGAAGATTCTTCTCNATGATANGTTGATCAGTTTTATGTCGAAACAGANATCCTCAGAGTCATCTGCTTGGAGCTAGTTCGTAGTAGATGAGTAGAAAGCATGACATGGGATCTCTTACCACAGNGCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCANAGTGGNACTCTGCTTGGNGATGGCAGACAGACANACAAANANNNNNC*</td>
</tr>
<tr>
<td><strong>IFNγ</strong></td>
<td>Sense</td>
<td>ATATCAGAGCTACTGATTTTCACCTCTCTTTAGGCAAACACTCTCTGAAGACAATGAA</td>
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<td>GTATACAAATGGTCATCTTGCAGCTCTTGATATCTATTGATTTTTTCTAG</td>
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<td></td>
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<td>CATGACATGGGACACCTTAAATGAGTGTATATACTCTATTGGAGCTAGT</td>
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<td>Antisense</td>
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<td></td>
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<td>ATGGCTGCACGAAGTTATCCTGTGAAATCTGATTCCGAGATTTGCTGAAAGAATG</td>
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<td></td>
<td></td>
<td>ATTTCCATTGTGAN*</td>
</tr>
<tr>
<td><strong>βactin</strong></td>
<td>Sense</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>AGATCATGTTGAGACTTTCTCAACACCNAAN*</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CAGACTGGGTCATCTCTCCTACGGTGGCTTGGGTTCAAGGGGAGCCCTCGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGCACAGCAGAGGCTGCTCATGAGGCCCACACGCAATTCTATTTAAGAGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGTCAGAGATTTCTCTCAGTCTCCAGTANNNNNNNNNN*</td>
</tr>
</tbody>
</table>

*The letter “N” shows the unreadable nucleotides. These reported sequences were aligned with relevant cytokine and housekeeping gene complementary DNA sequences for guinea pigs and the results were 99-100% match (not shown).
CHAPTER 6: MATERIALS AND METHODS

In this chapter the experimental design and the material and methods used in our major experiment will be described.

6.1 EXPERIMENTAL DESIGN

Four groups (n=12/group) of female juvenile, 1-month old Cam Hartley guinea pigs were studied as following:

Group 1: Uninfected, unsensitized controls
Group 2: High dose RSV infected + OA
Group 3: Low dose RSV inoculated + (High dose RSV infected + OA)
Group 4: CpG-ODN pre-treated + (High dose RSV infected + OA)

The terms "infected" and "inoculated" do not indicate different methods of RSV inoculation.

A schematic diagram showing time course of treatments is illustrated in Figure 6.1.

6.2 ANIMALS

Juvenile 1-month old, female Cam Hartley guinea pigs (body weight: 250-300g) were purchased from Charles River Laboratories (Montreal, QC) and housed under conditions of alternating 12-hour light-dark cycles, in plastic cages with hypoallergenic bedding, and with free access to food and water. Animals were acclimatized for five days before experimentation.

6.3 VIRUS PREPARATION

The Long strain of subgroup A human RSV (American Type Culture Collection; Manassas VA) was propagated on HEp-2 cell monolayers at 37°C in a humidified incubator containing 5% CO₂. Cell culture medium consisted of RPMI 1640 (Gibco, Grand Island, NY), supplemented with 5% fetal bovine serum (FBS) (Gibco), 1% L-glutamine (Gibco), and 1% Antibiotic-Antimyotic (Gibco). Working stocks of RSV for guinea pig inoculation were prepared by addition of autoclaved 3 mm diameter glass beads to infected HEp-2 cell monolayers and agitating with a vortex for 10 s. After agitation aliquots were prepared and kept in -70°C. One week before inoculation, an aliquot of frozen working stock was thawed and underwent plaque assay. On the designated day of inoculation another aliquot was thawed, centrifuged at 200 X g
for 5 min. The resulting supernatant was transferred to a sterile tube for inoculation into guinea pigs. Plaque assays were repeated on the day of inoculation to confirm the viral dose delivered to animals.

6.4 VIRUS INOCULATION

Animals were inoculated with virus preparations under light anesthesia induced by 3-5% halothane inhalation. Figure 6.2 shows the lung of a guinea pig 1 hour after inoculation with 150 μL of 1:10 diluted India ink in each nostril. The same volume and way of administration was used for the virus preparations. Low dose exposures consisted of $1 \times 10^2$ pfu of RSV, based on results of the preliminary experiment mentioned in Chapter 4.4. High dose viral exposures consisted of $4.5 \times 10^4$ pfu, an amount that reproducibly produces features of acute bronchiolitis in guinea pigs. Controls received normal saline instead of low dose RSV.

6.5 CpG-ODN ADMINISTRATION

The dose and route of CpG-ODN administration used was according to the regimen of the preliminary study described in Chapter 5. After anesthesia with 3-5% halothane, animals received 300 μL (containing 100 μg) of CpG-ODN intranasally such that 150 μL was inoculated to each nostril. The procedure was repeated biweekly for 4 weeks.

6.6 OA SENSITIZATION AND CHALLENGE

Guinea pigs were placed in polycarbonate chambers and sensitized with exposure to an aerosol of 1% OA (Sigma; St. Louis, MO) in sterile normal saline for 9 min. The aerosol was delivered by an ultrasonic nebulizer (DeVilbiss, Somerset, PA). Animals underwent sensitization 2h after inoculation with the high dose RSV. OA challenge started 7 d after the first exposure and consisted of 9 min exposure to 0.5% OA aerosols in saline and was repeated three times per week over 2 weeks. Twenty to thirty min before each challenge, animals received 40 mg.kg of body weight $^{-1}$ diphenhydramine hydrochloride (Sigma) $i.p.$ to prevent anaphylactic death.
6.7 SERUM COLLECTION

At the designated time point guinea pigs were killed by intraperitoneal injection of an overdose of barbiturate (MTC Pharmaceuticals). Heart blood was sampled and kept in serum separation tubes (SST) (Becton Dickinson, Franklin Lakes, NJ) and after 30-60 min sitting on bench at room temperature underwent centrifugation at 1000 X g for 15 min. The protocol for serum separation was obtained from Quantikine HS handbook (R&D SYSTEMS, Minneapolis, MN). Aliquots of serum were kept in −70°C.

6.8 LUNG TISSUE PROCESSING

Lungs were harvested after blood sampling. One randomly selected lobe was kept in RNAlater (Qiagen, Corp) for cytokine RT-PCR experiments and another lobe was inflated with OCT compound (Sakura Finetek, Torrance, CA), sliced and kept in formalin and embedded in paraffin. We used OCT for easier slicing of lung samples. OCT is a cryoprotective and normally is used for tissues that are going to be frozen.

6.9 MEASUREMENT OF OA-SPECIFIC IgG1 TITERS

OA-specific IgG1 was measured using ELISA method described by Dakhama et al. [75]. Each well of polypropylene 96-well microtitre plates (Corning; Corning, NY) was coated with 100 μL of 10 μg mL⁻¹ OA in carbonate/bicarbonate buffer (Sigma) and washed five times with Tween-containing phosphate-buffered saline (PBS-T; 10mM PBS, pH=7.4; with 0.05% Tween-20). Wells were blocked with 5% bovine serum (BSA; Sigma) in PBS-T, incubated for one hour at room temperature and washed five times with PBS-T. Guinea pig sera were 10-fold and then 2 fold serially diluted in PBS-T+1% BSA. From the serially diluted sera 100 μL were added to each well and incubated for 2 h at room temperature. Samples were washed five times with PBS-T before adding 200 μL of diluted (in PBS-T+1% BSA) HRP-conjugated Goat anti-Guinea Pig IgG1-heavy chain specific (Bethyl, Montgomery, TX). Plates were incubated for 2h and washed with PBS-T before adding 100 μL of O-phenylenediamine dihydrochloride (OPD) peroxidase substrate (Sigma) to each well. Half an hour after adding substrate the optical density (OD) was determined at a wavelength of 450 nm in an ELISA microtitre plate reader (Titertek, Multiskan Plus MK2; Huntsville, AL). Titers were reported as the greatest serum dilution to produce an OD at least 0.1 OD units higher than control (sera obtained from unsensitized, uninfected, naïve
animals) values. This cut off level was selected based on a lower limit of 1.5-2 times background values [75].

6.10 LUNG HISTOLOGY

Inflammation in membranous, muscular airways was evaluated by comparing the percentage of eosinophils and lymphocytes in animal groups [99].

6.10.1 Eosinophil and Lymphocyte Staining

Eosinophils and lymphocytes were stained on formalin-fixed paraffin-embedded lung sections mounted on glass slides by Hansel’s method and CD3+ immunostaining, respectively. For Hansel’s staining slides were flooded with Eosin solution for 60 s and buffer was added to slides for 5 min. After washing with distilled water, slides were counterstained in diluted Methylene blue and quickly washed in distilled water, dehydrated, cleared and mounted. For CD3+ immunostaining, after deparaffinization, slides underwent cell conditioning with EDTA based-buffer, conditioner #1 (CC1). The next step was adding 1:50 dilutions of anti-CD3 antibody (DAKO 0452, Dakopatts; Glostrop, Denmark) and then blocking the antibody and counterstaining. After this one drop of hematoxylin was added and coverslip was applied and sample was incubated for 2 min. Following a post counterstain step one drop of bluing reagent was added and after applying a coverslip the sample was incubated for another 2 minutes.

6.10.2 Image Capture and Cell Counting

Slides were coded such that the microscopist did not know from which study group a given slide was obtained. Images from 5-10 randomly selected membranous bronchioles were captured using a digital camera (Nikon, Japan). The Image Pro PLUS image analysis system (Media Cybernetic, Inc.; Silver Spring MD, USA) was used to place a grid over the microscopic field and count the positively stained points in all compartments of the airways. The ratio of these positively stained points to non-stained ones in the airway walls were reported as percentages.
6.11 CYTOKINE MESSENGER RNA EXPRESSION

6.11.1 RNA Extraction

From the lung samples kept in RNA later (Qiagen, Corp) in -20°C, RNA was extracted using RNeasy Midi Kit (Qiagen, Corp) and according to manufacturer’s instructions, as follows: 100-150g of RNA later stabilized lung tissue was placed in a 50 mL tube containing 4 mL of lysate buffer and after homogenization with Tissue-Tearor® underwent centrifugation for 10 min at 3000 X g. The supernatant was carefully transferred to a 15 mL tube by pipette and 4 mL 70% ethanol was added. After shaking the tube vigorously, the sample was transferred to RNeasy Midi column. The sample underwent spinning for 5 min at 3000 X g and the flow-through was discarded. RNase Free DNase Set (Qiagen, Corp) was applied at this stage and the column was washed one more time with RW1 solution and two times with RPE buffers both included in the kit. The RNA was then eluted in 150 μL of RNase-free water.

6.11.2 Reverse Transcription

The optical density of RNA at 260 nm was measured by spectrophotometer (Bodenseewerk Perkin-Elmer & Co.; Ueberlingen, Germany) and the RNA was quantified using Equation 1 (Appendix). Equal starting amounts of RNA (0.5 μg) underwent reverse transcription using random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Gibco) according to the manufacturer’s instruction as following: 250 ng of random hexamers and 10 mM of dNTP mix were added to 0.5 μg of RNA and distilled water was added to obtain a 12 μL solution. The mixture was heated to 65°C for 5 min and quickly chilled on ice. After a brief centrifugation 4 μL of 5X First-Strand Buffer and 2 μL of 0.1 M DDT (both prepared by the kit) and 1 μL of Ribonuclease Inhibitor (Invitrogen, Corp; Grand Island, NY), was added to the mixture. The contents were mixed gently and incubated at 25°C for 10 min and then 37°C for 2 min. The next step was adding 1 μL of M-MLV RT and gentle mixing by pipetting and then incubating at 37°C for 50 min. Finally, by heating the mixture at 70°C for 15 min the RT was inactivated. The obtained complementary DNA (cDNA) was then used as a template for amplification by PCR.

6.11.3: Polymerase Chain Reaction

Since the target messenger RNA (mRNA) was expressed at low levels, a PCR-based method of nucleic acid amplification was used. Two μL of complementary DNA (cDNA) (non-
diluted for IFNγ or IL-5, or 1:10-diluted in sterile distilled H₂O for β-actin PCR) from the reverse transcription reaction was used in a 25 μL PCR reaction mixture that consisted of 1X PCR buffer, 2.5 mM Mg²⁺, 200 μM of each dNTP, 0.5 μM each primer, 2.5 units per reaction HotStar Taq DNA Polymerase (Qiagen, Corp). Table 6.1 shows the denaturing, annealing, and extension temperature, time and cycles for amplifying β-actin, IL-5, and IFNγ cDNA on a Robocycler 96 (Stratagene; La Jolla, CA).

6.11.4: Gel Electrophoresis, Digital Image Capture and Densitometry

PCR products underwent electrophoresis on ethidium bromide-stained 2% agarose gels and were visualized under ultraviolet light. The intensity of bands was measured by densitometry using an Eagle Eye digital image capture and analysis system (Stratagene).

6.11.5: Normalization and Determining Copy Equivalents

The density of the bands obtained from different gels had to be normalized with a standard. To prepare this standard, diluted purified PCR products underwent PCR in 50 μL volumes, the maximum volume that our Robocycler 96 manufacturer recommended for each tube. Several 50 μL PCR products were mixed to provide a sufficient starting amount of standard. Five μL of this standard was run each time with samples to make normalization possible. Densities of the bands were normalized and the copy equivalents were obtained using standard curves drawn from known copy equivalents.

6.12: STATISTICAL ANALYSIS

SPSS version 11.0 statistical software was applied for our statistical analysis. One-way ANOVA was used to compare the airway T cell and eosinophil percentages as well as mean reciprocal OA-specific IgG₁ antibody titers and lung cytokine ratios among the experimental groups. In order to obtain a better approximation of a normal distribution, the mean reciprocal OA-specific IgG₁ antibody titers underwent logarithmic transformation. Any statistically significant result by ANOVA were further examined by post hoc t-tests where a $p$ value < 0.05 was considered to be statistically significant.
Fig 6.1 Schematic diagram showing time course of treatments. Group 1: uninfected, unsensitized; Group 2: high dose RSV infected OA-sensitized; Group 3: low dose RSV inoculated + high dose RSV infected OA-sensitized; Group 4: CpG-ODN pre-treated + high dose RSV infected OA-sensitized. □: intranasal saline inoculation; ◊: saline inhalation; ◊: high dose RSV infection + OA sensitization; ▲: OA-challenge; ■: low dose RSV inoculation; ◐: intranasal CpG-ODN administration; □: euthanization.
Fig. 6.2 Guinea pig whole lung (top) and sagittal sections of the lung lobes (bottom) one hour after intranasal administration of 300 µL of India ink. Note the distribution of the ink, indicative of successful delivery of inoculum to different regions of the lungs.
Table 6.1 Temperatures, Time, and Cycles during PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Activation (1 cycle)</th>
<th>Denaturing, Annealing, Extension</th>
<th>Final Extension (1 cycle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>15 min, 95°C,</td>
<td>1 min 94°C, 1 min 59°C, 1 min 72°C, (30 cycle)</td>
<td>10 min, 72°C,</td>
</tr>
<tr>
<td>IFNγ</td>
<td>15 min, 95°C,</td>
<td>1 min 94°C, 1 min 58°C, 1 min 72°C (40 cycle)</td>
<td>10 min, 72°C,</td>
</tr>
<tr>
<td>IL-5</td>
<td>15 min, 95°C,</td>
<td>1 min 94°C, 1 min 60°C, 1 min 72°C (40 cycle)</td>
<td>10 min, 72°C,</td>
</tr>
</tbody>
</table>
CHAPTER 7: RESULTS

As mentioned in Chapter 6 in our major experiments our animals were grouped as follows:

Group 1: Uninfected, unsensitized controls
Group 2: High dose RSV infected + OA
Group 3: Low dose RSV inoculated + (High dose RSV infected + OA)
Group 4: CpG-ODN pre-treated + (High dose RSV infected + OA)

During our major experiment two animals died in Group 3 during the second RSV inoculation procedure and one animal from group 2 underwent euthanasia two days after the second OA exposure because of decreased activity and poor feeding. Procedures were well tolerated by all other animals.

7.1 OA-SPECIFIC ANTIBODY TITERS

Table 7.1 shows the data obtained from serologic analysis. OA-specific IgG1 antibodies were detected in the sera of all animals who received repeated exposures of OA. Titers of OA-specific IgG1 were significantly lower in Group 4 than Group 2 (p<0.02) and Group 3 (p<0.03). Groups 2 and Group 3 showed no significant difference in circulating of OA-specific IgG1 antibody titers. No OA-specific IgG1 was detected in Group 1.

7.2 AIRWAY INFLAMMATION

Photomicrographs of airways stained for T cells and eosinophils are shown in Figures 7.1- 7.8. Tables 7.2 and 7.3 summarize the results of T cell and eosinophil percentages, respectively. Data shown in these tables are the ratios of positively stained to non-stained points by the grid applied with Image Pro PLUS software and are reported as percentages. Statistical analysis showed Group 1 had significantly lower airway T cell counts than other groups (p<0.001) and Group 4 had significantly lower airway T cell counts than Group 2 or Group 3 (p<0.001). There was no significant difference in airway T-cell counts between Groups 2 and 3 (p=0.76). Furthermore, Group 1 had significantly lower airway eosinophil counts than other groups (p<0.001) and Group 4 had significantly lower airway eosinophil counts than Group 2 and 3 (p<0.04 and p<0.001, respectively). There was no significant difference in airway eosinophil percentages between Groups 2 and 3. In summary, all the animals that received RSV
and OA had significant increases in T cell and eosinophil counts in their airway walls in comparison to uninfected unsensitized controls. Amounts of airway T cells in the Group 4 (the CpG-ODN pretreated group) were significantly lower than Groups 2 or 3. There were no significant differences in terms of T cell or eosinophil quantification between Group 2 and 3.

7.3 SEMI-QUANTITATIVE RT-PCR

Table 7.4 shows the mean and standard error for IFNγ/IL-5, IFNγ/β-actin, and IL-5/β-actin ratios. ANOVA revealed significant differences among groups for IFNγ/IL-5, IFNγ/β-actin, and IL-5/β-actin ratios (p<0.03, p<0.003, and p<0.02, respectively). The p values for post hoc t-tests of differences between pairs of groups are shown in Table 7.4. Data summarized in this Table show that, animals in Group 1 had very low baseline levels of IFNγ and IL-5 expression, with a Th1 dominant IFNγ/IL-5 ratio. Groups 2 and 3 showed similar results. The combination of high dose RSV + OA shifted the lung Th1/Th2 ratio toward Th2 and pre-treatment with low dose RSV did not affect patterns of cytokine gene expression resulting from combination of high dose RSV + OA alone. Group 4 (CpG-ODN pre-treated group) showed increased expression of IFNγ and IL-5 in response to high dose RSV + OA combination compared to Group 1, but IFNγ/IL-5 ratio was preserved in Th1 direction.
Table 7.1 Circulating Titers of OA-specific IgG₁ Antibodies in Sera of Guinea Pigs

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Log OA-IgG₁†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Uninfected, unsensitized)</td>
<td>None detected</td>
</tr>
<tr>
<td>2 (RSV + OA only)</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>3 (Low dose RSV + (RSV + OA))</td>
<td>6.0 ± 0.1*</td>
</tr>
<tr>
<td>4 (CpG-ODN + (RSV + OA))</td>
<td>5.3 ± 0.1**</td>
</tr>
</tbody>
</table>

† Data shown are mean ± SE

*Not significantly different from Group 2 (p=0.11).

** Significantly lower than Group 2 (p<0.002) and Group 3 (p<0.001).
Fig. 7.1 Photomicrograph of unsensitized, uninfected guinea pig (Group 1) bronchiole. Note the relatively low amount of T cells (brown stain) in the airway wall. Anti-CD3 immunostaining. Scale bar = 50 μm.
Fig. 7.2 Photomicrograph of RSV-infected, OA-sensitized guinea pig (Group 2) bronchiole. Note the extensive T cell infiltrates (brown stain). Anti-CD3 immunostaining. Scale bar = 50 μm.
Fig. 7.3 Photomicrograph of low dose RSV inoculated, RSV-infected, OA-sensitized guinea pig (Group 3) bronchiole. Note the extensive T cell infiltrates (brown stain). Anti-CD3 immunostaining. Scale bar = 50 μm.
Fig. 7.4 Photomicrograph of CpG-ODN pre-treated, RSV-infected, OA-sensitized guinea pig (Group 4) bronchiole. Note the T cell infiltrates, (brown stain). Anti-CD3 immunostaining. Scale bar = 50 μm.
Fig. 7.5 Photomicrograph of unsensitized, uninfected guinea pig (Group 1) bronchiole. Note the relatively low amount of eosinophils (red stain). Hansel's staining. Scale bar = 50 μm.
Fig. 7.6 Photomicrograph of RSV-infected, OA-sensitized guinea pig (Group 2) bronchiole. Note the extensive eosinophil infiltrates (red stain). Hansel’s staining. Scale bar = 50 μm.
Fig. 7.7 Photomicrograph of low dose RSV inoculated, RSV-infected, OA-sensitized guinea pig (Group 3) bronchiole. Note the extensive eosinophil infiltrates (red stain). Hansel’s staining. Scale bar = 50 μm.
Fig. 7.8 Photomicrograph of CpG-ODN pre-treated, RSV-infected, OA-sensitized guinea pig (Group 4) bronchiole. Note the eosinophil infiltrates (red stain). Hansel’s staining. Scale bar = 50 µm.
Table 7.2 Results of Lung Histology for Airway T Cells

<table>
<thead>
<tr>
<th>GROUP</th>
<th>T cell percentage†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Uninfected, unsensitized)</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>2 (RSV + OA only)</td>
<td>8.6 ± 0.6</td>
</tr>
<tr>
<td>3 (Low dose RSV + (RSV + OA))</td>
<td>8.3 ± 0.8</td>
</tr>
<tr>
<td>4 (CpG-ODN + (RSV + OA))</td>
<td>4.8 ± 0.4**</td>
</tr>
</tbody>
</table>

† Data shown are mean ± SE of the percentage of T cells (the ratio of positively stained points to non-stained ones) in the airway wall.

* Significantly lower than Groups 2-4 ($p<0.001$)

** Significantly lower than Group 2 or 3 ($p<0.001$)
Table 7.3 Results of Lung Histology for Airway Eosinophils

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Eosinophil percentage†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Uninfected, unsensitized)</td>
<td>3.6 ± 0.3*</td>
</tr>
<tr>
<td>2 (RSV + OA only)</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>3 (Low dose RSV + (RSV + OA))</td>
<td>10.4 ± 0.6</td>
</tr>
<tr>
<td>4 (CpG-ODN + (RSV + OA))</td>
<td>7.5 ± 0.5**</td>
</tr>
</tbody>
</table>

† Data shown are mean ± SE of the percentage of eosinophils (the ratio of positively stained points to non-stained ones) in airway wall.

* Significantly lower than Groups 2-4 (p<0.001)

** Significantly lower than Group 2 (p=0.037) or 3 (p<0.001)
### Table 7.4 Results of Lung Cytokine Gene Expression

<table>
<thead>
<tr>
<th>GROUP</th>
<th>IFNγ/IL-5 †</th>
<th>IFNγ/β-actin †</th>
<th>IL-5/β-actin †</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Uninfected, unsensitized)</td>
<td>9.8 ± 3.5</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>2 (RSV + OA only)</td>
<td>1.4 ± 1.0*</td>
<td>1.7 ± 0.9</td>
<td>3.2 ± 1.2 †††</td>
</tr>
<tr>
<td>3 (Low dose RSV + (RSV + OA))</td>
<td>1.0 ± 0.6**</td>
<td>4.6 ± 2.7</td>
<td>5.2 ± 1.0 †</td>
</tr>
<tr>
<td>4 (CpG-ODN + (RSV + OA))</td>
<td>8.8 ± 3.2</td>
<td>23.9 ± 8.5***</td>
<td>3.2 ± 0.9 ††</td>
</tr>
</tbody>
</table>

† Data shown are mean ± SE

* Significantly different from Group 1 (p<0.04) and Group 4 (p<0.05).

** Significantly different from Group 1 (p<0.03) and Group 4 (p<0.04).

*** Significantly different from Group 1 (p<0.02), Group 2 (p<0.03), and Group 3 (p<0.05).

† Significantly different from Group 1 (p<0.02)

†† Significantly different than Group 1 (p<0.03)

††† In comparison to Group 1, trend toward higher IL-5 observed (p<0.06).
CHAPTER 8: THE EFFECT OF CpG-ODN ON OA-SENSITIZED ANIMALS

The results of our major experiment suggest that CpG-ODN pre-treatment confers some benefit to the effects of combination of RSV infection plus repeated OA exposures. In murine models of allergen sensitization, some investigators have shown the effectiveness of administration of CpG-ODN alone (without antigen) on subsequent antigen exposures [40,41], while others have reported that CpG-ODNs are only effective when they are administered with the antigen [39,100]. The effects of CpG-ODN pre-treatment (using the regimen described in Chapter 5) on uninfected, OA-sensitized guinea pigs were not known. In order to explore this issue in guinea pigs, we performed the following experiment:

8.1 EXPERIMENTAL DESIGN

Three groups (n=4/group) of female juvenile, 1-month old Cam Hartley guinea pigs were studied as follows:

Group 1: Sham controls that received saline
Group 2: OA sensitized and challenged
Group 3: CpG-ODN pretreated and OA sensitized and challenged

8.2 SPECIMENS

Animals were killed 3 days after last OA or saline exposure by overdose of intraperitoneal sodium pentobarbital. Specimens for lung cytokines, serology, and histology were prepared as previously described in Chapter 6. IFNy, IL-5, and β-actin gene expression were measured using the same semi-quantitative RT-PCR method we used before. Levels of OA-specific IgG1 antibody were determined using our indirect ELISA method and the percentages of airway eosinophils were also measured as before.

8.3 DATA ANALYSIS

After normalization of IFNy and IL-5 OD to corresponding β-actin OD, the ratio of IFNy/IL-5 was calculated for each animal. Copy equivalents of cytokines and β-actin were
determined using normalized values and ratios and standard curves. Possible differences in mean levels of IFNγ/β-actin, IL-5/β-actin, and IFNγ/IL-5, OA-specific IgG1 antibody titer, and percentages of airway eosinophils among the 3 groups of animals were examined by one-way ANOVA (SPSS Statistical Software, v.11) and post hoc Student's t-tests were used to examine the differences between pairs of experimental groups. P value < 0.05 was set as level of statistical significance.

8.4 RESULTS

OA-specific IgG1 titers were undetectable in Group 1 and were significantly lower than the other two groups of animals (p<0.001) (table 8.1). The mean percentage of eosinophils was significantly lower in Group 1 than the other groups (p<0.001) (table 8.2). As shown in Table 8.1 and 8.2, there were no significant differences between Groups 2 and 3 in terms of OA-specific IgG1 antibody titer or eosinophil percentage. Levels of cytokine gene expressions were not significantly different among any of the three groups (table 8.3).

8.5 CONCLUSION

OA repeated exposures increased the titer of OA-specific IgG1 antibody and airway eosinophil percentage. CpG-ODN pre-treatment did not make any difference in these values after subsequent OA exposures. Lung cytokine gene expression also remained the same in all three groups.
Table 8.1 Circulating Titers of OA-specific IgG₁ Antibodies in Sera of Guinea Pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>Log OA-specific IgG₁ titers (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham controls</td>
<td>None detected</td>
</tr>
<tr>
<td>2. OA sensitized and challenged</td>
<td>5.7 ± 0.1</td>
</tr>
<tr>
<td>3. CpG-ODN + OA sensitized and challenged</td>
<td>5.9 ± 0.1</td>
</tr>
</tbody>
</table>
Table 8.2 Results of lung Histology for Airway Eosinophils

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham controls</td>
<td>1.8 ± 0.3*</td>
</tr>
<tr>
<td>2. OA sensitized and challenged</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>3. CpG-ODN + OA sensitized and challenged</td>
<td>5.2 ± 0.8</td>
</tr>
</tbody>
</table>

* Significantly different from Groups 2 or 3 (p<0.001)
### Table 8.3 Lung Cytokine Gene Expression Results

<table>
<thead>
<tr>
<th>Group</th>
<th>IFNγ/β-actin (mean ± SE)*</th>
<th>IL-5/β-actin (mean ± SE)*</th>
<th>IFNγ/IL-5 (mean ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham controls</td>
<td>0.3 ± 0.3</td>
<td>1.1 ± 1.0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>2. OA sensitized and challenged</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>10 ± 8</td>
</tr>
<tr>
<td>3. CpG-ODN + OA sensitized and challenged</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>9 ± 9</td>
</tr>
</tbody>
</table>

* p values NS
CHAPTER 9: DISCUSSION

The interaction between RSV infection and OA allergic sensitization has been studied in guinea pigs by Dakhama et al. [75], who showed that RSV infection at the time of first OA exposure was associated with increased circulating titers of OA-specific IgG1 antibodies. The purpose of our study was to determine whether interventions such as low dose RSV inoculation or CpG-ODN administration inhibits the development of subsequent RSV-enhanced allergic sensitization of guinea pigs to aerosolized OA in terms of development of allergic airway inflammation and production of OA-specific IgG1 antibodies. In this chapter we will discuss the results of our study for each intervention separately.

9.1 THE LOW DOSE RSV INTERVENTION

The idea that low dose of RSV can result in protection against RSV-enhanced allergic sensitization to OA was inspired by advances in tuberculosis [34] and Leishmaniasis [35] research, which showed that host Th1/Th2 balance is influenced by the first infectious dose to which the host is exposed and this first exposure to infection determines the response to subsequent exposures. In these studies exposures to comparatively low doses of mycobacterium or parasite showed similar responses, in which there was stimulation of a long lasting, protective Th1 response, whereas high infectious doses of these pathogens stimulated a mixed Th1+Th2 response. These results suggest the indicating possibility to design a generalized, effective intervention strategy for avoiding initial Th2 responses to pathogens.

On the other hand, in spite of the association between acute bronchiolitis and recurrent wheezing asthma and allergy in children [18-21], some investigators have postulated a protective role for early life viral infections in the development of allergy and asthma [77-79]. Part of the rationale in viruses being protective is based on the understanding that a Th1 response typically develops during viral infections, which can provide a milieu against allergy and asthma [79]. Overall, there is a considerable controversy with regard to the potential role of viral respiratory tract infections, including acute RSV bronchiolitis, in triggering allergic sensitization and airway inflammation in asthma. We postulated that the controversy in the role of RSV in the onset of asthma and allergy could be explained by differences in the first amount of infectious virus to which children are exposed. We proposed low dose viral exposures could have a protective role in post-bronchiolitis allergic sensitization.
Using a guinea pig model, we examined the effects of low dose RSV exposures on subsequent RSV-enhanced OA-sensitization. Allergic inflammation of the airways and production of allergen-specific IgG_{1} antibodies were studied as determinants of post-bronchiolitis allergic sensitization. In a study by Hamid et al. [99] the investigators reported that airways from patients with asthma demonstrated significant increases in the numbers of T cells and eosinophils compared with airways from nonasthmatic subjects. Accordingly, among all types of potential inflammatory cells to study, we focused on eosinophils and T cells. We also studied only the membranous airways because in the same study by Hamid et al. [99], they showed that there was a similar but more severe inflammatory process present in the peripheral compared with the central airways of patients with asthma. Moreover, RSV typically affects peripheral airways [44].

A 4-week interval between low dose RSV intervention and RSV infection + first OA exposure was chosen to let the guinea pigs recover from the first inoculation procedure [90].

In agreement with the study by Dakhama et al. [75] our results showed that high dose RSV infection plus OA repeated exposures in the high dose RSV infected + OA group (Group 2), led to production of OA-specific IgG_{1} antibody, a Th2-mediated response [101]. The animals in Group 2 also had significantly higher amount of airway eosinophils than uninfected-unsensitized controls. Increased numbers of eosinophils in guinea pig airways after RSV infection [97], OA sensitization [93], and combination of RSV infection and OA sensitization [75] have been reported before. In addition, animals in Group 2 had significantly increased numbers of T-cells in their airways, which has been reported in other strains of guinea pigs after experimental RSV infection [97].

Animals in the low dose RSV inoculated + (RSV+OA) group (Group 3) did not have any significant difference with Group 2 in terms of mean circulating titers of OA-specific IgG_{1} antibodies or airway inflammatory cells, indicating that the intervention of low dose RSV exposure did not protect guinea pigs against subsequent RSV-enhanced OA sensitization.

To characterize this further, we measured the lung cytokines by semi-quantitative RT-PCR and the results showed that in Group 2 the IFNγ/IL-5 ratio was significantly lower than sham controls (Group 1), along with trends in increased IL-5 gene expression (p = 0.06). Group 3 showed significantly higher IL-5 expression and lower IFNγ/IL-5 ratio than Group 1 and the expression of IFNγ was not significantly different among Groups 1, 2, and 3. Furthermore, Group 2 and Group 3 showed no difference in levels of IL-5 expression and IFNγ/IL-5 ratio. Taken together, these results indicate that Group 2 and Group 3 are similar in terms of shift of
the lung Th1/Th2 balance towards Th2, such that low dose RSV does not affect patterns of cytokine gene expression resulting from a combination of high dose RSV and repeated OA exposures. Our finding that RSV infection was not associated with increased IFNγ expression indicates that RSV appears to be an exception to the general rule that virus infections stimulate Th1 responses, which is consistent with the idea of some investigators [102] but in contrast to that of the others [103,104]. Moreover, in a recent study by Bramley et al. [97], RSV infection in strain 2 and strain 13 guinea pigs increased the lung IL-5 expression but did not have any effect on lung IFNγ expression, consistent with our results in Cam Hartley guinea pigs. Finally, a combination of RSV infection and repeated OA exposures might have been responsible in the inability of RSV to enhance the IFNγ expression. Increased IL-5 expression in Group 3 and a trend toward higher IL-5 gene expression Group 2 could be because of the effect of RSV [97], or OA (as an allergen generates Th2 responses and results in pulmonary inflammation [105]), or the combination of both.

In summary OA-specific IgG1 antibody production and airway inflammation by eosinophils and T cells in association with a Th2 prominent cytokine milieu, were produced by the combination of RSV infection and repeated OA exposures in guinea pigs. We demonstrate here that low dose RSV inoculation 4 weeks before high dose RSV infection plus repeated OA exposures does not affect patterns of cytokine gene expressions, OA-specific IgG1 titers, or airway eosinophil and T cell percentage.

9.2 THE CpG-ODN INTERVENTION

The rationale for applying the CpG-ODN intervention for primary prevention of virus-enhanced allergic sensitization is based on several lines of evidence. Epidemiological studies suggest that mycobacterial infections are protective against later development of allergic conditions in later childhood [106]. The decline in incidence of childhood bacterial and mycobacterial infections in western countries is in parallel with the increasing incidence of asthma [81], suggesting that these infections possibly protect children from allergy and asthma by inducing Th1 responses. Recent advances in animal experiments using bacterial components, including CpG-ODNs, suggest that developing novel vaccines for protecting humans from allergic disorders is possible [107]. CpG-ODN can act as a “danger signal” and activate innate immune defenses [108]. The first evidence that CpG-ODN administration confers resistance to infection was reported by Krieg et al. [109]. These investigators showed that single dose pre-
treatment with CpG-ODN provides resistance to *L. monocytogenes* and the resistance developed within 48 h and persisted for at least 2 weeks. Elkins *et al.* [110] also reported that prior administration of CpG-ODN protected mice against *Francisella tularensis*. CpG-ODN also conferred protection against malaria [111] and *Leishmania major* [112]. There are also reports showing protective effects of CpG-ODN against some viral infections including herpes simplex virus type 1 (HSV-1) [113], HSV-2 [114].

Gramzinski *et al.* [111] reported that stimulation of the innate immune system by CpG immunostimulatory motifs can confer protection against malaria but that the protection is short lived (less than 7 days). Hence, they proposed repeated dosing for CpG-ODN could offer a long lasting protection. Klinman *et al.* [115] examined this and reported repeated administration of CpG-ODN 2 to 4 times/month before infection conferred a long time protection (at least 4 months) to *L. monocytogenes* and *F. tularensis*.

For different studies CpG-ODN has been delivered via systemic and mucosal routes [116]. In order to determine how route and dose of administration affects pulmonary Th1/Th2 balance in guinea pigs we performed a preliminary study (Chapter 5). Our dosing regimens for CpG-ODN administration were 30 μg and 100 μg, which are reasonably near to the doses applied for mice [36,41,115]. The dosing was repeated biweekly over 4 weeks to provide a long-term protection [115]. Subcutaneous and intranasal routes of administration were considered to compare a systemic route with a mucosal one. Guinea pigs tolerated the CpG-ODN administration very well and no sign of illness, poor feeding or weight loss was observed during the whole procedure. This is consistent with CpG-ODN being well tolerated, with toxicity observed in special conditions such as administration of repeated large doses, or the combination of CpG-ODN with lipopolysaccharide (LPS) [117]. Toxicity has resulted in wasting syndrome and death of mice [117]. In addition sepsis syndrome and pulmonary inflammation has been reported after exposure to large doses of DNA containing CpG motifs [109].

Animals who did not receive any CpG-ODN (Group 1) had significantly lower IFNγ gene expression than animals given CpG-ODN (Groups 2-5). There was a trend toward higher IL-5 gene expression in the guinea pig lungs after CpG-ODN treatment except for 100 μg intranasal group (Group 5), which had significantly lower levels of IL-5 gene expression than other CpG-ODN-treated groups (Groups 2-4), suggesting routes and doses of CpG-ODN administration are important in shifting the immune response toward Th1, Th2 or mixed Th1/Th2 responses. In agreement with our results in Group 5, in murine models of asthma, CpG-ODN administration increased lung lavage fluid levels of IL-12 and IFNγ and prevented the allergen-induced increase
in IL-4 levels, therefore up-regulated Th1 and down-regulated Th2 responses [39]. In addition, consistent with our results in Groups 2, 3 and 4, McCluskie et al. [118] reported the increase in both Th1 and Th2 responses after oral administration of CpG-ODN to BALB/c mice. These investigators found that oral administration of CpG-ODN along with hepatitis B surface antigen (HBs Ag) or tetanus toxoid (TT) increased IgG2a antibodies (Th1 responses) as well as IgG1 and IgA antibodies (Th2 responses).

All CpG-ODN pre-treated groups showed significant Th1 shift in pulmonary Th1/Th2 balance in comparison to untreated controls. The extent of Th1 shift was influenced by dose and route of administration of CpG-ODN; in that 100 μg intranasal group (Group 5) had significantly more Th1 shift in Th1/Th2 balance than the 30 μg subcutaneous group (Group 2). As the goal of CpG-ODN treatment of guinea pigs was to maximize increase in Th1 and minimize any possible increase in Th2 in the lungs, for each animal we used 100 μg CpG-ODN, delivered intranasally, biweekly over 4 weeks.

To examine the effect of CpG-ODN pre-treatment on subsequent RSV infection plus repeated OA exposures, we used a guinea pig model of RSV and OA sensitization. The results showed that in the CpG-ODN pre-treated + (High dose RSV infected + OA) group (Group 4) the level of IFNγ and IL-5 gene expressions were significantly higher than those of the sham control group (Group 1), but the IFNγ/IL-5 ratios were shifted in the Th1 direction. Since CpG-ODN is supposed to induce the Th1 response [36] the increase in the IFNγ gene expression level was expected. The higher level of IL-5 gene expression could be the result of RSV infection, OA exposures or both.

As mentioned earlier, in the high dose RSV infected + OA group (Group 2), the combination of high dose RSV infection and repeated OA exposures shifted the Th1/Th2 balance towards Th2, with the IFNγ gene expression remaining unchanged but there was a trend toward higher IL-5 gene expression (p=0.06). When we compared the Group 4 with Group 2 IFNγ/IL-5 ratio and the IFNγ gene expression were significantly higher in the CpG-ODN pre-treated animals. The levels of IL-5 gene expression in Groups 2 and 4 were higher than Group 1, but Groups 2 and 4 did not have any significant difference in IL-5 gene expression level. Hence, CpG-ODN induced the Th1 response but could not suppress the Th2 response resulting from the combination of RSV infection plus OA exposures.

We next evaluated the effect of CpG-ODN on the development of airway inflammation in our model. In the CpG-ODN pre-treated animals levels of eosinophils and T cells were
significantly higher than sham controls and lower than the ones in Group 2, suggesting CpG-ODN pre-treatment confers a partial protection against the allergic inflammation of airways resulting from combination of RSV infection and OA exposures. Increases in airway T cells and eosinophils have been shown in RSV infected guinea pigs [97]. Moreover, OA as an allergen can result in T cell and eosinophil infiltration to the lungs [31]. Hence, increased airway inflammation by T cells and eosinophils after combination of RSV infection and OA exposure was not surprising.

It is possible that cytokines induced by CpG-ODN can disrupt viral replication and activate natural killer cells thereby lyse infected cells and limit the viral replication and disease [113]. This effect of CpG-ODN could be a reason that our CpG-ODN pre-treated group had a lower T cell inflammation than Group 2. The effect of CpG-ODN on allergen sensitization will be discussed in Chapter 9.3.

The shift of pulmonary Th1/Th2 balance toward Th2 after RSV infection plus OA exposures, suggests that many of infiltrating T cells in the airways of the animals were from the Th2 subset. The type of Th cells could be determined by in situ hybridization technique [105], in which target mRNA sequences are localized to individual cells in lung specimens.

We next examined serum titers of OA-specific IgG1 antibody, a marker for allergic sensitization. As expected, we could not detect serum OA-specific IgG1 antibody in the sham controls. The titer of serum OA-specific IgG1 antibody was significantly higher in Group 2 than the CpG-ODN pre-treated one. In murine models, it is well established that some cytokines can regulate the expression of immunoglobulin isotypes [119]. Initial activation of naive B-cells by antigens and T cells results in immunoglobulin class switching [31]. The Th cell cytokines determine the direction of this class switching. The Th2 cytokines such as IL-4 and also IL-13 are responsible for switching to IgG1 in mice, while IFNγ secreted by Th1 cells inhibits this [119]. In our animals we did not measure the level of IL-4 and IL-13 gene expression because the nucleotide sequences of these cytokines for guinea pigs were not available. The level of IL-5 gene expression (a Th2 cytokine) in the CpG-ODN-pre-treated animals did not have any difference with the RSV infected and OA exposed animals thereby, could not explain the lower titer of OA-specific IgG1 antibody in Group 4. However, the higher level of IFNγ gene expression in the CpG-ODN pretreated animals could be a reason for lower IgG1 antibody titers in Group 4, suggesting that in IgG1 antibody production, the Th1/Th2 balance is more important than the level of each Th class cytokine by itself.
In summary, we have demonstrated that CpG-ODN administration 4 weeks before high dose RSV infection plus repeated OA exposures confers partial protection against allergic sensitization and can affect patterns of cytokine gene expressions, OA-specific IgG1 titers, and number of airway eosinophils and T cells.

9.3 EFFECTS OF CpG-ODN PRE-TREATMENT REGIMEN ON OA-SENSITIZED GUINEA PIGS

To determine whether our CpG-ODN pre-treatment regimen had any effect on OA sensitization (without RSV infection) we performed another study, which is explained in more detailed in Chapter 8. Briefly, CpG-ODN pre-treated OA-sensitized animals were compared with OA-sensitized ones that did not receive CpG-ODN as well as sham controls. We measured the OA-specific IgG1 antibody titer as well as airway eosinophils. As we expected OA-sensitization resulted in antibody production and higher airway eosinophil counts in all OA-sensitized animals. However, there was no significant difference between the CpG-ODN pre-treated and non-CpG-ODN pre-treated OA-sensitized groups in terms of antibody titers or airway eosinophil counts. The phosphorothioate backbone of CpG-ODN 2007 (the CpG-ODN that we used) [120], provides more resistance to nucleases than phosphodiester CpG-ODNs [117] thereby, rapid degradation of CpG-ODN in our experiment is less likely. Animal studies have shown that i.p. or i.n. administration of CpG-ODN alone (given without antigen) led to suppression allergen induced asthma in mice [40,41]. However, the protocols used for CpG-ODN administration were different than ours such that other investigators administered the CpG-ODN before antigen challenges to presensitized animals. In contrast to these investigators, Kline et al. [39] reported that administration of CpG-ODN alone did not result in any significant protection against development of allergy and asthma. They administered CpG-ODN along with the allergen at the time of sensitization and observed the protective effects of CpG-ODN against asthma. Shirota et al. [100] also observed the inhibitory effects of CpG-ODN on airway hyperresponsiveness and eosinophilia of presensitized animals only when it was given in combination with allergen, suggesting allergen-specific immune responses were responsible for the inhibition. This interpretation was supported by results of another study, in which CpG-ODN combined with OA protected against the development of airway eosinophilia of mice, but that protection against OA sensitization did not protect the animals against schistosome sensitization [39]. Moreover, Tighe et al. [121] showed that conjugation of CpG-ODN to antigen intensifies the immunostimulatory
effects of CpG-ODN. In CpG-ODN conjugates, antigen is co-incorporated with the CpG-ODN during uptake by APCs, which results in high IL-12 expression and development of Th1 cells [122].

Taken together, several reasons could be proposed for our finding that CpG-ODN had no significant inhibitory effect on production of OA-specific IgG1 antibody titers or airway eosinophilia in guinea pigs. First, we gave CpG-ODN to unsensitized naïve animals and we stopped the administration one week before sensitization, therefore the timing of CpG-ODN administration was different from that of other studies. Secondly, CpG-ODN was administered alone and consistent with some of the aforementioned studies, CpG-ODN alone cannot offer a protective effect against antigens. It is possible that in the context of allergic sensitization, CpG-ODN acts as an immune enhancer only when it is combined with an allergen or at least it is given after the allergen sensitization. In this context, CpG-ODN has been used as an adjuvant for the RSV F protein adsorbed to aluminum hydroxide (F/AIOH), as a subunit candidate vaccine against RSV [123]. Compared with administration of F/AIOH alone, coadministration of CpG-ODN with F/AIOH resulted in significantly higher Th1 responses as well as significantly lower Th2 responses. By contrast in this thesis, the CpG-ODN was administered alone before OA-sensitization to determine if we could protect our animals in an antigen-independent way, thereby modeling the “real world” situation, in which there is an abundance of potential allergens (e.g., house dust mite, ragweed, animal dander, etc.) that might be deleterious to the patient. Moreover, our goal was to bring a protection against post-bronchiolitis allergy and asthma and as mentioned earlier, in naïve animals CpG-ODN alone has conferred protection against bacterial and viral infections.

In our experiment on effects of CpG-ODN on OA-sensitization alone (Chapter 8), we also measured Th1 and Th2 cytokine gene expressions. The results showed that there was no difference among the CpG-ODN pre-treated OA-sensitized group, animals that only received OA-sensitization, and sham controls. The result that CpG-ODN pre-treatment did not increase the IFNγ gene expression was consistent with the concomitant findings that CpG-ODN did not significantly affect airway eosinophilia or OA-specific IgG1 antibody titers. However, the finding of no significant differences in IL-5 gene expression between the OA-sensitized animals and sham controls was unexpected. To our knowledge, there is no other study on lung cytokines of OA-sensitized guinea pigs that we could compare with the present study. It is possible that the baseline levels of IL-5 gene expression in our guinea pigs were too high to let the OA-sensitization make a recognizable enhancement in its level. Furthermore, since we have used
total lung homogenates for our RT-PCR method, the amount of paranchymal IL-5 gene expression might have overwhelmed any significantly different airway IL-5 gene expression. It is also possible that in guinea pigs the effect of eotaxin on airway eosinophilia is greater than that of IL-5 [97]. Furthermore, the level of bioactive IL-5 protein in the guinea pig lung might be different from the level of its mRNA. Finally, the small sample size (n=4/group) and fairly large variances within the experimental groups might have increased the possibility of making type II error (i.e., accepting the null hypothesis when it is false) [124].

9.4 SUMMARY AND CONCLUSION

In summary, low dose RSV inoculation before RSV-enhanced OA-sensitization did not confer protection against manifestations of allergy and asthma in a guinea pig model. By contrast, CpG-ODN administration before RSV-enhanced OA-sensitization provided partial protection against production of OA-specific IgG1 antibodies, and airway inflammation by T cells and eosinophils. CpG-ODN also induced a predominantly Th1 pattern of immune activation in the guinea pigs. Investigating the effects of CpG-ODN on uninfected OA-sensitized animals revealed that our method of pre-treatment with CpG-ODN did not have any significant effect on ameliorating subsequent allergen-sensitization, suggesting CpG-ODN protected the animals against RSV only or the combination of both RSV infection and OA exposures. Alteration of protocols for CpG-ODN administration, such as combining antigen with CpG-ODN, may provide better protection against RSV-enhanced OA-sensitization. This work can be extended to other viruses and ultimately new approaches can be developed for the primary prevention of post-bronchiolitis asthma and allergy in children.
32. von Hertzen LC, Haathela T. Pro: Could the risk of asthma and atopy be reduced by a vaccine that induces a strong T-helper type response? Am J Respir Cell Mol Biol 2000;22:139-42.


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123. Hancock GE, Heers KM, Smith JD, Scheuer CA, Ibrahimbiov AR, Pryharski KS. CpG containing oligodeoxynucleotides are potent adjuvant for parenteral vaccination with the fusion (F) protein of respiratory syncytial virus (RSV). Vaccine 2001;19:4874–82.


Appendix

Equation 1 [125]:

\[
\text{RNA Concentration (\(\mu g / mL\))} = (\text{OD}_{260}) \times \text{Dilution Factor} \times \frac{40 \mu g \text{ DNA/mL}}{1 \text{ OD}_{260}\text{unit}}
\]

Equation 2 [125]:

\[
\text{DNA Concentration (\(\mu g / mL\))} = (\text{OD}_{260}) \times \text{Dilution Factor} \times \frac{50 \mu g \text{ DNA/mL}}{1 \text{ OD}_{260}\text{unit}}
\]

Equation 3 (Quanti Tect SYBR Green PCR Handbook, Qiagen, Corp):

\[
\left( \frac{X \text{ g/\(\mu L\) DNA}}{\text{[PCR Product length in base pairs} \times 660]} \right) \times 6.023 \times 10^{23} = Y \text{ molecules/\(\mu L\)}
\]