MANAGEMENT OF PULMONARY INFECTION

EXPERIMENTAL STUDIES ON ORAL IMMUNIZATION AND SURFACTANT REPLACEMENT THERAPY

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MANAGEMENT VAN LONGINFECTIE

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PREFACE

We are obliged to live in an infected world, along with hordes of pathogenic protozoa, bacteria, and viruses and their phages. Occasionally, when pathogen and host come together infection results. Infections of the respiratory tract are probably the commonest of all infections in otherwise healthy people. Symptoms ranging from mild to severe and serious illness may result from infection of the respiratory tract by a variety of viral and/or bacterial pathogens. Pulmonary infection may cause severe primary or secondary pneumonia with allied conditions and may eventually lead to severely impaired pulmonary function with life threatening hypoxemia. Furthermore, respiratory infection may cause exacerbations of preexisting disease, especially in patients with preexisting respiratory disease, e.g. chronic bronchitis or asthma.
Specific antibiotic treatment of pneumonia is usually guided by knowledge of the likely infecting organism. Apart from specific antibiotic treatment or when antibiotic treatment is not effective, e.g. in case of non-influenzal viral pneumonia or due to resistance of the microbial agent, therapy is entirely supportive and often requires artificial ventilation.

This thesis aims at evaluating two "new" concepts for the management of pneumonia. The first concept, *oral immunization*, seems a promising approach towards prevention of pneumonia. Extensive programs have been developed for active immunoprophylaxis to immunize against specific microbial pathogens, and vaccines introduced into clinical practice since the 1970s include those against H. influenzae, meningococs, pneumococs, pseudomonas, hepatitis B virus and rabies. However, controversies surrounding established vaccines and the search for vaccines against other pathogens constantly stimulate investigations on the development of new vaccines.
Therefore, and because oral administration of vaccines is convenient, much attention has been paid to the development of vaccines for oral administration. It is proven that oral administration of vaccine of viral or bacterial origin can induce a mucosal immune response, but the possibility of oral immunization with bacterial lysate for the prevention of viral pneumonia has not been well documented. The first part of this thesis presents

investigations on oral immunization with a new polyvalent bacterial lysate against both bacterial and viral pathogens that may cause pneumonia.

The second concept, *surfactant replacement therapy*, has already proven its value in the prevention and treatment of idiopathic respiratory distress syndrome (IRDS) of the newborn, which is caused by a surfactant deficient state of the premature lungs. Several studies on the origin and nature of the adult respiratory distress syndrome (ARDS) have demonstrated a surfactant deficient state of the lungs of ARDS-patients and first clinical trials of surfactant replacement in ARDS patients have proven the beneficial effects of surfactant replacement. Pneumonia may lead to a clinical syndrome closely resembling ARDS. Therefore, the second part of this thesis presents investigations on surfactant replacement therapy during ARDS-like viral pneumonia.

One

Overview of the study

CHAPTER I

OVERVIEW OF THE STUDY

PART I. ORAL IMMUNIZATION

Chapter 2

Oral immunization may prove a convenient and effective way for the induction of protection against infections. Oral administration of vaccines of viral or bacterial origin - also referred to as oral immunomodulators - to immunize against infectious disease is not widely utilized in the Netherlands. To my knowledge the only oral vaccine currently in use in the Netherlands is the oral polio-vaccine. The so-called "common mucosal immune system" plays an important role in oral immunization. Therefore, to introduce the concept of oral immunization and the mechanisms involved this chapter gives a general survey of the "common mucosal immune system" and presents studies on oral administration of vaccine.

Chapter 3

In this chapter, a new polyvalent bacterial lysate (Paspat oral®) for use as an oral immunomodulator is presented. The potential efficacy of oral immunization with this lysate was studied in a model of intranasal S. pneumoniae infection in mice.

Chapter 4

In the previous chapter a significant protective effect of oral immunization with Paspat oral® against S. pneumoniae infection in mice was reported. Thereafter, the pulmonary response to oral immunization with Paspat oral® was investigated by means of bronchoalveolar lavage (BAL). Inflammatory changes in BAL fluid were measured during oral immunization, before and after infection with S. pneumoniae. Details of the study are presented in this chapter.

Chapter 5

In chapter 4 a potential stimulation of nonspecific immune mechanisms by oral immunization was reported. To further investigate the potency of this stimulation a study was designed in which mice were orally immunized with Paspat oral® and subsequently infected with influenza A virus aerosol. Furthermore, the role of T-helper lymphocytes and alveolar macrophages was investigated.

PART II. SURFACTANT REPLACEMENT THERAPY

Chapter 6

A short introduction to pulmonary surfactant is given. Surfactant replacement therapy has found its application in the prevention and treatment of the idiopathic respiratory distress syndrome (IRDS) in newborns and is currently being investigated for treatment of the adult respiratory distress syndrome (ARDS).

Chapter 7

Several experimental models have been developed to study the etiology and treatment of ARDS. The use of surfactant replacement therapy has been evaluated to a certain limit in experimental ARDS-models. Inevitably, variations exist concerning the techniques for induction of pulmonary damage and concerning development of the characteristic features of ARDS in these models. In this chapter a new model of acute respiratory failure, in rats, induced by infection with Sendai virus aerosol, - closely resembling ARDS - is presented.

Chapter 8

In the previous chapter a new ARDS model in rats was presented. In this chapter the influence of different ventilator settings and surfactant replacement therapy on arterial blood gases was measured in the endstage of the infection.

Chapter 9

In chapter 8 significant beneficial effects of surfactant replacement therapy during endstage respiratory failure during Sendai virus pneumonia were reported. It is conceivable that improved gas exchange following surfactant replacement therapy is a result of improved pulmonary mechanics. To adequately measure the effects of surfactant replacement therapy on pulmonary mechanics it is essential to measure functional residual capacity (FRC) and pulmonary compliance. In this chapter the influence of influenza A virus infection on pulmonary mechanics is described by measuring FRC and thorax-lung compliance in mice. Furthermore, the effects of surfactant replacement therapy on pulmonary mechanics was measured in the endstage of the disease. Basically, the same experimental model was used as described in chapter 5.

Two

Induction of a mucosal immune response by oral immunization. An overview.

GJ van Daal, B Lachmann

Z Erkr Atmungsorgane (in press)

CHAPTER II

INDUCTION OF A MUCOSAL IMMUNE RESPONSE BY ORAL IMMUNIZATION. AN OVERVIEW

Mucosal surfaces, which represent a vast area of contact with the environment, are the most frequent portals of entry of viral, bacterial and parasitic disease and other environmental antigenic substances. Via this route, antigens of diverse microbial and food origin continuously stimulate the entire immune system. Specific humoral as well as cell-mediated immune responses are continuously induced, not only at the site of stimulation but also in draining lymph nodes, spleen and bone marrow. The primary function of the immunoglobulins, produced in the mucosal tissues, is to prevent the entry of environmental pathogens into the internal milieu. The major mucosal immunoglobulin, secretory IgA (sIgA) has been shown to be correlated with protection against a variety of mucosal pathogens (53). Compared to other immunoglobulin isotypes, sIgA does not effectively fix complement or promote phagocytosis. These mechanisms, however, except for alveolar macrophages, do not to a significant extent operate in external secretions. sIgA functions as a so called "antiseptic paint" at mucosal surfaces. Invasion of pathogens is effectively neutralized by inhibition of the adherence of these organisms to surfaces by sIgA.

It would, therefore, seem to be highly desirable to induce protective antibody- and cell-mediated immunity at mucosal surface (111). Numerous attempts have been made to induce immune responses in mucosal tissues, mucosa-associated lymphoid tissues (MALT) and external secretions (for review see 80). Mucosal tissues of the respiratory tract, conjunctiva, vagina or intestinal tract have been exposed to live or attenuated or killed viruses or bacteria in attempt to induce a local immune response and concurrent protection against the pathogens under investigation. Considerable attention has been paid to the induction of sIgA and studies on the origin of IgA-producing cells and routes of immunization led to the concept of a so-called "common mucosal immune system".

THE "COMMON MUCOSAL IMMUNE SYSTEM"

The concept of the "common mucosal immune system" describes the relationship between gut-associated lymphoid tissue (GALT) and other mucosal tissues and exocrine secretory tissues, in which local immunization at one place in the mucosal system also induces a humoral immune response at distant mucosal sites. Of all exocrine secretory tissues - i.e mucosa and MALT - the intestinal tract is quantitatively the most active organ engaged in the immunoglobulin production, particularly those of the IgA isotype (32, 81). For example, 70-80% of all Ig-producing cells of the body are located in the intestinal mucosa (23, 129). Submucosal B-cells synthesize predominantly J-chain containing IgA. It is not fully understood how these phenotypic features are favored by the mucosal microenvironments (16). Positive immune regulation must favor the development of B-cell clones which can also produce J-chains, because this is a prerequisite for external translocation of locally produced immunoglobulins (22). Before secretion an additional polypeptide chain, the secretory component (SC), is coupled to the dimeric IgA and this complex (secretory IgA, sIgA) is transported into external secretions (22).

An important basis for mucosal immunity is the migration so-called "homing" of specific B- and T-cells after stimulation with antigen, from BALT and GALT to the submucosa and to various other secretory tissues (for reviews see 14 and 80). IgA plasma cells that produce antibodies specific for orally administered antigens were also detected in extraintestinal lymphoid organs (60). Further investigations revealed that antibody-forming cells disseminate to other organs after topical application of antigen (87) and studies on oral immunization confirmed that in addition to milk, sIgA antibodies can be induced in secretions of almost all other glands as well (40, 82, 139). This "homing" phenomenon requires the local presence of T-helper cells for IgA (15). The nature of the "common mucosal immune system" and the induction of a mucosal immune response have been the object of many studies on oral immunization. However, the particular immunoregulatory requirements of mucosal immunity are still obscure. Under appropriate conditions, oral administration of protein antigens may even lead to systemic

tolerance (107, 118, 122, 130). Genuine forms of systemic tolerance can be induced by oral administration of common antigens like ovalbumin, ß-lactoglobulin or erythrocytes (3, 108, 140). It is not clear what causes the different immune responses to microbial and nonmicrobial antigens but systemic tolerance and mucosal immunity may appear concomitantly by development of regulatory antigen-specific suppressor and helper T-cells after oral immunization (25, 26, 38, 39). Furthermore, it is suggested that MALT may be able to discriminate between microbial and nonmicrobial antigens and deviate in this respect from its systemic counterpart (140).

HUMORAL IMMUNE RESPONSE AFTER ORAL IMMUNIZATION

A sizable number of publications on the induction of mucosal antibody by oral immunization has been generated (for review see 10). Most studies were performed in rodents and show induction of IgA in external secretions like saliva, colostrum, milk, tears, bile and on the mucosal surfaces of the respiratory and female genital tract. Antigens of bacterial, viral or other origin have been investigated widely (Table 1). In some studies, a concurrent serum response was observed (IgG and IgM) and in some cases increased IgG antibodies were also found in mucosal tissues or external secretions (43, 102). Generally it was concluded from these studies that oral administration of antigen is an attractive method for the induction of specific mucosal immunity and that live vaccines induce a stronger antibody response. Furthermore, that the physical or chemical nature of the antigens may be decisive for the strength of the mucosal immune response and the type of antibody produced (99). Therefore, currently much attention is paid to the development of sophisticated antigen-delivery systems for oral immunization.

Table 1. Mucosal IgA production after oral administration of antigen.

Antigen	Species	Tissue/fluid	Reference
S. mutans	rat	saliva/milk saliva/milk	82, 83
S. mutans	mouse	saliva	26
E. coli	rat	milk/lung urine/bile	56, 74
P. aeruginosa	rat	BAL	51
B. gingivalis fimbriae	mouse	saliva	94
S. pneumoniae	mouse	BAL/tear/saliva	88
C.trachomatis	mouse	BAL	34
Gastroenteritis virus	pig	milk	20
Influenza A virus	mouse monkey	BAL/bile nose/saliva	7, 123
RSV	rabbit	milk/gut	102

Antigen delivery

Induction of an effective mucosal immune response by the oral route requires relatively high doses of antigen due to the digestion of antigens by gastrointestinal enzymes, limited absorption of antigens from the gastrointestinal tract and coupling to preexisting antibodies and other substances in the intestinal tract (70, 116, 120, 126). Furthermore, oral use of inactivated microbial preparations does not stimulate the immune system as strong as live attenuated vaccines (80). Therefore, several approaches to improve antigen delivery into the gastrointestinal tract are currently under investigation. Several studies report the successful use of cholera toxin to enhance a mucosal uptake of antigen and enhanced immune response (11, 38, 66, 121). To protect antigenic determinants from acid pH and proteolysis by gastric enzymes, antigens have been given orally in a large amount of sodium bicarbonate (17), coatings consisting of gelatin capsules that are soluble only in alkaline pH (17, 37, 136) or liposomes, in some cases combined with adjuvant (9, 84, 133). From these studies it becomes clear that protection of orally

delivered antigens against intragastric degradation significantly improves the delivery of antigens into the gut and consequently improves the stimulation of GALT which, in turn, significantly enhances the immune response of the mucosal immune system.

Oral induction of a humoral mucosal immune response in humans

The evidence for a "common mucosal immune system" in humans is indirect, because experiments in which labeled cells from donors are transferred to irradiated syngeneic recipients cannot be performed in humans (33). However, substantial information exists that indicates that a "common mucosal immune system" is also functionally present in humans. Secretions from glands that are not directly stimulated may contain sIgA antibodies against ingested or inhaled antigen (4, 79); this antibody has to originate from local production because circulating polymeric IgA is not efficiently transported into human external secretions (64).

Several studies (Table 2) have now demonstrated the possibility of antibody induction by oral immunization with vaccines of different bacterial or viral origin in humans. After the first successful results with oral immunization obtained with killed bacteria (S. mutans) in humans (76, 78), Clancy and coworkers could demonstrate the specific protective effect of oral immunization with killed non-typable H. influenzae (NTHI), which reduced the colonization with NTHI of patients with chronic obstructive lung disease (COPD) concurrently with a reduced incidence of respiratory infection (30, 31). Other studies have also investigated the influence of oral immunization on patients prone to frequent respiratory infection (Table 3). The overall conclusion from these studies is that oral immunization can significantly reduce the number and duration of infectious episodes in both infant and adult patients with chronic bronchitis. It is, however, not entirely clear whether the protective effects of oral immunization in some of the clinical studies may entirely be attributed to antibody induction or whether activated nonspecific immune mechanisms also play a significant role.

Table 2. Examples of induction of antibody response by oral immunization in humans.

Antigen	Response	Reference
Bacterial vaccines		
S. mutans	↑ sIgA in saliva, milk and tears ↑ IgA and IgM producing cells in blood	37, 76, 78
E. coli	↑ sIgA producing cells in milk ↑ sIgA in saliva, IgG and IgM response in serum	55, 62
H. influenzae	↑ sIgA in saliva	29, 30
S. typhi	↑ IgA, IgG and IgM producing cells in blood no specific antibody in saliva or faeces	63
V. cholerae + B-subunit	serum antitoxin, IgA, IgG and IgM producing cells obtained from blood	68
Mixed bacterial vaccines		
H. influenzae/ S. aureus vaccine	IgA, IgG and IgM antibodies specific for H. influenzae in saliva, not in serum. No increase in antibodies against S. aureus	28
polyvalent (lysate of 8 strains)	↑ sIgA, ↓ IgE in saliva ↑ IgA, ↓ IgE in serum, ↑ urinary IgA	36, 45, 46, 105
ribosomal vaccine (from 4 strains)	antibody-forming cells in tonsils	48
Viral vaccines		
Poliovirus	↑ nasal sIgA, serum IgG ↑ nasal sIgA, genital IgG, ↑ serum IgA, IgG, IgM ↑ milk IgA, milk and saliva sIgA	57, 96, 97 98, 119
Rotavirus	↑ serum IgG and IgM (external secretions not examined)	131
Influenza A virus	↑ sIgA in saliva, tears and nasal secretions	8, 135, 137
Adenovirus type 1, 2, 5 and 21	↑ coproantibody (IgA), neutralizing antibodies in serum	114, 115

Table 3. Clinical results, in patients with frequent infection of the respiratory tract, obtained with oral immunization with different preparations of bacterial origin (trade names).

Antigen	Results	Reference
Patients with recurrent respiratory infection		
Non-typable H. influenzae	↓ colonization with NTHI and of ↓ incidence of infections in patients with COPD	30, 31
Glycoprotein extract of K. pneumoniae (Biostim®)	↓ exacerbations and ↓ infections in patients with stage 2 and 3 chronic bronchitis	21, 24, 131
Ribosomes from 4 different bacterial strains (Ribomunyl®)	↓ number and duration of respiratory infections, ↓ clinical symptoms	85, 110
Lysate from 8 different bacterial strains (Broncho-Vaxom®)	↓ clinical symptoms and antibacterial therapy, ↑ respiratory function in infants, adults and elderly with chronic bronchitis	1, 2, 36, 41 45, 46, 47, 54 69, 71, 77, 100
Capsular extract from K. pneumoniae and E. coli (Diribiotine CK)	↓ incidence and duration of infectious episodes and antibiotics in patients with COPD	109
ENT disorders		
Lysate from 8 different bacterial strains (Broncho-Vaxom®)	↓ major symptoms in infants and adults with chronic rhinosinusitis and chronic purulent sinusitis	58, 113, 144

NONSPECIFIC EFFECTS OF ORAL IMMUNIZATION

From the previous sections it becomes clear that oral immunization may enhance the specific antiviral or antibacterial resistance of mucosal tissues. Another important issue, however, is whether the protective effects observed in studies on oral immunization must be attributed to nonspecific immune mechanisms. Nonspecific effects of oral immunization may involve activation of innate immunity (50) or enhancement of specific immune responses by so-called immunological adjuvant present in the immunomodulatory preparations (13). Stimulation of nonspecific immune mechanisms

like phagocytosis in patients with recurrent respiratory infection may be of equal importance as the induction of antibody. Alveolar macrophages (AMs) make up over 90% of cells in bronchoalveolar lavage fluid (BAL) of normal subjects. These cells form the first line of defense against inhaled pathogens, mainly through phagocytosis but also through the production and release of lysosomal enzymes, proteases, O_2 metabolites and inflammatory mediators and their function may be affected in patients with COPD (12, 49).

Antigen presenting cells (APC's) like macrophages and monocytes can enhance a humoral antibody response by presenting antigens on their surface, associated with class II molecules of the major histocompatibility complex, to T-helper lymphocytes. Presentation of antigen can also be enhanced by addition of adjuvant to a, possibly weak antigen to increase its immunogenicity. A long list of natural substances exists which increase the activity of weak immunogens, but to date most immunoadjuvants are of bacterial origin (for review and list of adjuvants see 134). Adjuvants may theoretically enhance antigen presentation through increased expression of Ia antigens or indirectly through increased secretion of interferons or interleukins. It is assumed that Freund complete adjuvant, presently the most powerful adjuvant, enhances both the expression of Ia and the production of interleukin-1 (IL-1) whereas muramyl dipeptide (MDP), a synthetic adjuvant originally derived from the cell wall of gram positive bacteria, primarily induces high levels of IL-1 (6).

However, the use of adjuvants is not without problems and various side effects have been reported. Cysts, local granuloma's and the development of tumors were observed with the use of adjuvants (90, 95) and the obtained results were conflicting with respect to efficacy (106).

Supposedly by activation of macrophages to a cytotoxic state and induction of lymphokine release from macrophages, parenterally administered MDP can induce protection against viral, bacterial and fungal infection in different animal models (27, 35, 42, 73, 101, 142, 143). Adjuvants like murabutide, endotoxic lipopolysaccharide (LPS) and monophosphoryl A (MPL) have been shown to directly influence different kinds of cells involved in the immune response like B-cells, T-cells, NK cells and macrophages (65, 91,

93, 89, 124). Although MDP's do not seem to have a direct effect on interferon production (61, 112), the mechanism of action of adjuvants like MPL and LPS, appears to be a direct action on T-helper cells and thereby induction of interferon-gamma (IFN-gamma) production (93, 124). Nagao and coworkers suggested that MDP injected intraperitoneally in guinea pigs stimulates macrophages resulting in the attraction of neutrophils (91). Furthermore, apart from stimulation of macrophages, MDP derivates augment the IgG antibody response against influenza hemagglutinin and enhance the nonspecific resistance against P. aeruginosa (52). Direct adjuvant activity has also been described for RU 41740 (Biostim®), a glycoprotein extract from Klebsiella pneumoniae (104). In this study RU 41740, given simultaneously during parenteral vaccination of elderly (over the age of 65 years) against influenza, significantly increased the specific antibody response in otherwise unresponsive volunteers. Considering the nonspecific activation of different kinds of cells by oral immunomodulators, adjuvant activity may be an important and clinically useful property of oral immunomodulators.

Several studies have shown changes of cellular activity of macrophages, lymphocytes or neutrophils after stimulation with oral immunomodulators in vitro (Table 4) or in vivo (Table 5). In general it can be concluded from these studies that oral immunomodulators not only show immunological adjuvant activity but induce a multitude of nonspecific cellular reactions, including activation of neutrophils, monocytes, macrophages and NK cells, and the production of lymphokines and interferons.

Considering the above mentioned, it is not surprising that a few studies have now reported possible beneficial effects of oral immunization with different preparations in patients with malignancies (5, 18, 67, 138). Increased NK cell activity, increased T-cell proliferation, induction of tumor-inhibitory macrophages, declined T-suppressor cells and increased production of interferon and interleukin-2, as reported in these and other studies on oral immunization, may significantly contribute in the treatment of malignancies. Therefore, immunomodulation for the treatment of cancer by oral immunization may become a complementary approach to immunomodulation with cytokines like interferon, interleukins and others.

Table 4. Effects of in vitro stimulation of cells with oral immunomodulators.

Cell	Effect	Reference
Glycoprotein extract of K. pneumoniae (Biostim®)		
Human monocytes	↑ release of antitumor factors	19
Murine macrophages	↑ IL-1 and TNFα production	125
Human PMN's	↑ respiratory burst	44
Human neutrophils	↑ chemiluminescence upon stimulation	92
Murine bone marrow precursor cells	NK generation from precursors	86
Murine NK cells	↑ of activity	92, 117
Lysate from 8 different bacterial strains (Broncho-Vaxom®)		
Macrophages and monocytes	↑ oxidative metabolism, ↑ intracellular killing capacity of murine peritoneal macrophages	75
Human peripheral blood mononuclear cells	↑ TNFα, IL-2 and IFN-gamma production, ↑ NK activity	141
Human T-suppressor cells	↓ antibody-dependent allergic autocytotoxicity of white blood cells of bronchial asthma patients	103

CONCLUDING REMARKS

By comparison with the functions of IgM and IgG, the functions of sIgA antibodies do not seem very spectacular; nevertheless, under physiological conditions sIgA is produced in considerable quantities. The function of secretory antibody is to prevent or limit the entrance of pathogens into the internal milieu, whereas systemic immunity generally removes pathogens from the internal milieu. It is important to realize that the mucosal immune system may be exploited in the prevention of infectious disease by stimulation with orally applied immunomodulators. Furthermore, it is important to note that other, non-humoral immune mechanisms, may significantly attribute to mucosal defense.

Table 5. Effects of oral immunization in vivo.

Cell	Effect	Reference
Glycoprotein extract of K. pneumoniae (Biostim®)		
Murine bone marrow precursor cells	↑ reconstitution of NK cell activity after transplantation of bone marrow in irradiated mice	86
Murine NK cells	↑ activity	92, 117
Lysates from 8 different bacterial strains (Broncho-Vaxom®)		
Human lymphocytes and macrophages	↑ CD4/CD8 ratio and ↑ IFN-gamma production, ↑ total cell counts and ↑ macrophageal activity in patients with chronic nonobstructive bronchitis	45, 46
Human lymphocytes	↑ T-lymphocyte counts in patients with chronic bronchitis	36
Human lymphocytes	↑ interferon production by T-lymphocytes, obtained from infants with recurrent respiratory infection	72
Lysates from 7 different bacteria (Paspat oral)		
Rabbit neutrophils	↑ oxidative burst after stimulation in vitro	59
Murine lymphocytes	increased pulmonary interferon-gamma	Own observations submitted
Murine alveolar macrophages	altered reactivity to stimulation with histamine and PGE_2, ↑ activity after infection with influenza A	Own observations submitted
Survival of mice	Increased survival after intranasal infection with S. pneumoniae	127, 128
	Increased survival after infection with influenza A virus aerosol	127, Own observations submitted

Enhanced activity of phagocytotic cells, intraepithelial T-lymphocytes and other T-cell populations after oral immunization may prove to be of equal importance as the induction of antibody in the development of antiviral, antibacterial, and even antiparasitic immunity. Improved antigen packaging, the use of immunological adjuvants and the use of synthetic peptides of relevant antigens may improve antigen delivery and significantly

reduce the cost of vaccine production. Obviously, oral administration of immunomodulators offers attractive possibilities with respect to the simplicity of administration of vaccines. Oral immunization circumvents the stringent criteria applicable for injectable vaccines and large populations can be simultaneously immunized without the assistance of highly trained personnel. Oral immunization may improve our chances in eliminating or alleviating severe respiratory, diarrhoeal, and venereal diseases that yearly afflict millions of individuals and cause significant mortality both in developed and developing countries. Furthermore, enhancement of innate immune mechanisms, like stimulation of NK-cells or induction of tumor-inhibitory activity of macrophages, may prove effective as an immunomodulatory therapy against malignant disease.

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Oral immunization with bacterial lysate against infection with Streptococcus pneumoniae in mice.

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CHAPTER III

ORAL IMMUNIZATION WITH BACTERIAL LYSATE AGAINST INFECTION WITH STREPTOCOCCUS PNEUMONIAE IN MICE

SUMMARY

The protective effect of oral immunization against infection with streptococcus pneumoniae was investigated in mice. Two bacterial lysates, one with an additional lysate of C. albicans, were investigated. Intranasal inoculation of adult Balb/c mice with a S. pneumoniae type I strain resulted in a lethal infection, with deaths occurring from the second day after infection until the sixth day after infection. Oral immunization resulted in a significant decrease in mortality rate (18 - 48% reduction). No significant difference in mortality rates was observed between groups immunized with different lysates, in the same concentrations.

INTRODUCTION

An important effect of immunization protocols should be enhanced antibody- and cell-mediated immunity at mucosal membranes, because this large surface area is the porte d'entrée of the majority of infectious diseases. Repeated attempts have been made to develop methods for nonparenteral immunization against viral as well as bacterial pathogens in humans [1].

Studies on local immunization, using different antigens and routes of administration, reported very good local antibody responses [2,3,4,5]. However, this method has many practical disadvantages including e.g. the need for aerosol delivery of vaccine, cooperative patients and highly trained personnel.

Studies on oral administration of antigen to protect against intestinal infections have resulted in the development of several effective vaccines for the prevention of cholera

and typhoid fever [6,7,8]. A significant reduction in acute episodes with fever and consumption of antibiotics was described in several studies on oral immunization with bacterial lysates in patients susceptible to respiratory infection [9,10,11]. These results suggest that it is possible to immunize against respiratory pathogens, circumventing the adverse reactions often accompanying parenteral immunization.

The aim of this study was to investigate whether oral administration of a bacterial lysate has any protective effect in mice infected with a high dose of S. pneumoniae, using the respiratory tract for inoculation. Further, to investigate whether the addition of C. albicans has any influence on the protective effect of the bacterial lysate in this model of respiratory infection in mice.

MATERIALS AND METHODS

Animals.

125 Male Balb/c mice (mean body weight 25.3 ± 2.1 g) from Harlan/CPB (Zeist, The Netherlands) were used. Animals were kept under conventional conditions; food and water were given ad libitum.

Infection procedure.

Animals were anesthetized by placing them in a container, through which a mixture of nitrous oxide and oxygen (2:1) with 1.5 % halothane was led.

Inoculation was performed intranasally by holding the mice vertically while the bacterial suspension was applied dropwise into the nostrils [12]. All animals were infected with 0.05 ml from the same bacterial suspension, to prevent differences in size and quantity of the inoculum. Animals fully recovered within two minutes after application of the inoculum.

Bacteria.

A S. pneumoniae type I strain (ATCC 6301) was used in all investigations. This strain was selected for its pathogenicity for Balb/c and NMRI mice. Bacteria were grown on

mucin blood agar plates, lyophilized and stored in batches of $6.8x10^7$ colony forming units (cfu) at 4° C. On the day of inoculation, 1 ml sterile water was added to one of the batches before use.

Immunization and challenge scheme.

Two bacterial lysates {LW 50020 A and Paspat oral®; Luitpold-Werk, Munich, FRG} containing lysates of at least 10^{10} of each of the following bacteria per g: S. aureus, S. pneumoniae, S. pyogenes, S. viridans, K. pneumoniae, B. catarrhalis and H. influenzae were used in two different concentrations.

LW 50020 A contains an additional lysate of C. albicans, which is currently being considered as a possible adjuvant for immunomodulator, whereas Paspat oral® is without C. albicans. The spray-dried lysates were resuspended in sterile saline before application. Anesthetized animals received 0.1 ml of the suspension after intraesophageal catheterization. Control animals were sham-immunized with 0.1 ml saline.

Application was done on days 1, 2 and 3 followed by a second challenge period on days 7, 8 and 9. The animals were inoculated on day 10 followed by a 14 day observation period.

Immunization protocol.

Immunization with LW 50020 A resulted in the following groups:

I 19 animals immunized with 12.5 mg doses

II 19 animals immunized with 6.25 mg doses

III 15 controls, receiving saline

Immunization with Paspat oral® resulted in the following groups:

IV 23 animals immunized with 12.5 mg doses

V 24 animals immunized with 6.25 mg doses

VI 23 controls, receiving saline

Statistical analysis.

Statistical evaluation of collected data was performed using Fisher's exact test. Statistical significance was accepted at $P \leq 0.05$ (two-tailed).

RESULTS

At the end of the immunization period no differences in bodyweight, behaviour or appearance existed between treated and control groups.

Deaths occurred from the second until the sixth day after inoculation. The loss of bodyweight was linear and significant from the first day after infection to the respective moments of death. In both the treated and untreated groups deaths occurred on almost the same days. Surviving animals remained unchanged and showed no signs of morbidity whatsoever.

Figure 1 shows survival rates for the different groups. In the group receiving 12.5 mg LW 50020 A the mortality rate (5%) proved to be significantly lower than in the control group (53%), while mortality rate in the group receiving 6.25 mg doses (42%) was not significantly different from the control group. Both groups (12.5 and 6.25 mg) receiving Paspat oral® showed a significantly decreased mortality rate (13 and 25% respectively) when compared with controls (43%).

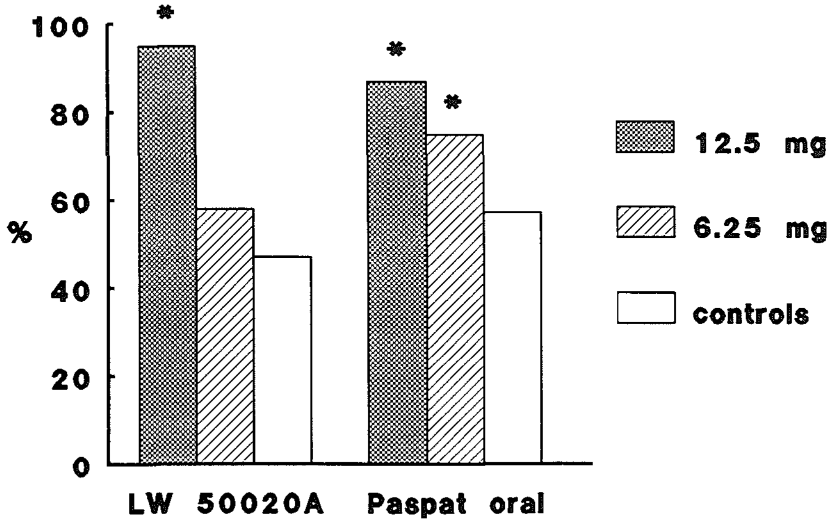

Figure 1. Survival rates (%) of mice immunized with different concentrations of LW 50020 A and Paspat oral®, 14 days after infection with S. pneumoniae. * indicates $p \leq 0.05$ Fisher's test, immunized group compared to controls.

DISCUSSION

This study indicates that animals can be protected against high-dose infection with S. pneumoniae by oral immunization with a bacterial lysate. The protective effect might be dose-dependent, because higher lysate concentrations resulted in increased protection in this study. Scientific acceptance of oral immunization is limited because results often seem to be contradictory. Several studies reported the induction of genuine forms of systemic tolerance after oral feeding of antigens like ovalbumin, ß-lactoglobulin or erythrocytes [13,14,15]. Systemic tolerance and mucosal immunity may appear concomitantly after oral immunization and antigen feeding is associated with the development of regulatory antigen-specific suppressor and helper T-cells [16,17]. The type of antigen may also play an important role because a difference in mucosal immunogenicity between bacterial and food antigens has been reported [15].

Most orally applied immunomodulators, currently used in many countries, are developed empirically and consist of crude lysates of one or more different bacteria or bacterial components. Also, concentrations and treatment protocols may differ widely.
The concentrations of the bacterial lysate used in this animal study are relatively high compared to concentrations generally used in humans and, therefore, may not be of comparable clinical relevance. Nevertheless, we aimed to achieve a maximal protective effect of oral immunization because the inoculum concentration was also very high compared to clinical situations. Several groups are currently investigating the combination of various adjuvants, like cholera toxin B unit or avridine [17,18] with antigens to enhance the protective effects of oral immunization.
There were no significant differences in mortality rate between groups immunized with LW 50020 A or Paspat oral® at the same concentrations, therefore it seems that addition of C. albicans does not result in increased protection against S.pneumoniae and thus can be excluded from the lysate.
The protective effect of oral immunization against S. pneumoniae might be explained in two ways. First, oral immunization with so called immunomodulators, containing various combinations of bacterial and viral antigens, causes priming of precursor cells in the gut and the subsequent occurrence of specific secretory IgA (S-IgA) antibody in saliva, milk, tears and the respiratory and genital tracts [19,20,21]. As immunological cross-activity between antigens of the streptococci in the bacterial lysate and the S. pneumoniae used for inoculation was not tested in this study we cannot assess whether this plays a role in the protective mechanism observed in these investigations.
Second, gamma-interferon is thought to have an active role in the nonspecific defense against respiratory tract pathogens. Natural killer cells might be stimulated through a rise in interferon titer as observed after oral immunization in lung lavage fluids and lung tissues [22]. Polyvalent bacterial lysates can induce interferon production by lymphocytes in vitro [23].
One of these mechanisms, an increase in S-Ig A or an increase in interferon titers, or both, might explain the observed protective effect of oral application of the lysates in this study. Because second challenge with antigens in humans produced high titers of S-Ig A,

which lasted for at least three months [24], we used an immunization scheme that contained two separate periods for application of the bacterial lysate in mice.
It is concluded that oral application of the bacterial lysate used in this study can protect mice against infection with S. pneumoniae. Further investigations are needed to elucidate the exact mechanism of oral immunization against bacterial respiratory pathogens.

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Influence of oral immunization and infection with S. pneumoniae on interferon-gamma and PMN-elastase concentrations in murine bronchoalveolar lavage fluid.

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CHAPTER IV

INFLUENCE OF ORAL IMMUNIZATION AND INFECTION WITH S. PNEUMONIAE ON INTERFERON-GAMMA AND PMN-ELASTASE CONCENTRATIONS IN MURINE BRONCHOALVEOLAR LAVAGE FLUID

SUMMARY

Oral immunization with a polyvalent bacterial lysate (Paspat oral®) can significantly reduce mortality rates in mice, infected with S. pneumoniae or influenza A virus. In this study it is demonstrated that oral immunization with the same bacterial lysate reduces the pulmonary inflammatory reaction to infection with S. pneumoniae. Furthermore, it is demonstrated that oral immunization increases pulmonary IFN-gamma production.

INTRODUCTION

We recently demonstrated that oral immunization with a bacterial lysate can significantly reduce mortality in mice infected with S. pneumoniae [1, 2] or influenza A virus [2]. One of the protective mechanisms of oral immunization, apart from induction of specific immunity [for review see 3], might be limitation of the alveolar inflammatory reaction after infection and thereby reduction of pulmonary damage. Furthermore, it is claimed that immunomodulators for oral application enhance nonspecific innate defense mechanisms at various sites, including the lungs [4-13]. Emmerich and coworkers have reported increased interferon-gamma (IFN-gamma) concentrations and less damage to pulmonary tissues due to reduced pulmonary inflammatory reactions in the lungs of patients with chronic bronchitis, following oral immunization with a polyvalent bacterial lysate [3, 4]. Considering the immunoregulatory properties of IFN-gamma [14-16], we

designed a study to investigate whether oral immunization increases pulmonary IFN-gamma production and concurrently reduces the inflammatory reaction in mice after infection with S. pneumoniae.

MATERIALS AND METHODS

Male Balb/c mice (n=120; bodyweight 22.1 ± 0.8 g, from Harlan/CPB, Zeist, The Netherlands) were randomly assigned to twelve groups. Eight groups were treated with a polyvalent bacterial lysate (Paspat oral®, Luitpold-Werk, München, FRG) [13]; four groups served as controls and were sham-immunized with 0.1 ml saline. Anesthetized mice ($N_2O:O_2$ 2:1 with 1.5% halothane) daily received 12.5 mg or 6.25 mg of the lysate, dissolved in 0.1 ml sterile saline, via an intraesophageal tube. Application was done on days 1, 2 and 3, and again on days 7, 8 and 9.
Intranasal infection with of S. pneumoniae (Type I, ATCC 6301, > LD_{100}) was done on day 10. On four occasions, twice before (days 5 and 10) and twice after (days 12 and 14) infection, three groups (one 12.5 mg group, one 6.25 mg group and one control group, n=10/group) were lavaged according to the following protocol. Animals were anesthetized (pentobarbitone sodium 60 mg/kg i.p.), tracheotomized and a small metal cannula was inserted. Lavage was done three times with a total of 3 ml sterile saline preheated to body-temperature (average recovery 2.4 ± 0.1 ml). Bronchoalveolar lavage (BAL) fluid was centrifugated and cells were collected for total cell count and evaluation of macrophages, lymphocytes and granulocytes concentrations.
IFN-gamma concentrations in BAL fluid were measured with a solid phase enzyme-linked immunosorbent assay (ELISA); units are defined according to NIH standard number Gg 02-901-533 [17], one unit corresponds with 500 pg pure recombinant murine IFN-gamma. The detection level of the IFN-gamma ELISA was 3U/ml. PMN-elastase concentrations in BAL were measured with an ELISA for the specific determination of elastase from polymorphonuclear leukocytes (PMN's) in complex with alpha-1 proteinase inhibitor [18].

RESULTS

Cell count data are shown in Table 1. Immunization did not significantly change the total amount of cells recovered from the lung; neither did it influence concentrations of subpopulations (Table 1). IFN-gamma measurements are summarized in Figure 1. After the first immunization period IFN-gamma concentrations in BAL fluid were significantly higher in both immunized groups and increased even further after the second immunization period, whereas all concentrations in the control groups were below the detection level. Infection with S. pneumoniae did not influence the quantity of IFN-gamma recovered.

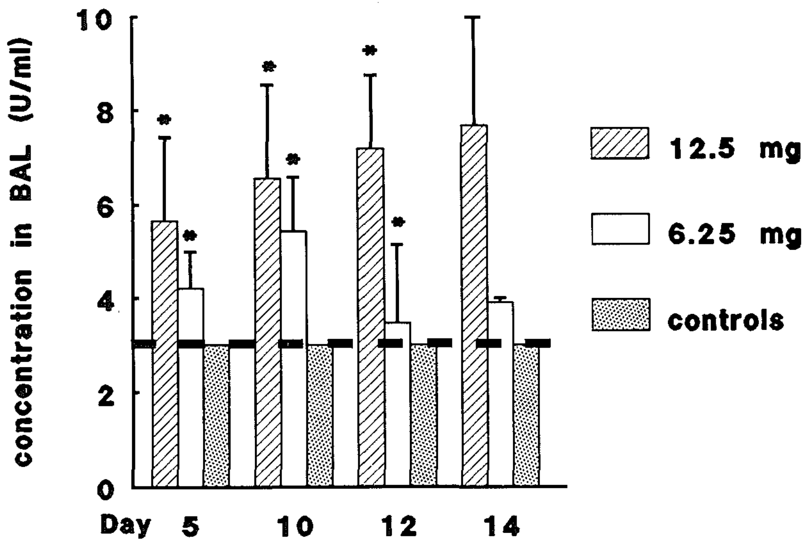

Figure 1. IFN-gamma concentrations (mean ± SD) in BAL fluid before and after infection. Due to technical problems only three samples per group could be measured on day 14, therefore levels of statistical significance could not be reached. The detection level of the ELISA is 3 U/ml. All controls remained below the detection level. * indicates $p \leq 0.05$ compared to controls, Mann-Whitney U test.

Table 1.

Total number of cells in BAL (x 100.000)

	Day 5	Day 10	Day 12	Day 14
12.5 mg	6.2	7.5	5.2	4.2
	(4.5)	(9.3)	(4.4)	(3.7)
6.25 mg	1.3	2.3	5.2	2.5
	(0.7)	(3.0)	(4.1)	(0.8)
controls	1.0	1.4	2.0	2.3
	(0.2)	(0.7)	(0.8)	(2_w)

Macrophages (%)

	Day 5	Day 10	Day 12	Day 14
12.5 mg	68.0	79.2	54.3	76.4
	(27.7)	(18.4)	(20.8)	(12.2)
6.25 mg	88.8	83.9	68.5	80.8
	(6.3)	(17.6)	(18.5)	(4.6)
controls	89.3	82.7	60.8	73.4
	(1.6)	(10.8)	(18.1)	(19.9)

Lymphocytes (%)

	Day 5	Day 10	Day 12	Day 14
12.5 mg	11.3	8.3	17.5	17.8
	(6.2)	(3.7)	(6.0)	(7.0)
6.25 mg	8.4	8.6	14.6	15.0
	(2.9)	(2.9)	(10.3)	(3.6)
controls	8.9	9.3	12.6	19.3
	(1.5)	(4.1)	(4.8)	(10.3)

PMN's (%)

	Day 5	Day 10	Day 12	Day 14
12.5 mg	21.3	14.0	28.1	4.6
	(28.0)	(20.0)	(15.6)	(3.3)
6.25 mg	2.9	7.2	17.9	3.8
	(5.1)	(14.2)	(13.1)	(2.0)
controls	1.8	5.8	27.1	7.1
	(0.8)	(8.8)	(11.4)	(7.2)

Cells (mean ± SD) in BAL fluid from orally immunized mice (12.5 mg, 6.25 mg and controls) on two moments before (days 5 and 10) and two moments after infection (days 12 and 14) with S. pneumoniae. Infection of the day 12 and day 14 groups was done on day 10. No significant differences were observed.

PMN-elastase measurements are summarized in Table II. Before infection, concentrations in all groups were below the detection level. Two days after infection (day 12) PMN-elastase concentration was still undetectable in the group immunized with the highest lysate dose (12.5 mg). In the other two groups (6.25 mg and controls), however, concentrations were significantly higher. On the fourth day after infection (day 14), concentrations were still below detection level in the group immunized with the high lysate dose and concentrations in the group immunized with the low lysate dose were significantly lower compared to controls.

Table 2.

	Day 5	Day 10	Day 12	Day 14
12.5 mg	nd	nd	nd *	nd *
6.25 mg	nd	nd	23.2 (1.8)	2.5 * (3.5)
controls	nd	nd	17.5 (16.8)	17.0 (6.9)

PMN-elastase (mg/ml) in BAL fluid from orally immunized mice (12.5 mg, 6.25 mg and controls) on two moments before and two moments after infection with S. pneumoniae. nd = nondetectable, mean (SD), * indicates $p \leq 0.05$ compared to controls, Mann-Whitney U test.

DISCUSSION

Oral immunization had no significant effect on concentrations of subpopulations of cells in BAL fluid, which is consistent with the findings of other workers in chronic bronchitis patients [4]. However, an increase in the helper/suppressor T lymphocyte ratio, mainly due to decreased CD8+ cell concentrations, was observed in BAL fluid after oral immunization, which might increase activity of T-helper cells [4]. This might account for the increased IFN-gamma concentrations in BAL fluid after oral immunization in this study and other studies on oral immunization [4, 5]

The interferon system is an integral part of the defense system of the body, mediating a large variety of biological effects whereas IFN-gamma has potent immunoregulatory effects on a variety of cells. Increased alveolar concentrations of IFN-gamma after oral immunization, as observed in this study, suggest enhancement of nonspecific pulmonary immunity which might result in increased antibacterial and antiviral capacity of the pulmonary defense system. IFN-gamma has antiviral properties [19-21] and can also be considered a lymphokine, since it is exclusively a product of lymphocytes upon stimulation by antigen-presenting cells. IFN-gamma is a potent macrophage-activating factor which might significantly attribute to pulmonary defense after oral immunization [4, 22, 23]. T-helper cells are considered the main producers [24, 25]. Therefore, to evaluate the induction of nonspecific defense mechanism of the respiratory tract by oral immunization, we measured IFN-gamma concentrations in BAL fluid. Simultaneously we demonstrated that oral immunization can reduce the pulmonary inflammatory reaction after infection with S. pneumoniae. The inflammatory reaction can damage the alveolar membrane and the intensity of alveolitis during pulmonary infection can be assessed by measurement of PMN-elastase in BAL fluid [26]. Although normally macrophages are the resident alveolar phagocytic cells, pulmonary infection causes influx of PMN's. Proteolytic enzymes, like PMN elastase, have the potential to destroy the elastin as well as the other macromolecular components of pulmonary extracellular matrix [26]. Disruption of the epithelial barrier of the lung by proteolytic enzymes may allow invading mechanisms, in this case S. pneumoniae, to penetrate the interstitium and migrate into the bloodstream more easily. A similar effect was observed during P. aeruginosa infection in guinea pigs [27]. Other groups reported that orally applied immunomodulators increase the oxidative burst of stimulated peripheral blood PMN's [6, 11, 13, 21]. Infection would thus result in increased PMN-elastase production in immunized animals. Our, seemingly contradictory, observations might be attributed to either different reaction of PMN's to different stimuli or to a compartimentalization of the effects of oral immunization because in this study alveolar PMN-elastase was measured.

Summarizing, we conclude that oral immunization with a bacterial lysate reduces the pulmonary inflammatory reaction after infection with S.pneumoniae and simultaneously may enhance nonspecific pulmonary defense mechanisms by increasing IFN-gamma production.

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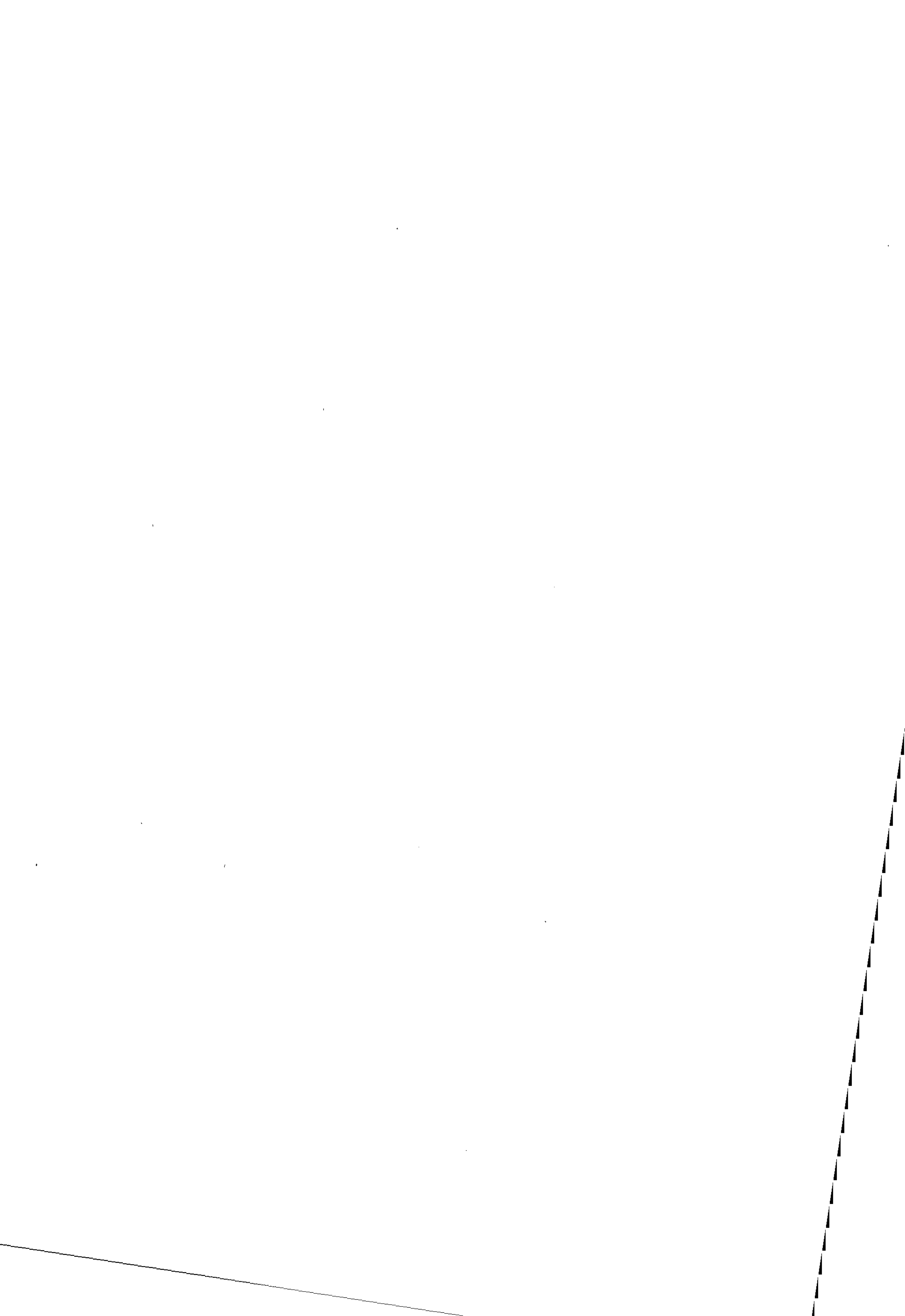

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Protection against influenza A virus infection in mice by oral immunization with a bacterial lysate.

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CHAPTER V

PROTECTION AGAINST INFLUENZA A VIRUS INFECTION IN MICE BY ORAL IMMUNIZATION WITH A BACTERIAL LYSATE

SUMMARY

A study is presented which investigated whether oral immunization with a polyvalent bacterial lysate (Paspat oral®) can sufficiently enhance cell-mediated defense mechanisms to protect mice against influenza A virus infection. It was found that oral immunization reduced mortality due to influenza A infection with 15-70%, depending on the quantity of virus administered and the moment of infection. Cyclosporin A severely reduced the protective effect of oral immunization, suggesting that a major effect of oral immunization in these studies is T-cell activation. The effect of oral immunization on macrophageal activity was evaluated by measuring cyclic-AMP in alveolar macrophages (AMs) obtained by bronchoalveolar lavage. Before infection, basal activity levels of AMs in immunized mice were significantly lower than in controls. Five days after infection, however, basal activity level of AMs in immunized mice was significantly higher than AM activity in controls. Stimulation of AMs with PGE_2 significantly reduced cellular activity in both groups, before and after infection. However, cellular activity of AMs from immunized animals was less reduced than cellular activity of control macrophages. Activity of AMs of immunized animals was significantly more reduced by histamine than activity of control macrophages. It is concluded that oral immunization with Paspat oral® stimulates T cell dependent immune mechanisms, resulting in protection against influenza A virus infection in mice.

INTRODUCTION

Apart from parenteral immunization protocols, alternative approaches have been considered extensively. Studies on local immunization, using different antigens and routes of administration (e.g. aerosols or nasal sprays), result in a very good local antibody response [Ganguly, Ogra, Regas & Waldman (1973); Schwartz, Toyo & Hornick (1974)]. Besredka introduced oral administration of antigen to protect against intestinal infections [Besredka (1927)]; this concept has led to the development of several effective vaccines for the prevention of cholera [Clemens, Sack, Harris, Chakraborty, Khan, Stanton, Kay, Khan, Yunus, Atkinson, et al. (1986)] and typhoid [Germanier (1984)] fever. Oral challenge with so-called immunomodulators, containing various combinations of bacterial and viral antigens, results not only in the occurrence of specific sIgA antibody in the intestinal tract [Ogra & Karzon (1969)] but also in saliva [Michalek, McGhee, Mestecky, Arnold & Bozzo (1976)], milk [Hanson, Ahlstedt, Andersson, Carlsson, Cole, Cruz, Dahlgren, Ericsson, Jalil, Kahn, Mellander, Schneerson, Svanborg Eden, Soderstrom & Wadsworth (1983)] and mucous membranes of the respiratory [Clancy, Cripps, Husband & Buckley (1983)] and urogenital [Mestecky (1987)] tracts. We recently demonstrated that Balb/c mice are protected against a lethal infection with S. pneumoniae (Type I) by oral immunization with a polyvalent bacterial lysate [Van Daal, de Jong, Tenbrinck, Mouton, Petzoldt, Bergmann & Lachmann (1990); Van Daal, So, Mouton, Van 't Veen, Tenbrinck, Bergmann & Lachmann (1990)].
Furthermore, we recently demonstrated that oral immunization significantly increases IFN-gamma production in the lung [submitted] which was also described in other studies on oral immunization [Emmerich, Emslander, Pachmann, Hallek, Milatovic & Busch (1990a); Emmerich, Emslander, Milatovic, Hallek & Pachmann (1990b)]. Therefore, it can be concluded that oral immunization has a direct influence on the activity of several types of cells involved in the immune response.
Already in the mid-Seventies, Raettig observed that the most important advantages of oral immunization are its nonspecific effects. He reported that mice can be protected against infection with a virulent strain of S. typhimurium by oral immunization with a

heat-inactivated strain of S. enteridis [Raettig (1975)]. Altogether, these results suggest enhancement of both antibody-and cell-mediated defense mechanisms by oral immunization.

Therefore, we designed a study to investigate whether oral immunization with a polyvalent bacterial lysate can sufficiently enhance cell-mediated defense mechanisms to protect mice against influenza A virus infection.

MATERIALS AND METHODS

Animals

The experiments were carried out in 6-8 week old (SPF, 18.0 - 23g) male Balb/c or Swiss-bred mice obtained from Harlan/CPB (Zeist, The Netherlands). Animals were kept under conventional conditions; food and water were given ad libitum. Bodyweight was registered each day at the same time during the entire immunization period and during twenty days after the day of infection.

Virus

The influenza virus (A/PR8/34, H1N1) used, was passed once in 10 day embryonated chicken eggs. The allantoic fluid was clarified by centrifugation and stored at -70°C in small aliquots. The stock solution was diluted in sucrose and had a hemagglutination (HA) titer 1:400.

Treatment protocol

A polyvalent bacterial lysate [Helmberg, Böck, Wolf & Wick (1989)] (Paspat oral®; Luitpold-Werk, Münich, FRG) was applied in three different concentrations. The lysate contains at least 10^{10} of each of the following bacteria per g: Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus viridans, Klebsiella pneumoniae, Branhamella catarrhalis and Hemophilus influenzae. The mechanically disintegrated bacteria were resuspended in sterile saline before application. For intraesophageal catheterization, animals were anesthetized by placing them in a container

through which a mixture of oxygen and nitrous oxide (1:2), containing 1% halothane, was led. Animals received 0.1 ml of the suspension through this catheter. Control animals were sham-immunized with 0.1 ml saline. Animals recovered within 2 min after this procedure. Application was done on days 1, 2 and 3 followed by a second application period on days 7, 8 and 9. Animals were infected on day 10 or 24, followed by a 20 day observation period.

Infection procedure

All animals were exposed once, at the same time, to nebulized live influenza virus suspension in an 11 liter multichamber aerosol box [Loosli, Robertson & Puck (1943)]. Aerosol was produced with an Ultravent nebulizer (Ultravent, Malinckrodt Diagnostica, The Netherlands) with a flow of 5 l/min. This device produces particles with a size of 0.6 - 2 μm [Dahlbäck, Nerbrinck, Arborelius & Hansson (1983)], which allows for deposition of the particles in the peripheral airways and alveoli [Brain & Valberg (1979)].

Five studies were performed

Study 1. The effect of oral immunization against a high-dose influenza A virus infection was tested. Eighty male Balb/c mice were randomly divided into four groups and treated daily with 6.25 mg, 12.5 mg or 25 mg Paspat oral• whereas controls were sham-immunized with saline according to the above mentioned scheme. On day 10 the animals were challenged for 10 min with influenza virus, 1:80 dilution in PBS of stock solution ($> LD_{100}$).

Study 2. A low virus dose was applied. Sixty-eight male Balb/c mice were randomly divided into four groups and immunized in the same way as described in study 1. On day 10 the animals were challenged for 10 min with influenza virus, 1:160 dilution of stock solution in PBS ($\pm$ 0.5x LD_{100}).

Study 3. The long-term protective effect of oral immunization against a high-dose of influenza infection 1:80 dilution in PBS of stock solution (> LD_{100}) was investigated to establish whether the protective effects would persist. Thirty-one male Balb/c mice were randomly divided into two groups and treated daily with 25 mg Paspat oral• or saline. After the nine-day immunization period, animals were stored for two weeks in a separate room to avoid contact with antigen and were challenged fourteen days after the immunization period (day 24).

Study 4. The effect of immuno-suppression by Cyclosporin A (CyA) on the course of a high-dose influenza infection was tested in orally immunized animals and controls. This study was performed to evaluate the significance of T-cells in the protective mechanisms against influenza virus, activated by oral immunization. Sixty male Swiss-bred mice were randomly divided into four groups. Two groups were treated with 25 mg Paspat oral• dosages, of which one group received additionally 100 mg/kg CyA solved in olive oil (1:10) subcutaneously every other day, starting one day before infection. Two sham-immunized groups served as controls: of which one group received CyA in the same way as the immunized group treated with CyA. All animals were challenged directly after the immunization period (day 10) with a high-dose influenza virus, 1:80 dilution in PBS of stock solution (> LD_{100}).

Study 5. The effect of oral immunization on lung function was evaluated. Thorax-lung compliance, protein concentrations in broncho-alveolar lavage (BAL) fluid, surface active properties of BAL fluid and cellular activity of alveolar macrophages (AMs) were measured directly after the immunization period (day 10) and five days after infection (day 15) with a high-dose influenza virus, 1:80 dilution in PBS of stock solution (> LD_{100}). Eighty male Balb/c mice were randomly divided into four groups. Two groups were treated daily with 25 mg Paspat oral• whereas two groups served as sham-immunized control groups. Directly after the immunization period (day 10) thorax-lung compliance was measured in one immunized group and one control group without being infected. Next, BAL was performed three times with 1 ml sterile saline (total 3 ml),

preheated to body temperature (average recovery 2.4 ± 0.1 ml). Also, on day 10, one immunized group and one control group were infected with a high-dose influenza virus 1:80 dilution in PBS of stock solution (> LD_{100}). On the fifth day after infection (day 15), thorax-lung compliance was measured and lung-lavage was performed.

Lung mechanics

For measuring lung mechanics, animals were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital (Nembutal®, Algin BV, Maassluis, The Netherlands) and tracheotomized, using a metal cannula as tracheal tubing. The mice were paralyzed by intramuscular injection of 0.05 ml pancuronium bromide (Pavulon®, Organon Technika, Boxtel, The Netherlands), transferred to a multichambered body plethysmograph [Lachmann, Grossmann, Nilsson & Robertson (1981)] heated to 38° C, and connected to a ventilator system for pressure-controlled ventilation. The initial ventilator frequency was set at 30/min, I/E ratio 1:2, peak pressure 15 cm H_2O and FiO_2 = 1; the mice were ventilated under these circumstances for 5 min to allow stabilization. Then thorax-lung compliance was recorded at a frequency of 6/min.

Macrophageal activity

The effect of oral immunization on macrophageal activity was evaluated by measuring cyclic-AMP (cAMP) in AMs obtained by BAL. A rise in macrophageal intracellular cAMP levels is reflected by a reduction in cellular activity [Beusenberg, Adolfs, Van Schaik, Van Amsterdam & Bonta (1989)]. Basal cellular activity levels and reactivity to stimulation with the inflammatory mediators prostaglandin E_2 (PGE_2) and histamine were measured. Activity was measured according to the following protocol. BAL was filtered through surgical gauze and BAL cells were recovered from the lavage fluid by centrifugation (800 x g, 10 min at 4° C) and were resuspended in Gay Balanced Salt Solution (pH 7.4); thereafter a Ficoll-Isopaque gradient centrifugation was carried out (400 x g, 30 min at 4° C).

Milliliter samples containing 2.10^5 macrophages were incubated for 15 min at 37° C in the presence of 400 μM IBMX (a phosphodiesterase inhibitor), 10^{-5} M PGE_2 or 10^{-4} M

histamine. After incubation the cAMP concentrations (expressed as pmol/1.10^5 cells) were determined using a high-affinity protein binding method [Bonta, Adolfs & Fieren (1984)]. Total protein concentrations of BAL fluid were measured with the Biuret method [Doumas, Bayse, Borner, Carter, Peters & Schaffer (1981)].
Surfactant activity [Clements (1957)] in BAL fluid was measured with a modified Wilhelmy balance (Biegler Electronic, Vienna, Austria) by applying 400 µl of BAL fluid onto a 71.3 cm^2 trough. For measuring minimal surface tensions the area was compressed to 20% of the initial value.

Statistical analysis and data presentation

Statistical evaluation of collected data was performed using the Kaplan-Meier method (product-limit survival estimates) for survival rate curves [SAS® User's guide (1985)] or the Mann-Whitney U-test for analyzing between-groups differences. Statistical significance was accepted at $p \leq 0.05$ (two-tailed). All data are expressed as mean ± SD.

RESULTS

Study 1. Deaths occurred from 6 - 14 days after infection. Figure 1 shows survival rates for the different groups until day 16 after infection; no more animals died after this period. Survival was significantly improved (15 - 70%) in all three immunized groups compared to the control group.

Study 2. No animals died in the immunized groups, except for one animal in the 12.5 mg dose group which died on day 17 after infection (figure 2). In the control group, deaths occurred from 9 - 16 days after infection, whereas 39% of the control animals survived after 16 days. This resulted in a significantly reduced (55 - 61%) mortality in the immunized groups.

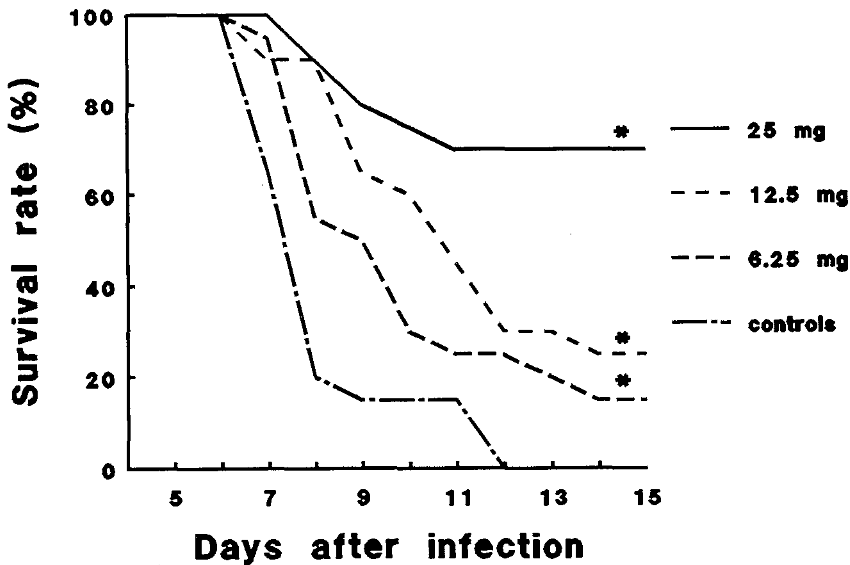

Figure 1. Survival rates of study 1. Animals were infected directly after the immunization period (day 10) with high-dose influenza A. * $p \leq 0.05$, survival of immunized animals vs survival of controls, Kaplan-Meier product-limit survival estimates.

Study 3. Fifteen days after infection all animals in both groups had died (figure 3). Survival time in the immunized group (8 - 15 days), however, was significantly longer than in the control group (8 - 10 days).

Study 4. Figure 4 shows survival rates of the four different groups from days 6 - 16 after infection. After fourteen days, no more animals died. Survival in the group immunized with 25 mg Paspat oral® was significantly better than in the saline treated control group and was comparable to the results obtained in the first survival study (study 1). In the groups treated with CyA, survival was also significantly better in the immunized group than in the saline treated control group receiving CyA. However, immunosuppression with CyA significantly reduced the protective effect of Paspat oral®. No significant difference was found between the two saline treated control groups.

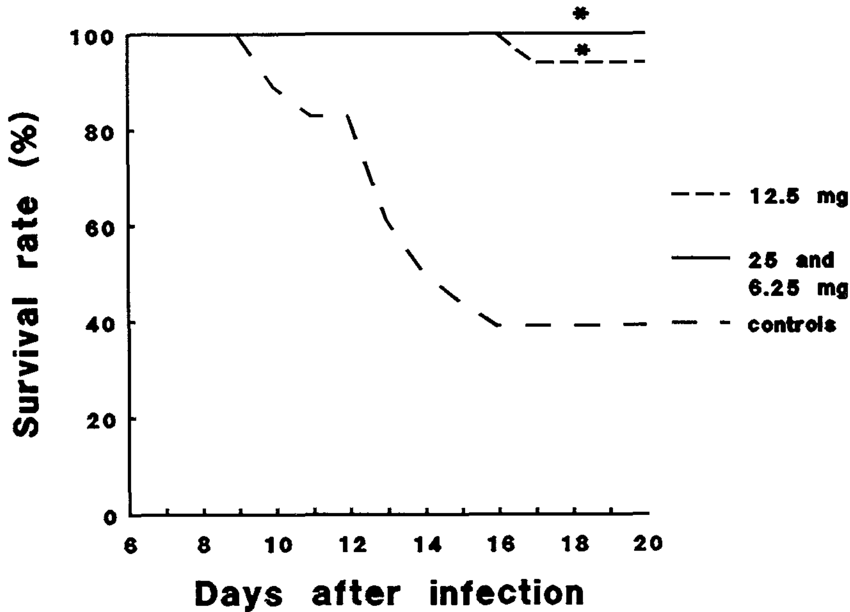

Figure 2. Survival rates of study 2. Animals were infected directly after the immunization period (day 10) with low-dose influenza A. * $p \leq 0.05$, survival of immunized animals vs survival of controls, Kaplan-Meier product-limit survival estimates.

Study 5. Immediately after immunization (day 10), there was no difference between the immunized and control group with respect to thorax-lung compliance, protein concentrations in BAL fluid or surface tensions of BAL fluid (table 1). On the fifth day after infection (day 15) thorax-lung compliance was significantly decreased in the control group compared to day 10 and was significantly lower than in the immunized group. At day 15, protein concentrations in BAL fluid were significantly increased in both groups compared to day 10. However, concentrations remained significantly lower in the immunized group compared to controls indicating a more increased permeability of the alveolo-capillary membrane in the control group (table 1).

Also on day 15, minimal surface tension of BAL fluid was significantly higher in the control group compared with immunized animals, indicating loss of surfactant in the control group (table 1).

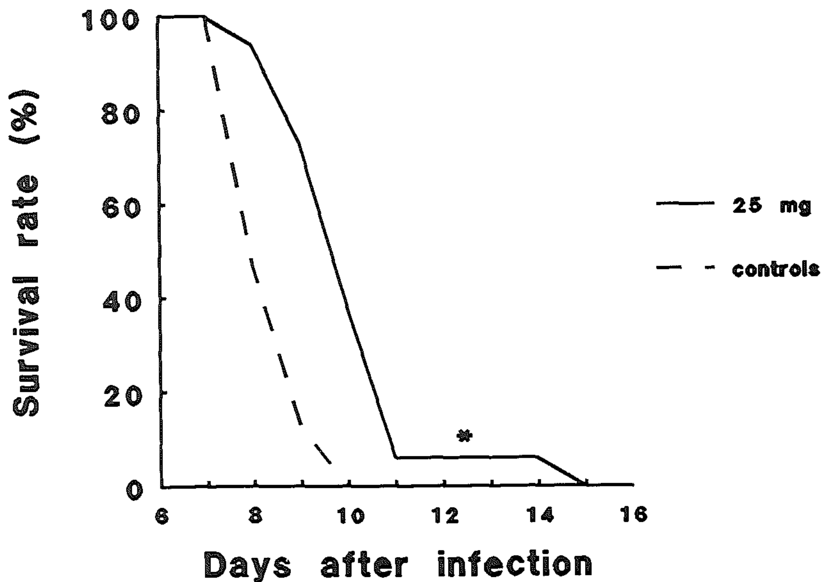

Figure 3. Survival rates of study 3. Animals were infected two weeks after the immunization period (day 24) with high-dose influenza A. * $p \leq 0.05$, survival of immunized animals vs survival of controls, Kaplan-Meier product-limit survival estimates.

Viability of the alveolar macrophages in BAL fluid (tested by trypan blue exclusion) ranged from 85-93%. Table 2 shows basal concentrations of cAMP of AMs before and after infection. Directly after immunization (day 10) AMs obtained from immunized animals showed less cellular activity, as concluded from significantly higher cAMP levels compared to AMs collected from control animals (table 2). Five days after infection, macrophageal activity in both groups had significantly increased as indicated by reduced cAMP concentrations in both groups. At this time, however, AMs collected from immunized animals showed significantly higher cellular activity than the AMs of control animals (table 2).

Table 1. Thorax lung compliance and protein concentrations in BAL fluid.

	Day 10	Day 15
C/kg (ml.cm $H_2O^{-1}.kg^{-1}$)	0.90 (0.24)	0.89 (0.19)
Protein conc. in BAL (mg/ml^{-1})	0.72 (0.26)	0.67 (0.10)

Surface active properties of 400 μl BAL fluid measured with a modified Wilhelmy Balance.

	Day 10		Day 15	
	immunized	controls	immunized	controls
Area (mN)	0.21 (0.16)	0.18 (0.10)	0.28 # (0.08)	0.27 # (0.10)
Maximal surface tension ($mN.m^{-1}$)	58.1 (5.9)	55.0 (5.4)	65.5 # (4.0)	64.7 # (2.7)
Minimal surface tension ($mN.m^{-1}$)	22.6 (4.7)	22.4 (3.7)	23.5 * (3.7)	27.1 # (4.0)

Thorax-lung compliance, protein concentrations in BAL fluid and surface active properties of BAL fluid obtained from immunized and control animals, directly after the end of the immunization period (day 10) and five days after infection (day 15). * $p \leq 0.05$, concentrations in macrophages from immunized animals vs controls, # $p \leq 0.05$, day 10 vs day 15. Mann-Whitney U test.

Incubation of AMs with PGE_2 (table 2), directly after immunization, significantly reduced cellular activity compared to basal levels in both groups. However, on the fifth day after infection, activity of AMs from immunized animals was significantly higher after incubation with PGE_2 than activity of AMs from control animals. Incubation with histamine (table 2) also resulted in significantly reduced macrophageal activity in both groups, although to a lesser extent than PGE_2. Directly after the immunization period cellular activity of AMs obtained from immunized animals was significantly more reduced than cellular activity of AMs from control animals. Five days after infection no difference could be observed between both groups.

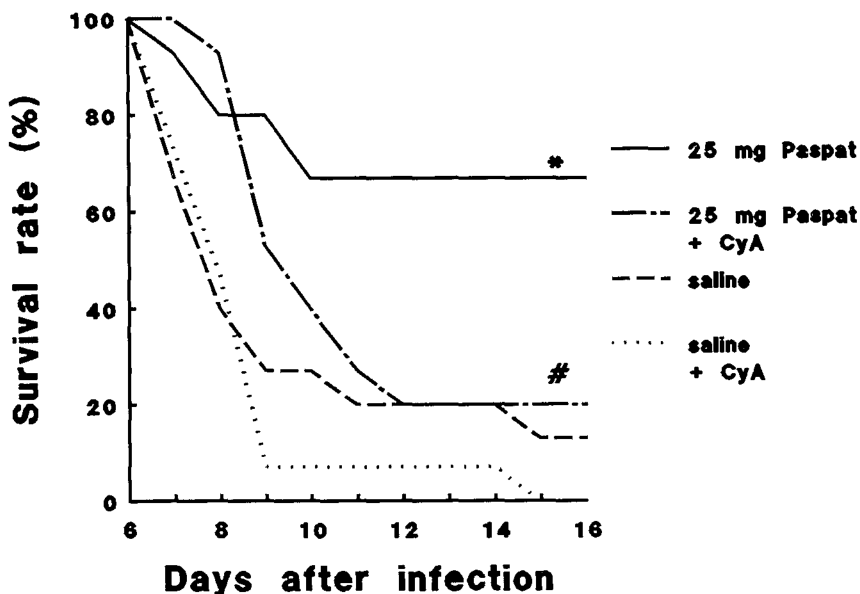

Figure 4. Survival rates of study 4. Animals were infected directly after the immunization period (day 10) with high-dose influenza A. * $p \leq 0.05$, survival of immunized animals vs survival of controls, # $p \leq 0.05$, survival of immunized immunosuppressed animals vs saline treated immunosuppressed animals, Kaplan-Meier product-limit survival estimates.

DISCUSSION

Most orally applied immunomodulators are developed empirically and consist of lysates of one or more different bacteria or bacterial components. Such preparations, applied orally, result in protection against exacerbation of chronic obstructive lung disease in infants [Maestroni & Losa (1984)] as well as in adults prone to frequent respiratory infection [Clancy, Cripps, Murree-Allen & Yeung (1985)]. Scientific acceptance of these preparations, however, is limited due to poor characterization of the active components and poor understanding of underlying mechanisms.

Table 2. Macrophageal activity.

	Day 10 immunized	controls	Day 15 immunized	controls
Basal conditions	0.43 * (0.01)	0.40 (0.01)	0.16 *,# (0.02)	0.19 # (0.03)
Histamine	1,21 * (0.29)	0.68 (0.15)	0.23 # (0.05)	0.26 # (0.05)
PGE_2	7.24 (0.93)	8.46 (1.44)	0.44 *,# (0.08)	0.80 # (0.07)

Concentrations ($pmol.10^{-5}$ cells) of cyclic-AMP (cAMP) in alveolar macrophages, obtained by bronchoalveolar lavage (BAL) from immunized and control animals, directly after the end of the immunization period (Day 10) or 5 days after infection with influenza A virus (Day 15). Concentrations of cAMP were measured directly after BAL (basal concentrations) or after stimulation with histamine or prostaglandin E_2 (PGE_2). * $p \leq 0.05$, concentrations in macrophages from immunized animals vs controls, # $p \leq 0.05$, day 10 vs day 15. Mann-Whitney U test.

Concentrations, administered in experimental and clinical studies on oral immunization, differ widely. The concentrations of Paspat oral® used in these studies are the same as those found to have a protective effect against S. pneumoniae in our previous studies on oral immunization [Van Daal, et al. (1990a); Van Daal, et al. (1990b)]. These high concentrations, also empirically found, are without adverse side effects as evidenced by a similar gain in bodyweight during the immunization period in both immunized and control groups (data not shown). Furthermore, immunization itself did not influence thorax-lung compliance, protein concentrations or surfactant activity in BAL fluid.
The dosages and time schedules used in these studies may not be directly applicable to the clinical situation. We do presume, however, that the presented results may help to further clarify the underlying principles of oral immunization.
The relative contribution of different immune mechanisms to resistance to infection is difficult to identify. It is, however, reasonable to suggest that both mucosal and systemic immune responses [Murphy & Clements (1989)] participate in this resistance. During the

first few days of influenza infection in nonimmunized hosts, the virus must inhibit or avoid nonspecific, cell-mediated defense mechanisms on, within, and below the surface epithelium of the respiratory tract [Sweet & Smith (1980)]. Therefore, in this study we wanted to investigate whether these cell-mediated mucosal defense mechanisms can be sufficiently enhanced, by oral immunization with a bacterial lysate, to protect mice against infection with influenza A.

It was found that oral immunization with Paspat oral® can reduce mortality due to influenza A infection with 15-70%, depending on the quantity of virus administered. Furthermore, protection by oral immunization seems to be dose-dependent, at least with a high virus dose. With a low virus dose, the protective effect of the lysate does not seem to be dose-dependent but lower concentrations of the lysate might have resulted in similar dose-dependent protection. Although in the groups infected two weeks after immunization (study 3) a significant increase was found in the immunized group with regards to survival time, oral immunization did not result in a reduced mortality at the end of the study, 20 days after infection. These findings suggest an important role for cell-mediated defense in the protective mechanisms stimulated by oral immunization with a bacterial lysate. It is improbable that specific antibody formation against influenza plays a major role in our experiments, considering the time schedule we applied in this study. Mice used in this study had not previously encountered influenza antigens and cross-reactivity of the bacterial lysate with influenza hemaglutinin antigens seems unlikely too, because we could not demonstrate hemaglutinating activity in BAL fluid of mice orally immunized with Paspat oral® (unpublished observations). In addition, the protective effect of oral immunization, as achieved in this study, almost entirely disappeared within two weeks and we opine that the protective effect must be looked for in nonspecific mechanisms, such as activation of cytotoxic or phagocytotic cells.

In several animal models, injection of a nonspecific immunomodulator, muramyl dipeptide (MDP), resulted in resistance against bacterial, viral and fungal infections. The involved mechanisms are thought to include interferon production [Sakuma, Suenaga, Yoshida & Azuma (1983)] activation of macrophages and amplification of numerous immune responses by release of lymphokines from macrophages [Chedid, Parant, Parant,

Lefrancier, Choay & Lederer (1977); Cummings, Pabst & Johnson (1980); Wyde, Six, Ambrose & Throop (1990)]. We therefore chose to suppress T-cell function with CyA to investigate the importance of T-cells in the protective effect of oral immunization in mice infected with influenza A. Cytotoxic T cells play an important role in the clearing of influenza virus [Ennis, Hua & Schild (1982)] and protect the infected host by recognizing and killing virus-infected target cells [Taylor & Askonas (1986)]. CyA completely blocks anti-influenza A cytotoxic T-cells [Amerding (1981)], reduces primary and secondary response of T_{helper}-cells [Hess, Tutschka & Santos (1982)] and thereby IFN-gamma production [Kalman & Klimpel (1983); Reem, Cook & Vilcek (1983)] whereas T-cell mediated suppression is not altered [Hess, Tutschka & Santos (1981); Leapman, Filo, Smith & Smith (1980)].

CyA severely reduced the protective effect that could be achieved by oral immunization in our survival studies, which implies that a major effect of oral immunization in these studies is T-cell activation and increased IFN-gamma production as described earlier [own observations (submitted); Emmerich, et al. (1990a); Emmerich, et al. (1990b)]. Oral immunization enhances NK cell cytotoxicity both in vitro and in vivo [Normier, Pniel, Dussourd D'Hinterland, Ramstedt & Wigzell (1985)], it might therefore be that enhanced activity of NK cells is responsible for part of the observed protective effect against influenza A infection in this study. Reduced IFN-gamma production by CyA results in reduced natural killer (NK) cell activity [Gui, Ho & Camp (1982)]. However, is has also been observed that NK activity after CyA immunosuppression in influenza infected mice was comparable to control levels. This state of tolerance for CyA was specific for influenza virus infection [Schiltknecht & Ada (1985)].

Numbers of AMs increase during the cellular response to influenza infection in humans and mice [Raut, Hurd, Cureton, Blandford & Heath (1975)]. IFN-gamma primes macrophages to react more violently to stimulation. Suppression of T-helper cells with CyA will result in decreased production of IFN-gamma [Kalman & Klimpel (1983)] and may thereby decrease macrophageal activity, although other authors found that activity of guinea pig peritoneal macrophages was not influenced by CyA [Thomson, Moon, Geczy & Nelson (1983)]. It is conceivable that, in this study, macrophages were already

activated to a cytotoxic state by oral immunization, before the application of CyA was started (one day before infection) and could therefore react more strongly to virus infection than control macrophages, which might also explain the observed difference in survival between immunized animals receiving CyA and control animals receiving CyA.
In patients with chronic bronchitis, oral immunization enhanced metabolism of alveolar macrophages [Munteanu, Schötler, Milatovic, Emslander, Daum & Emmerich (1986)]. Other authors described increased activity of murine peritoneal macrophages after stimulation in vitro with oral immunomodulators [Normier, et al. (1985); Vacheron, Périn, Kodari, Smets, Zalisz & Guenounou (1989)]. Our findings extend these observations to AMs of orally immunized animals after infection with influenza A.
Before infection basal activity levels of AMs in immunized mice were significantly lower than in control animals. However, five days after infection basal activity level of the AMs in immunized mice was significantly higher than AM activity in controls. Inflammatory pulmonary processes induce release of arachidonic acid metabolites [Peters, McGlashan, Schleimer, Hayes, Adkinson & Lichtenstein (1985); Rankin, Hitchkock, Merrill, Bach, Brashler & Ashkenase (1982)]. These metabolites (like PGE_2) stimulate adenylate cyclase and thereby increase intracellular cAMP concentrations, reflecting decreased cellular activity and decreased phagocytotic capacity [Adolfs & Bonta (1982); Ashby (1986)]. Stimulation of AMs with PGE_2 significantly reduced cellular activity in both groups, before and after infection. However, cellular activity of AMs from immunized animals was less reduced than cellular activity of control macrophages. This finding might indicate that AMs from orally immunized animals are less sensitive to the inactivating properties of arachidonic acid metabolites like PGE_2 and may, therefore, better maintain phagocytotic capacity than control macrophages.
Cellular activity of AMs of immunized animals was significantly more reduced by histamine than activity of control macrophages. These observations might indicate that cellular activity of AMs in orally immunized individuals might be reduced after histamine release during anaphylactoid reactions. This might prove especially important in asthmatics when the secretory functions of AMs are considered. Amongst a variety of other mediators, AMs secrete potent proinflammatory mediators [Henderson (1987)] like

thromboxane A_2, a constrictor of pulmonary vessels [Samuelsson, Goldyne, Granstrom, Hamberg, Hammarstrom & Malmsten (1978)], and leukotriene B_4, a potent chemoattractant for eosinophils [Goetzl, Pickett (1981)] which increases pulmonary vessel permeability [Dahlen, Bjork, Hedqvist, Arfors, Hammarstrom, Lindgren & Samuelsson (1981)] and constricts pulmonary airways [Whittle & Moncada (1983)].
Therefore, oral immunization might prove to be an interesting method to modulate cellular reactivity to allergenic stimuli, and further investigations in this direction seem necessary.

Summarizing, we conclude that oral immunization with Paspat oral• stimulates T cell dependent immune mechanisms, resulting in efficient protection against influenza A virus infection in mice. The survival rate of orally immunized mice infected with influenza A virus seems to be dependent on: lysate concentrations applied, inoculum dose, and time between the moment of infection and the end of the immunization period. Furthermore, AMs appear to play an active role in protection against influenza A infection resulting from oral immunization with Paspat oral•.

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Six

Introduction to pulmonary surfactant

CHAPTER VI

INTRODUCTION TO PULMONARY SURFACTANT

In the adult lung, alveoli are coated with a film of surfactant consisting mainly of phospholipids and surfactant-associated proteins. Surfactant "floats" on the hypophase like a soap-molecule and reduces the surface tension at the air-liquid interface of the alveolus which prevents the airspaces from collapsing at end-expiration [Clements '77, King '82]. Surfactant is produced by, stored in and excreted from the type II pneumocytes [Goerke '74, King '82]. The major intracellular pool of surfactant is found in the form of "lamellar bodies" in the type II cells [Chevalier '72]. When lamellar bodies are excreted by exocytosis they transform into so-called tubular myelin [Wright '87], an intermediate structure from which the surfactant spreads to the air-liquid interface of the alveolus to form the active film [Hallman '81]. Clearance of surfactant from the alveolar space occurs mostly by reuptake and reutilization of the surfactant lipids by the type II cells [Jacobs '83].

Composition of surfactant

Extracellular surfactant, obtained from dogs by bronchoalveolar lavage, consists of 8% protein, 90% lipid and about 2% carbohydrate [King '72]. Over 85% of surfactant lipids is phospholipid; phosphatidylcholine (PC), of which generally over 60% is disaturated (DPPC), makes up 70-85% of the phospholipid fraction. The remainder consists of other phospholipids of which phosphatidylglycerol (± 10% of total phospholipid fraction) makes up the largest fraction [King '84]. Relatively little interspecies difference is found with respect to the phospholipid fraction of pulmonary surfactant [King '84]. Without the presence of specific surfactant-associated proteins DPPC, which is the major surface tension reducing component of surfactant, does not adsorb and spread at a sufficient rate to the surface of the air-liquid interface to support normal lung function [Goerke '74, Notter '84, Van Golde '88]. At least four surfactant-associated proteins (Sp-A, B, C and D) are produced by type II cells [Dobbs '82, Wartell '83, Weaver '89, Liley '88, Persson

'89] which have, apart from functions such as the regulation of phospholipid metabolism, an important function in the formation of the surfactant monolayer [King '86, Possmayer '90].

Functions of surfactant

The primary functions of surfactant are stabilization of alveoli of different size, and reduction of the work of breathing by reducing surface tension [Clements '77]. Without the presence of a functional surfactant layer, small alveoli will empty into larger ones according to Laplace's law ($P = 2\Gamma/r$; P = pressure in the alveolus, Γ = surface tension at the air-liquid interface, r = radius of the alveolus). Surfactant changes the net surface tension in a manner related to the actual size of the alveoli. With decreasing size of the alveolus the surfactant film is concentrated and certain components are "squeezed out" into the hypophase until, at the end of maximal expiration, a densely-packed lining of phospholipids remains which exerts a pressure gradient that counteracts the surface tension of the alveolar walls with the hypophase. By the same mechanism, surfactant is partly responsible for maintaining functional residual capacity [Goerke '74, Clements '77]. Furthermore, surfactant functions as an anti-edema factor, by stabilizing the fluid balance of the lungs [Guyton '84]. Alveolar flooding will not occur as long as the suction force in the pulmonary interstitium exceeds the pressure gradient (which is dependent on the size of the alveolus) generated by the surface tension in the alveolar liquid interface.
Other functions of the surfactant system comprise involvemelx in pulmonary defense mechanisms [Huber '76, Jarstrand '84, Coonrod '86, van Iwaarden '90], non-ciliary and mucous clearance of particles from the airways [Green '73] and protection of the cardiocirculatory system against inhaled pharmacologically active substances [Smit '87].

Consequences of surfactant deficiency

Functional impairment of the surfactant system has far-reaching consequences for the functioning of the lung. Independent of the cause, decreased surfactant function will directly or indirectly lead to [Lachmann '84]:

- decreased pulmonary compliance
- decreased functional residual capacity of the lung
- atelectasis
- pulmonary edema with decreased gas exchange and respiratory acidosis
- hypoxemia with anaerobic metabolism and metabolic acidosis
- further inactivation of surfactant by plasma constituents
- enlargement of the functional right-to-left shunt of the lung

However, in some cases it is difficult, if not impossible, to prove that a surfactant deficiency is the cause for decreased lung function or presenting clinical symptoms.

Surfactant replacement therapy

Based on the above mentioned, a simple but rational philosophy was developed to manage the problems caused by surfactant deficiency [from Lachmann '88]:

1 If surfactant is necessary for keeping retractive forces as low as possible and for optimal gas exchange, any disturbance in the surfactant system will result in abnormalities in lung distensibility and gas exchange.

2 If 1 is correct, surfactant replacement will restore lung distensibility and gas exchange to normal.

Idiopathic respiratory distress syndrome (IRDS)

It has been established that IRDS/hyaline membrane disease results from prematurity of the newborn and its surfactant system [Avery '59]. Surfactant replacement therapy,

with different preparations, has been used with considerable clinical benefits in several studies on the treatment of IRDS [Fujiwara '80, Hallman '85, Gitlin '87] and in studies on the prevention of IRDS [Enhorning '85, Kwong '85, Shapiro '85, Merritt '86, Morley '87]. Although the beneficial effects of surfactant replacement therapy have not always been consistent [Avery '86], the overall effects comprise reduced mortality of IRDS and reduced morbidity of IRDS including reduced bronchopulmonary dysplasia.

Adult respiratory distress syndrome (ARDS) and ARDS-like lung diseases

Ashbaugh and coworkers introduced the concept of ARDS [Ashbaugh '67],which comprises respiratory failure resulting from sepsis, polytrauma, liquid aspiration, multiple organ failure, burns, pneumonia and many other diseases [Hopewell '77]. An operational definition of ARDS comprises the following features: an appropriate risk factor, severe hypoxemia refractory to increased inspiratory oxygen concentrations ($P_aO_2/F_iO_2 < 150$ mm Hg), decreased lung compliance, bilateral diffuse infiltration on the chest X-ray and severe pulmonary edema. In studies on the development of ARDS and in studies on established ARDS, compositional changes in surfactant and decreased surfactant content of the lungs were observed [Ashbaugh '67, Lachmann '77, Von Wichert '77, Petty '79, Hallman '82, Lachmann '84, Seeger '90]. Furthermore, biochemical abnormalities of lung surfactant in ARDS patients were present early in respiratory failure but tended to normalize during recovery [Hallman '82].

First clinical trials [Lachmann '87, Richman '89] and experimental studies [for review see Holm '89] showed that surfactant replacement therapy can restore lung function in ARDS of different etiology. Therefore, surfactant replacement therapy seems a promising approach for the treatment of acute respiratory failure in ARDS and ARDS-like syndromes.

To date, surfactant replacement therapy has been studied in only a limited number of ARDS-patients, whereas well over 15,000 babies suffering from IRDS have been treated with surfactant. The non-homogeneity of ARDS patients dictates further investigations to evaluate the efficacy of surfactant replacement therapy in animal models and patients

with ARDS from different etiology. Therefore, the usefulness of surfactant replacement therapy in ARDS-like respiratory failure caused by viral pneumonia is evaluated in the following chapters.

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Seven

Acute respiratory failure during pneumonia induced by Sendai virus.

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CHAPTER VII

ACUTE RESPIRATORY FAILURE DURING PNEUMONIA INDUCED BY SENDAI VIRUS

SUMMARY

In this study a model of acute respiratory failure due to viral pneumonia in rats, closely resembling ARDS, is presented. Severe respiratory failure with lethal outcome in four days was induced by infection concentrated Sendai virus aerosol. This model permits evaluation of different therapeutical approaches for improving gas exchange during ARDS. Furthermore, preliminary results of surfactant substitution therapy in this model are presented.

INTRODUCTION

Although there is no universal agreement on the criteria for the diagnosis of the adult respiratory distress syndrome (ARDS), the syndrome is characterized by distinct clinical and pathophysiological features (Ashbaugh et al., 1967; Petty and Ashbaugh 1971). ARDS can be the outcome of a number of disorders including shock of any etiology, infectious causes, trauma, drug overdose and many others (Hopewell and Murray, 1977). During the past 20 years little has changed in the possibilities for the treatment of ARDS and the mortality rate is still extremely high (50 - 70%) (Shale, 1987).
In this study a model of acute respiratory failure due to viral pneumonia in rats, closely resembling ARDS, is presented. This model permits evaluation of different therapeutical approaches for improving gas exchange during ARDS. Furthermore, preliminary results of surfactant substitution therapy in this model are presented.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (n=32, SPF, 170-200 g) were used. Rats were housed under filter bonnets and autoclaved food and water were available ad libitum. Infected animals were removed and kept separately in another facility. Twenty-four non-anesthetized animals were randomly assigned to four groups and were exposed for 90 min in an 11 liter aerosol chamber, through which an aerosol (1 : 2 dilution in PBS of stock solution) flow of 5 l/min was led. Aerosol was produced with an air jet nebulizer (Ultravent, Malinckrodt Diagnostica, The Netherlands). This device produces small particles, size 0.6 - 2 μm (Dahlbäck et al., 1986), which allows for alveolar deposition of the aerosol (Brain and Valberg 1979). Two animals were infected in the same way for histological examination of the lungs. Six additional animals served as healthy controls.

Virus

Sendai virus (Myxovirus parainfluenza type 1) is a single-stranded RNA virus (150-200 nm diameter) belonging to the family of the paramyxoviruses, to which several important human viruses also belong (e.g. mumps, measles and respiratory syncytial virus). Sendai virus generally replicates in respiratory epithelium (Ito et al., 1982; Ito et al., 1983; Tyrrell and Coid, 1970), primarily in bronchial and bronchiolar epithelium (Blandford and Heath, 1972). Sendai virus was propagated in 11-day embryonated chicken eggs. The hemagglutination (HA) titre of the stock solution was determined to be approximately 1:3000.

Evaluation of arterial blood gases and surfactant substitution

Each day one group of 6 animals was anesthetized (pentobarbital sodium 60 mg/kg i.p.), tracheotomized and a metal cannula was inserted into the trachea. A catheter (0.8 mm outer diameter) was inserted into the right carotid artery for drawing arterial blood samples. Animals were paralyzed (pancuronium 0.1 mg/kg i.m.) and mechanically ventilated with a Siemens 900c ventilator, in a pressure controlled mode, at a rate of

35/min, P_{peak} = 15 cm H_2O (15/0), I/E ratio 1:2 and FiO_2=1. Then peak-airway pressure was increased to 20 cm H_2O (20/0). Next, peak-airway pressure was increased to 25 cm H_2O and positive-end-expiratory-pressure (PEEP) of 4 cm H_2O (25/4) was introduced. At each different ventilator setting 15 min was allowed for stabilization before 0.3 ml blood was collected and arterial blood gases were determined (ABL 330, Radiometer, Copenhagen, Denmark). On the second day after infection, two animals received 1.5 ml exogenous natural, bovine surfactant (phospolipids 50 mg/ml) intratracheally and were monitored for two hours after surfactant substitution; airway pressures 25/4, rate 35/min, I/E ratio 1:2 and FiO_2=1.

Thorax-lung compliance registration

After evaluation of the influence of different ventilator settings on blood gases, animals were transferred to a body-plethysmograph (for details see Lachmann et al., 1980) and thorax-lung compliance was registered at a rate of 6/min, P_{peak} = 15 cm H_2O, I/E ratio 1:2 and FiO_2=1.

Statistical evaluation of data

Data are presented as mean ± standard deviation (SD). Statistical analysis of data was done with the Wilcoxon test for within-group comparison and with the Mann-Whitney U test for between-group comparison. Statistical significance was accepted at $p \leq 0.05$ (two-tailed).

RESULTS

Bodyweight of infected animals (182.8 ± 35.3 g, day 3 and day 4 group, n=12) increased during the first day after infection (202.1 ± 52.3 g); animals suffered significant weightloss during the second (197.3 ± 51.3 g) and the third day (173.0 ± 44.8 g) after infection. Furthermore, animals showed clinical signs of illness like ruffled fur, tachypnea, rhinitis and reduced motility from the first day after infection. Figure 1 shows mean arterial oxygen (P_aO_2) and carbon dioxide (P_aCO_2) tension of control animals and three

groups of infected animals, on three successive days after infection, during artificial ventilation (P_{peak} = 15 cm H_2O, I/E ratio 1:2 and FiO_2 = 1). With these ventilator settings P_aO_2 dramatically decreased from the first day after infection whereas P_aCO_2 significantly increased.

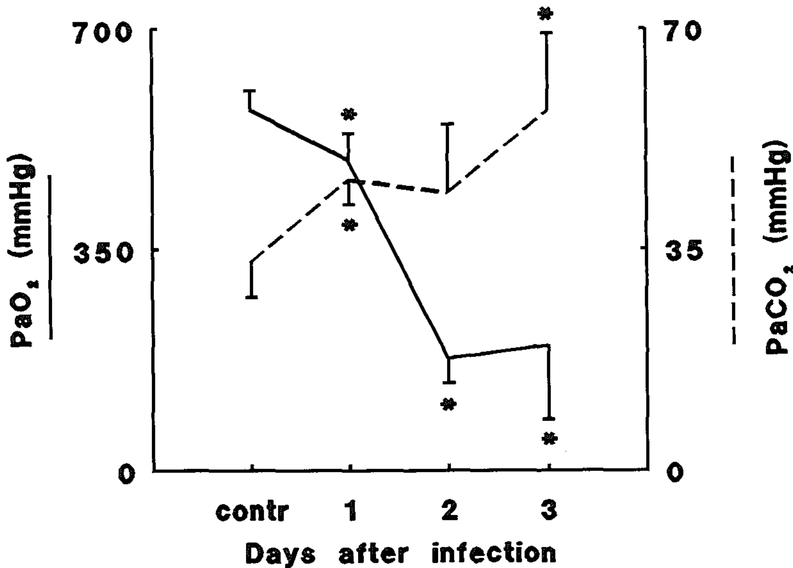

Figure 1. Mean arterial oxygen (P_aO_2) and carbon dioxide (P_aCO_2) tension of control animals and three groups of rats, infected with Sendai virus, on three successive days after infection, during artificial ventilation (P_{peak} = 15 cm H_2O, I/E ratio 1:2 and FiO_2 = 1). * $p \leq 0.05$, vs controls, n=6/group.

Arterial pH (Table 1) was significantly decreased in all groups of infected animals whereas arterial bicarbonate concentration (HCO_3^-) was not significantly different compared to controls. The remaining six animals (day 4 group) died spontaneously between the third and the fourth day after infection and blood gases and thorax-lung compliance could, therefore, not be evaluated.

Thorax-lung compliance was significantly decreased on the second and the third day after infection (Fig. 2) whereas protein concentrations of bronchoalveolar lavage (BAL) fluids were significantly increased on all three days after infection (Fig. 2).

The effect of increased peak airway pressures on P_aO_2 is shown in figure 3. Only on the second day after infection P_{peak} of 25 cm H_2O with PEEP of 4 cm H_2O significantly

Table 1.

	P_aCO_2 (mmHg)			pH			HCO_3^- (mmol/l)		
	15/0	20/0	25/4	15/0	20/0	25/4	15/0	20/0	25/4
contr.	32.7 (5.6)			7.49 (0.03)			24.4 (2.6)		
day 1	45.7# (3.9)	36.1* (3.7)	38.6* (3.0)	7.31# (0.02)	7.40* (0.03)	7.36* (0.03)	22.6 (1.3)	21.6 (1.1)	21.3 (1.2)
day 2	44.3# (10.8)	43.9 (10.4)	38.3 (8.1)	7.32# (0.1)	7.32 (0.09)	7.35 (0.07)	21.5 (1.0)	21.5 (1.5)	19.9 (1.5)
day 3	56.8# (12.3)	54.9 (12.6)	57.6 (14.0)	7.29# (0.07)	7.30 (0.08)	7.29 (0.08)	26.3 (2.0)	25.8 (1.9)	26.7 (1.7)

Influence of different ventilator settings on arterial PCO_2, pH and HCO_3^-, during the course of infection in rats infected with Sendai virus. Animals were mechanically ventilated with a Siemens 900c ventilator, pressure controlled, at different pressures at a rate of 35/min, I/E ratio 1:2 and $FiO_2=1$. Data are presented as mean (SD), n=6 per group. # $p \leq 0.05$, MW U-test, infected animals vs controls. * $p \leq 0.05$, Wilcoxon test, vs controls.

increased P_aO_2. Increased airway pressures did not result in significantly increased P_aO_2 on days one and three after infection. However, increased airway pressures significantly reduced P_aCO_2 and increased pH on the first day after infection whereas levels of significance were not reached on days two and three (Table 1). Increased airway pressures did not significantly influence HCO_3^- (Table 1).

The increase of airway pressures from 15/0 to 25/4 only slightly increased P_aO_2, whereas instillation of surfactant restored P_aO_2 almost to normal (Fig. 4).

Histological examination of lungs of two infected animals two days after infection showed alveolar flooding with protein rich fluid and atelectatic areas.

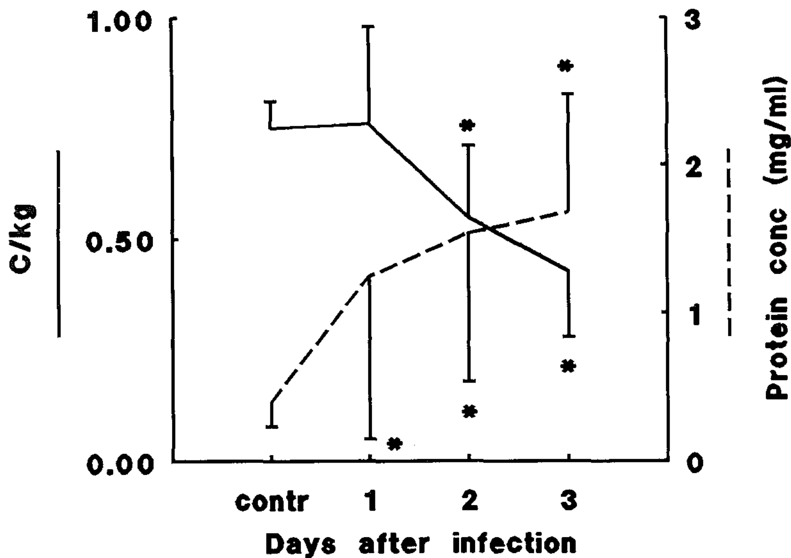

Figure 2. Thorax-lung compliance (C/kg) and protein content (mg/ml) of BAL fluid of rats infected with Sendai virus. * $p \leq 0.05$, vs controls, n=6/group.

DISCUSSION

We wanted to investigate whether we could induce severe respiratory failure in rats by infection with Sendai virus. Studies of *intranasal* inoculation of rats with high dose Sendai virus, as described by Giddens et al. (Giddens et al., 1987), performed earlier in our laboratories, did not result in hypoxemia or acidosis. This in spite of the fact that animals showed clinical signs of illness like ruffled fur, rhinitis, reduced motility and loss of bodyweight. However, animals spontaneously recovered within one week after infection. Therefore, we decided to employ concentrated live Sendai virus *aerosol* during an extended period of time. In this way we were able to induce severe respiratory failure with lethal outcome in four days. During the progression of the infection animals suffered significant loss of bodyweight and showed severe signs of illness.
At ventilator settings which resulted in hyperventilation and respiratory alkalosis in control animals, infected animals showed hypoventilation and respiratory acidosis as a result of mismatching between ventilation and perfusion of the lungs; P_aCO_2 of infected animals increased to 135% (day 2) and 173% (day 3) of P_aCO_2 of control animals.

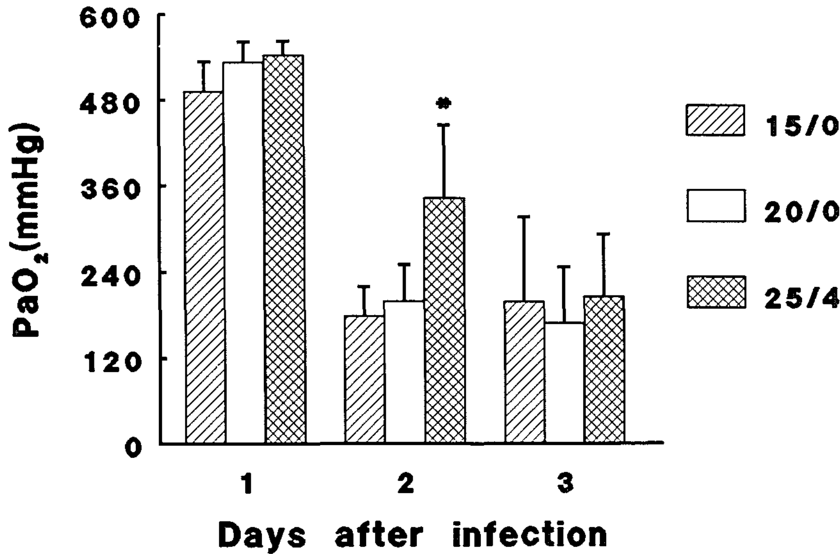

Figure 3. Effect of increased airway pressures on arterial oxygen tension (P_aO_2) during artificial ventilation of rats infected with Sendai virus. * $p \leq 0.05$, vs 15/0, n=6/group.

Furthermore, infected animals developed severe hypoxemia as a result of atelectasis, flooded alveoli and, as mentioned above, mismatching between ventilation and perfusion; P_aO_2 of infected animals decreased to 31% (day 2) and 35% (day 3) of P_aO_2 of control animals in three days.

An intact surfactant system is indispensable for the maintenance of proper lung function and therefore any type of surfactant deficiency, whether primary or secondary, will contribute to the development of severe pulmonary pathology.

It appears that loss of surfactant function is not the primary pathogenic factor for the occurrence of the severe respiratory failure in this study, but it may contribute significantly.

Infection with Sendai virus may lead to destruction of type I cells and loss of integrity of the alveolar-capillary membrane, allowing albumin and other macromolecules to enter the alveoli, as observed in this study. One of the common characteristics of ARDS is the accumulation of protein-rich edema in the alveolar space (Hopewell and Murray, 1977; Rinaldo and Rogers, 1982; Shale, 1987; Bradley, 1987).

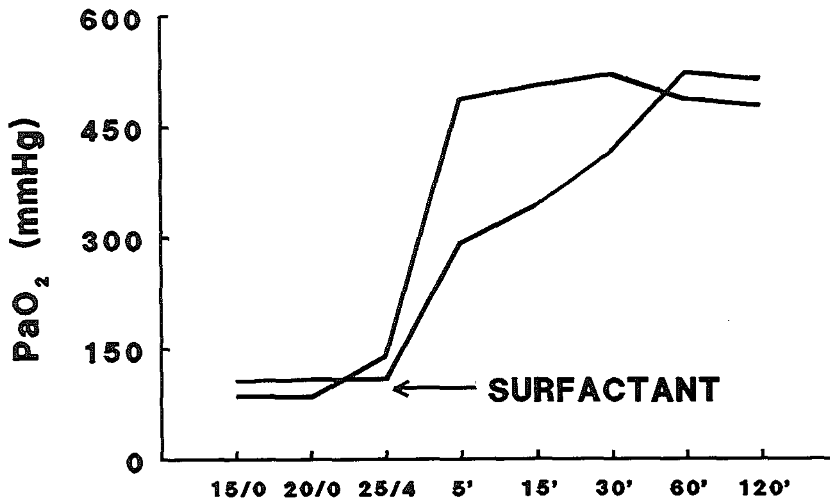

Figure 4. Effect of increased airway pressures and surfactant instillation on arterial oxygenation during artificial ventilation of 2 rats infected with Sendai virus.

Edema components like plasma proteins and cell membrane constituents inhibit the biophysical activity of the surfactant system (Holm et al., 1985; Holm and Notter, 1987; Ikegami et al., 1984; Fuchimukai et al., 1987; Seeger et al., 1985). Furthermore, destruction of type II cells may decrease surfactant synthesis and secretion.

Increasing ventilatory pressures to 20/0 did not significantly increase P_aO_2. Only P_{peak} = 25 cm H_2O with PEEP (4 cm H_2O) could significantly increase P_aO_2 of infected animals (92% increase) on day 2, which is evidence for a surfactant deficiency. Any tendency for alveolar collapse during endexpiration - which can be compensated only by counterpressure e.g. PEEP - speaks for a surfactant deficiency in these areas. To prove this, we performed surfactant replacement in two animals with severely reduced arterial oxygenation. Surfactant substitution restored arterial oxygenation to normal whereas increased airway pressures (25/4) alone did not; this indicates that surfactant deficiency is one of the major pathogenic causes of respiratory failure in this model of ARDS. However, since the applied ventilator settings were not very effective in restoring gas exchange, higher pressures or different modes of ventilation might have improved arterial oxygenation and reduced acidosis in infected animals.

Although there is no overall consensus on the criteria for the diagnosis of ARDS, the essential features of ARDS are met in this animal model (Pepe et al., 1982; Fowler et al., 1983; Stevens and Raffin, 1984). Infection of rats with Sendai virus led to acute respiratory failure with severe hypoxemia, increased intrapulmonary shunting, stiff lungs and pulmonary edema.

Surfactant replacement therapy seems to be a promising approach for the treatment of ARDS-like syndromes caused by viral pneumonia, as can be concluded from the preliminary results following surfactant replacement in this study. Several animal models of ARDS have demonstrated some form of surfactant deficiency or dysfunction and in seven of these models the beneficial effects of exogenous surfactant has been demonstrated: pulmonary oxygen toxicity (Holm et al., 1985), pulmonary damage induced by xanthine oxidase instillation (Saugstadt et al., 1984), in vivo lung lavage (Lachmann et al., 1980), bilateral cervical vagotomy (Berry et al., 1986), anti-lung serum infusion (Lachmann et al., 1987), induced damage to bronchial surfactant by artificial ventilation combined with saline instillation (Lachmann, 1985), and finally influenza A virus pneumonia in mice (Lachmann and Danzmann, 1984).

Although most of these models show characteristic features of ARDS, inevitable variations exist concerning the technique employed for the induction of pulmonary damage. Therefore, we opine that this virus model may help clarify the underlying principles of ARDS and from the various therapeutic approaches available elucidate which approach is most appropriate.

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Eight

Intratracheal surfactant administration restores gas exchange in experimental ARDS associated with viral pneumonia

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CHAPTER EIGHT

INTRATRACHEAL SURFACTANT ADMINISTRATION RESTORES GAS EXCHANGE IN EXPERIMENTAL ARDS ASSOCIATED WITH VIRAL PNEUMONIA

SUMMARY

The effect of intratracheal surfactant administration was studied in rats with adult respiratory distress syndrome (ARDS) associated with infection with nebulized Sendai virus. Thirty-six hours after infection animals (n=7) showed severely impaired gas exchange and acidosis during artificial ventilation (P_aO_2 = 152.2 ± 18.7, P_aCO_2 = 65.3 ± 19.2, pH = 7.26 ± 0.11) with a pressure- controlled mode, standard frequency 35/min, P_{peak} = 15 cm H_2O (15/0), I/E ratio = 1:2 and F_iO_2 = 1. Gas exchange improved (p=0.02) with increased ventilator pressures with PEEP (25/4). Fourty-eight hours after infection blood gas tensions could no longer be significantly improved by these same ventilator settings (P_aO_2 = 123.8 ± 31.0, P_aCO_2 = 95.1 ± 43.6, pH = 7.12 ± 0.16, n=9). At this time, surfactant replacement dramatically increased arterial oxygenation within five minutes (P_aO_2 = 389.4 ± 79.9) and resulted in a fourfold increase in P_aO_2 within two hours. It is concluded that intratracheal surfactant administration is a promising approach in the treatment of respiratory failure during ARDS associated with viral pneumonia.

INTRODUCTION

First conceptualized in the late Sixties (1), the adult respiratory distress syndrome (ARDS) is now recognized as the result of lung damage from a number of causes (2), including sepsis, polytrauma, aspiration, multiple organ failure, burns, pneumonia and many other conditions (3). Although there is no agreement on criteria for the diagnosis of ARDS, an operational definition of ARDS includes at least the following features: an

appropriate risk factor; severe hypoxemia refractory to increased inspiratory oxygen concentrations ($P_aO_2/F_iO_2 < 150$ mmHg); decreased lung compliance; bilateral diffuse infiltration on the chest radiograph and severe pulmonary edema. Presently, despite increased sophistication in methods of respiratory support, mortality associated with ARDS is still around 50 - 70%, whatever the criteria of diagnosis (4-6).

Development of respiratory failure in ARDS patients is highly correlated with surfactant abnormalities and the progressive time course suggests that compositional changes of alveolar phospholipid in ARDS reflect type II cell injury in the course of posttraumatic pulmonary insufficiency (7, 8). Furthermore, decreases in surfactant content are found in lung specimens from ARDS patients and abnormalities in lung elastic properties and increased surface tension of alveolar surfactant are present in postmortem bronchoalveolar lavage fluid (1, 9, 10, 11).

Clinical trials (12, 13) and data from experimental studies indicate that exogenous surfactant therapy can restore lung function in ARDS of different etiology (for reviews see 14 and 15). Therefore, although surfactant deficiency does not appear to be the primary pathogenic factor in ARDS, surfactant substitution may be a promising approach in the treatment of ARDS (12, 13).

Because ARDS is often associated with pneumonia of different etiology, experimental models are required to investigate surfactant replacement therapy in this type of respiratory failure. There are indications that surfactant deficiency plays a role in the development of respiratory failure due to viral pulmonary infection (16, 17). These findings are supported by data obtained from mice with influenza A pneumonia which demonstrate positive therapeutic effects of surfactant replacement on lung mechanics (18). No other information is available, to our knowledge, concerning the therapeutic effects of surfactant substitution during respiratory failure due to viral pneumonia. Therefore, we recently developed a rat model of acute respiratory failure, closely resembling ARDS, caused by infection with Sendai virus (19).

We report the effects of exogenous surfactant therapy and artificial ventilation at different ventilator pressures on arterial blood gas tensions during respiratory failure.

MATERIALS AND METHODS

Male Sprague-Dawley rats (n=41, 180-200 g, SPF) housed under filter bonnets, with autoclaved food and water available ad libitum, were used. Approval was obtained from the institutional animal investigation committee. Infected animals were removed and kept in a separate facility under identical conditions. Sendai virus (Myxovirus parainfluenza type 1) was propagated in 11-day embryonated chicken eggs. The thus prepared stock solution had a hemagglutination (HA) titre of approximately 1:3000.

For inoculation, non-anesthetized animals were exposed for 90 min in an aerosol chamber, through which an aerosol (1:2 dilution of stock solution) with a flow of 5 l/min was led. Aerosol was produced with an air jet nebulizer (Ultravent, Malinckrodt Diagnostica, The Netherlands). This device produces small particles, 0.6 - 2 μm in diameter (20), which allows for alveolar deposition of the aerosol (21).

For measurement of arterial blood gas tensions, animals were anesthetized (pentobarbital 60 mg/kg i.p.), tracheotomized and a metal cannula was inserted into the trachea. A catheter (0.8 mm outer diameter) was inserted into the right carotid artery for drawing arterial blood samples. Animals were paralyzed (pancuronium bromide 0.1 mg/kg i.m.) and mechanically ventilated (Siemens Servo Ventilator 900C, Siemens Elema, Sweden) in a pressure controlled mode, standard frequency 35/min, peak-airway pressure (P_{peak}) of 15 cm H_2O (15/0), inspiratory/expiratory ratio (I/E ratio) of 1:2 and 100% oxygen ($F_iO_2=1$). These ventilator settings result in slight hyperventilation with moderate respiratory alkalosis in healthy Sprague-Dawley rats (19).

To assess the severity of respiratory failure blood gas tensions were also measured at higher ventilatory airway pressures at different times after infection. P_{peak} was first increased to 20 cm H_2O (20/0), then to 25 cm H_2O with a positive end-expiratory pressure (PEEP) of 4 cm H_2O (25/4). At each ventilator setting 15 min was allowed for stabilization before a 0.3 ml arterial blood sample was collected for measurement of blood gas tensions (ABL 330, Radiometer, Copenhagen, Denmark).

Treatment with surfactant or saline (decided by prior randomization) was performed only in animals with arterial oxygen tension (P_aO_2) below 150 mm Hg ventilated at 15/0 and

in which increased ventilatory airway pressures (25/4) did not increase P_aO_2 above 175 mm Hg.

The effect of surfactant substitution with a natural bovine surfactant, extracted in basically the same way as described by others (22), was evaluated 48 h after infection; 22 animals met the preset criteria for treatment. After randomization, 9 animals were treated with 1.5 ml surfactant (200 mg phospholipids/kg bodyweight) intratracheally, whereas 7 control animals received 1.5 ml saline intratracheally and 6 other control animals received no treatment whatsoever except for continuation of the same ventilatory support that was used in the treated animals (25/4). After treatment, ventilator settings remained unchanged and blood gas tensions were monitored for 2 h afterwards.

After the experiment, the chest of each animal was opened and a cannula inserted into the pulmonary artery. The lungs were inflated with a pressure of 20 cm H_2O and maintained at this pressure while being perfused with formalin (4%) through the pulmonary artery. After perfusion, the lungs were removed and kept in formalin (10%) for at least 48 h. Paraffin sections from the right middle lobe were then stained with hematoxylin and eosin and examined microscopically.

All variables were analyzed by the Mann-Whitney U test for between-group comparisons or the Wilcoxon signed rank test for within-group comparisons. All tests of statistical significance were two-tailed and data were tested at the 0.05 significance level. All data are presented as mean ± standard deviation.

RESULTS

At 36 h after infection (table 1), during artificial ventilation (15/0), animals already showed severe signs of respiratory insufficiency with reduced P_aO_2, increased arterial carbon dioxide tension (P_aCO_2) and acidosis. Increasing the ventilatory airway pressures to 20/0 did not significantly change arterial blood gases; further increase of peak airway pressure and introduction of PEEP (25/4), however, almost completely restored arterial blood gas tensions to normal values.

Table 1.

	15/0	20/0	25/4
pO_2 (mmHg)	152.2 (18.7)	162.1 (50.7)	456.5 * (65.6)
pCO_2 (mmHg)	65.3 (19.2)	60.5 (14.3)	46.8 (8.2)
pH	7.26 (0.11)	7.27 (0.08)	7.35 (0.08)

Blood gas tensions and acid-base status, mean (SD), of seven animals after infection with a lethal dose of Sendai virus, during artificial ventilation. Animals were mechanically ventilated with a Siemens 900c ventilator, in a pressure controlled mode, at a rate of 35/min, I/E ratio 1:2 and $FiO_2=1$. Different ventilator pressures were applied: P_{peak} = 15 cm H_2O (15/0), P_{peak} = 20 cm H_2O (20/0) or P_{peak} = 25 cm H_2O with PEEP = 4 cm H_2O (25/4). * $p < 0.05$, vs 15/0.

At this stage during the course of the infection, criteria for treatment were not met and therefore surfactant was not injected. Twelve hours later (48 h after infection), six animals had died spontaneously and five animals died during ventilation at 15/0. Therefore, immediately after tracheotlxy, higher airway pressures were applied but reduced P_aO_2 {101.8 ± 20.7 (20/0, n=22) and 123.7 ± 34.1 (25/4, n=22), respectively} persisted. These animals were then randomly assigned to three groups. Before treatment there existed no significant differences in P_aO_2, P_aCO_2, arterial pH and arterial bicarbonate concentrations (HCO_3^-) between these groups (table 2).
Five minutes after surfactant instillation, P_aO_2 and pH had already significantly increased (figure 1) while P_aCO_2 decreased significantly below pretreatment values. During the entire observation period, P_aO_2 in the surfactant treated group was significantly higher than in the saline group and in untreated controls. At the end of the observation period (2 h after treatment) P_aO_2 in the surfactant treated group had increased fourfold compared with pretreatment values. P_aO_2 values in the saline group and the untreated control groups increased slightly during the 2 h ventilation period. These increases, however, were not statistically significant.

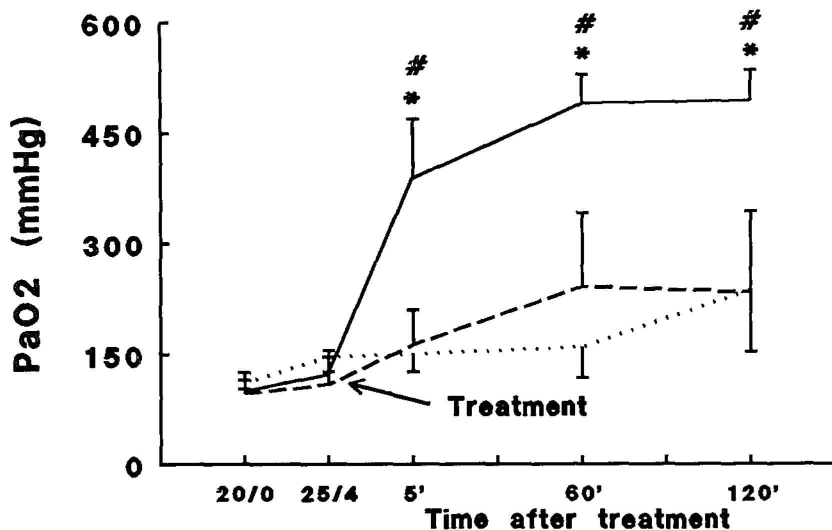

Figure 1. P_aO_2 values (mean ± SD) 48 h after infection with a lethal dose of Sendai virus, during artificial ventilation and after treatment with surfactant (solid line, n=9), saline (broken line, n=7) or respiratory support only (dotted line, n=6). Animals were paralyzed (pancuronium bromide 0.1 mg/kg i.m.) and mechanically ventilated with a Siemens 900c ventilator, in a pressure controlled mode, at a rate of 35/min, I/E ratio 1:2 and $FiO_2=1$. Different ventilator pressures were applied: P_{peak} = 20 cm H_2O (20/0) or P_{peak} = 25 cm H_2O with PEEP = 4 cm H_2O (25/4). Treatment with surfactant or saline was done at 25/4 ventilatory pressures and ventilator settings were kept unchanged during the entire observation period. * $p < 0.05$, surfactant treated animals vs saline treated and untreated controls, Mann-Whitney U test. # $p < 0.05$, vs pretreatment values at the same ventilator settings (25/4).

In the surfactant treated group and in both control groups P_aCO_2 decreased and pH concurrently increased during the observation period, whereas P_aCO_2 reached physiological values in the surfactant treated group only and pH did not reach normal values (table 2). There were no significant differences in P_aCO_2 or pH between the surfactant treated group and control groups.

Histological examination of rat lungs infected with Sendai virus (figure 2a) showed swelling of the alveolar walls and alveolar edema containing inflammatory cells. Furthermore, swelling of bronchial epithelial cells and atelectatic areas were present. Examination of lungs of infected animals 2 h after surfactant administration showed clearly improved lung aeration compared to the saline group and untreated controls (figure 2b and c).

Table 2.

	Before		After treatment		
$PaCO_2$ (mmHg)	20/0	25/4	5 min	60 min	120 min
surfactant n=9	105.8 (57.0)	95.1 (43.6)	61.9 # (14.9)	46.9 # (8.2)	42.6 # (8.3)
saline n=7	74.5 (21.0)	76.7 (17.6)	68.1 (21.2)	49.2 # (12.7)	54.6 (15.9)
untreated n=6	122.9 (27.1)	97.6 (23.2)	80.4 (11.3)	63.7 # (5.3)	55.9 # (7.3)

	Before		After treatment		
pH	20/0	25/4	5 min	60 min	120 min
surfactant n=9	7.13 (0.18)	7.12 (0.16)	7.22 # (0.09)	7.33 # (0.06)	7.33 # (0.11)
saline n=7	7.22 (0.11)	7.19 (0.11)	7.22 (0.14)	7.32 # (0.08)	7.31 (0.09)
untreated n=6	7.03 (0.05)	7.09 (0.07)	7.13 (0.04)	7.23 (0.03)	7.29 # (0.03)

P_aCO_2 and pH values, mean (SD), 48 h after infection with a lethal dose of Sendai virus, during artificial ventilation and after treatment with surfactant, saline or respiratory support only. Animals were mechanically ventilated with a Siemens 900c ventilator, in a pressure controlled mode, at a rate of 35/min, I/E ratio 1:2 and $FiO_2=1$. Different ventilator pressures were applied: P_{peak} = 20 cm H_2O (20/0) or P_{peak} = 25 cm H_2O with PEEP = 4 cm H_2O (25/4). Treatment with surfactant or saline was done at 25/4 ventilatory pressures and ventilator settings were kept unchanged during the entire observation period. * $p < 0.05$, surfactant treated animals vs saline treated and untreated controls, Mann-Whitney U test. # $p < 0.05$, vs pretreatment values at the same ventilator settings (25/4).

DISCUSSION

One of the main functions of the alveolar surfactant system is prevention of end-expiratory collapse of alveoli and small airways (23, 24). Furthermore, the surfactant system is essential for balancing lung water content. Pulmonary injury that impairs surfactant production or disturbance of the arrangement of phospholipids and surfactant-associated proteins in the alveolar lining, may, therefore, result in pulmonary edema. Clements predicted in the early Sixties, that, on theoretical grounds, alveolar surfactant plays an important role in the prevention of pulmonary edema (25) and later experimental studies clearly demonstrate that dysfunction of the surfactant system causes pulmonary edema (26, 27).

Findings in the present study suggest that surfactant deficiency is also an important factor in the pathogenesis of respiratory insufficiency due to Sendai virus pneumonia. Thirty-six hours after infection, animals show respiratory insufficiency which could only be overcome by a combination of high ventilatory peak airway pressure and PEEP. These ventilator settings almost completely restored blood gas to tensions to normal. However, that end-expiratory pressure - i.e. PEEP - is necessary to prevent alveolar collapse indicates the presence of surfactant deficiency 36 h after infection with Sendai virus.

The decreased thorax-lung compliance two days after infection (19) and our present findings that gas exchange can be restored to near normal 48 h after infection by exogenous surfactant are further confirmation of the surfactant deficiency in the presence of Sendai virus-induced ARDS.

The surfactant deficient state observed during Sendai virus pneumonia can be explained in several ways. In viral pneumonia, quantitative surfactant deficiencies are most likely to occur secondary to type II cell destruction (16). Destruction of type II cells by the inflammatory reaction to Sendai virus or by virus replication itself may result in depletion of surfactant stores in type II cells and decreased surfactant production by type II cells. Qualitative surfactant deficiency may result from biophysical inhibition of the surfactant material in the alveolar space. Protein rich edema fluid accumulated in the alveoli of infected animals contains potent inhibitors of the surfactant system, including plasma

proteins and red blood cell components. Studies on biophysical inhibition of surfactant activity have demonstrated that whole serum, hemoglobin and serum proteins such as albumin and fibrinogen can inhibit the function of lung surfactant (28-33). From these studies, it is clear that protein-induced inhibition of surfactant activity is enhanced by low surfactant concentrations. This indicates that the surfactant system in an already surfactant deficient lung - i.e. a surfactant deficiency as a result of destruction of type II cells by Sendai virus - is especially at risk for biophysical inhibition of surfactant function by the constituents of the alveolar exudate.

Loss of surface active material from the alveolar space via lymphatics or directly into the bloodstream may occur when the integrity of the alveolar-capillary membrane is lost during viral pneumonia. Normally, little surface active material is lost from the lung in this manner, but this situation might change when the alveolar epithelium is disrupted in the presence of lung injury (11, 34, 35). Furthermore, loss of surfactant-associated proteins by leakage from the alveoli or breakdown of surfactant-associated proteins during the inflammatory reaction to Sendai virus will seriously disturb surfactant function as these proteins are indispensable for proper functioning of the surfactant system (36-40).

One of the main goals in treatment of ARDS patients, adequate arterial and tissue oxygenation, can often be achieved by artificial ventilation with PEEP and increased inspiratory oxygen concentrations. In this study, increased ventilatory airway pressures plus PEEP could maintain arterial oxygenation for at least 36 h after infection with the applied ventilator settings.

After 48 hours, however, high inspiratory peak pressures together with PEEP (25/4) resulted in only minor (20%) increases in P_aO_2 (n=22), compared with low inspiratory peak pressure without PEEP (15/0). This can be explained by alveolar collapse resulting from increased intrapulmonary surface tensions. Higher peak-airway pressures are needed to open up the lungs and a PEEP of 4 cm H_2O is not high enough to prevent end-expiratory collapse, demonstrating the small benefits of the applied ventilatory strategy.

Instillation of surfactant almost instantaneously increased P_aO_2 significantly (320% increase within 5 min) and during subsequent 2 hours of observation P_aO_2 increased even further. In the saline and in the nontreated control groups P_aO_2 gradually increased whereas P_aCO_2 gradually decreased during the observation period, probably due to the application of PEEP.

Our findings indicate that, initially, a surfactant deficient state and resulting hypoxemia in the acute phase of viral pneumonia can be overcome by application of PEEP. In a later stage of the disease, however, a higher airway peak-airway pressure with PEEP has no significant effects because the opening pressure of the lungs is increased. Intratracheal instillation of exogenous surfactant, however, reduces the opening pressure, prevents alveolar collapse and, combined with ventilatory support, restores gas exchange to almost normal.

We conclude that lethal infection with Sendai virus in rats results in a surfactant deficiency of the lungs, which, initially, can be treated with respiratory support with high peak airway pressures and PEEP. When respiratory insufficiency is fully developed, artificial ventilation at high airway pressures with PEEP is not sufficient to restore gas exchange. At this stage of the disease, however, intratracheal instillation of exogenous surfactant can almost completely restore gas exchange to normal.

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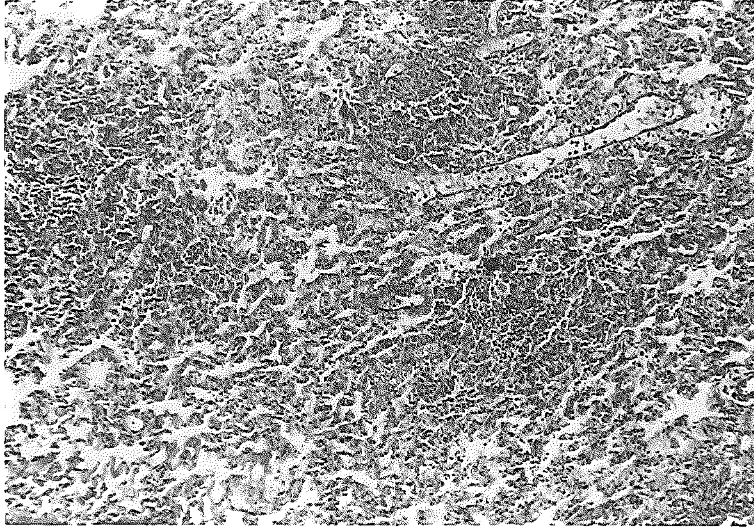

Figure 2a. Rat lung 48 h after infection with Sendai virus shows pneumonitis, interstitial swelling, alveolar exudate and atelectasis. Before fixation, lungs were inflated at a pressure of 20 cm H_2O. Original magnification x 120, Hematoxylin/eosin staining.

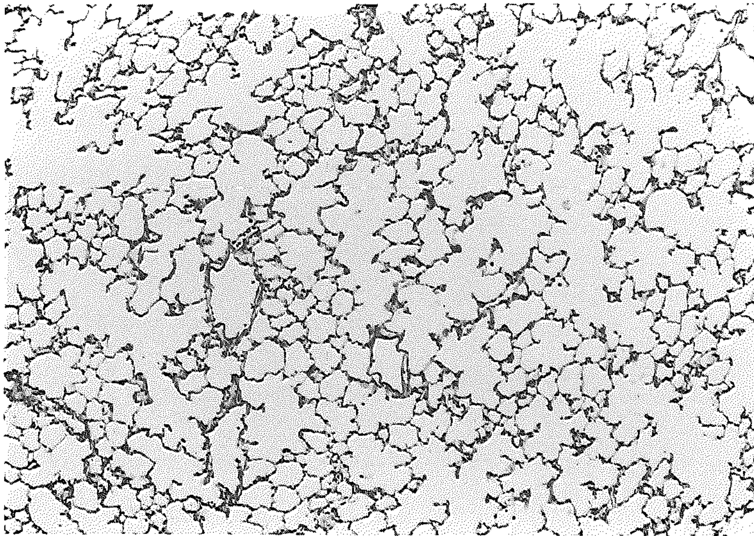

Figure 2b. Rat lung 48 h after infection with Sendai virus and 2 h after treatment with surfactant and artificial ventilation at 25/4. Before fixation, lungs were inflated at a pressure of 20 cm H_2O. Original magnification x 120, Hematoxylin/eosin staining.

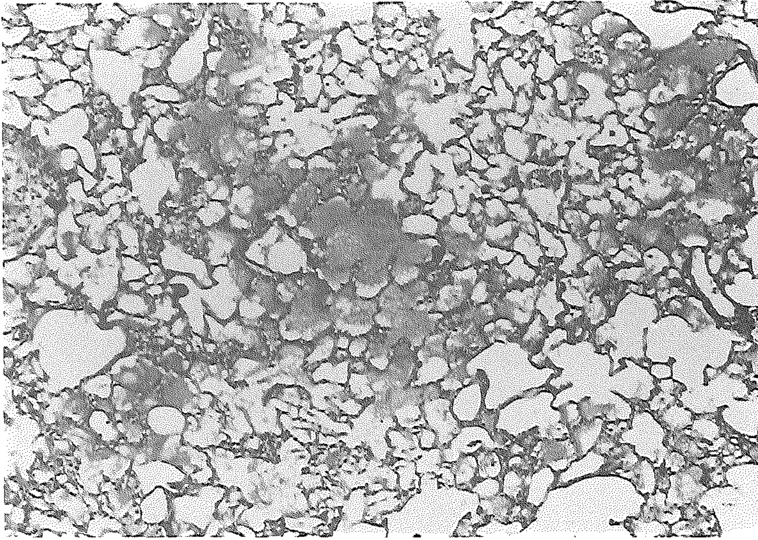

Figure 2c. Rat lung 48 h after infection with Sendai virus and 2 h after treatment with saline and artificial ventilation at 25/4. Before fixation, lungs were inflated at a pressure of 20 cm H_2O. Original magnification x 120, Hematoxylin/eosin staining.

Nine

Surfactant replacement therapy improves lung compliance and functional residual capacity in endstage influenza A pneumonia in mice

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Chapter IX

SURFACTANT REPLACEMENT THERAPY IMPROVES LUNG COMPLIANCE AND FUNCTIONAL RESIDUAL CAPACITY IN ENDSTAGE INFLUENZA A PNEUMONIA IN MICE

SUMMARY

Surfactant replacement therapy may be a promising approach for treatment of respiratory failure caused by virus pneumonia. This study in mice demonstrates that during the development of lethal influenza A pneumonia, thorax-lung compliance (C/kg) and functional residual capacity (FRC/kg) significantly decrease (28% and 54%, respectively) whereas lung water content significantly increases (25%). Furthermore, surface tension of broncholaveolar lavage fluid significantly increased, indicating loss of pulmonary surfactant. Surfactant replacement therapy during the endstage of pneumonia significantly increased C/kg by 31% and FRC by 21%. Instillation of solvent in control animals did not significantly affect pulmonary compliance (5% decrease) but significantly decreased FRC (25% decrease). Further, a new method for postmortem measurement of FRC in small laboratory animals - based on Archimedes' principle - is presented.

INTRODUCTION

Severe damage to the alveolar-capillary membrane caused by influenza A virus infection can lead to pulmonary edema, hemorrhage and death from respiratory failure and shock [1, 2]. Injury of type II cells decreases synthesis and excretion of surfactant [3, 4]. Therefore, disturbance of the pulmonary surfactant system may be an important factor in the etiology of respiratory failure during viral pneumonia, which might be treated by surfactant replacement therapy. If exogenous surfactant could restore lung mechanics in

influenza A virus pneumonia it may be a promising approach for treatment of respiratory failure caused by virus infection.

To appropriately evaluate the effect of surfactant replacement on lung mechanics it is essential to measure thorax-lung compliance (C_{tl}) ánd functional residual capacity (FRC). In most studies on the effect of surfactant replacement in animals with respiratory failure [for review see 5] FRC was not determined because an effective method for measuring FRC in small laboratory animals was not available. In this study we report the effects of lethal influenza A pneumonia in mice on C_{tl}, FRC and surfactant activity in bronchoalveolar lavage (BAL) fluid, and the effects of surfactant replacement on C_{tl} and FRC in endstage influenza A pneumonia. Further, a simple method for postmortem measurement of FRC in small laboratory animals is described.

MATERIALS AND METHODS

Eighty animals were randomly divided into 10 groups (scheme 1, n=8/group). Two groups served as controls, whereas eight groups were infected with a lethal dose of influenza A virus. In the control groups and in infected animals on the first, third and fifth day after infection the following protocol was executed. Bronchoalveolar lavage (BAL) was performed in one group for measurement of surface tensions of BAL fluid whereas in a second group C_{tl}, postmortem FRC and the lung wet/dry weight ratios were measured ($ratio_{w/d}$). Furthermore, on the fifth day after infection, in the two remaining groups C_{tl} was registered before and 10 min after intratracheal instillation of 0.15 ml surfactant or solvent. In these groups, postmortem FRC was obtained after the second registration of C_{tl}.

Scheme 1. Experimental setup.

controls BAL	day 1 BAL	day 3 BAL	day 5 BAL	day 5 surfactant, FRC, C_{tl}
controls C_{tl}, FRC, $ratio_{w/d}$	day 1 C_{tl}, FRC, $ratio_{w/d}$	day 3 C_{tl}, FRC, $ratio_{w/d}$	day 5 C_{tl}, FRC, $ratio_{w/d}$	day 5 solvent, FRC, C_{tl}

Animals

Male Swiss-bred mice (6-8 weeks old, SPF, obtained from Harlan/CPB, Zeist, The Netherlands) were used. Animals were housed under conventional conditions; water and food were given ad libitum. Infected animals were housed under the same conditions in a separate facility. Animals were kept under close surveillance and body weight (BW) was registered on the day of infection and on the day of the actual measurements.

Infection procedure

Influenza virus (A/PR8/34, H1N1) was passed once in 10 day embryonated chicken eggs. The allantoid fluid was clarified by centrifugation and stored at -70°C. The stock solution was diluted in sucrose and had a hemagglutination (HA) titer 1:400.
Animals were exposed once for 30 min to nebulized live virus suspension (8x dilution in PBS of stock solution) in a 11 liter multichamber aerosol box [6]. Aerosol with a flow of 5 l/min, particle size 0.6-2 μm [7], was produced with an Ultravent nebulizer (Ultravent, Malinckrodt Diagnostica, The Netherlands).

Thorax-lung compliance (C_{tl})

For measurement of C_{tl}, animals were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg, Nembutal®, Algin BV, Maassluis, The Netherlands) and tracheostomized, using a metal cannula as tracheal tube. Animals were paralyzed by intramuscular injection of 0.05 ml pancuronium bromide (Pavulon®, Organon Technika,

Boxtel, The Netherlands), transferred to a multichambered body plethysmograph [for details see 8] heated to 38°C, and connected to a ventilator system for pressure-controlled ventilation. The initial ventilator frequency was set at 30/min, I/E ratio 1:2, peak pressure 25 cm H_2O (25/0) and FiO_2 = 1; the mice were ventilated under these circumstances for 10 min to allow stabilization. Then C_{tl} was recorded at frequency 10/min. For calculation of compliance per kg BW (C_{tl}/kg), BW registered on the day of infection was used.

Postmortem FRC measurement

After registration of C_{tl}, the animals were killed with an overdose of pentobarbital and skinned. The thorax was prepared free, carefully leaving the diaphragm intact, whereas the intratracheal cannula was left in place. After this procedure the lungs were reexpanded with 0.5 ml of air to reopen atelectatic areas induced by the surgical procedure. Next, the lungs were connected for 10 min to 100% nitrogen at a pressure of 5 cm H_2O to allow for absorption of the remaining oxygen in the lungs. The trachea was then bound and the metal cannula removed, maintaining the positive pressure of 5 cm H_2O in the lungs.

First the total weight (W) of the thorax was registered and then the thorax was immersed in saline at a preset depth to measure the upward force (F) caused, according to Archimedes' principle, by displacement of a volume of saline. FRC was calculated by the following formula*:

$$FRC = 0.99 * F - 0.94 * W$$

For calculation of the FRC per kg BW (FRC/kg), BW on the day of infection was used. After the FRC measurement, the lungs were excised for determination of the $ratio_{w/d}$. Dry lung weight was obtained after drying the lungs in a conventional microwave oven at 250 W for 1 h [9].

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* For deductive procedure of FRC formula see Appendix.

Bronchoalveolar lavage (BAL) and surface tension measurement

BAL was performed three times with a volume of 1 ml sterile saline preheated to 38° C (total volume 3 ml, recovery 2.3 ± 0.2 ml). BAL cells were removed from the lavage fluid by centrifugation (800 x g, 10 min at 4° C). Surfactant activity in BAL fluid was measured with a modified Wilhelmy balance (E. Biegler GmbH, Mauerbach, Austria) by applying 400 µl of BAL fluid onto a 71.3 cm^2 trough. For measurement of minimal surface tensions the area was compressed to 20% of the initial value.

Surfactant/solvent instillation

On the fifth day after infection one group received treatment with surfactant and one group was sham-treated with solvent.

After measurement of C_{tl} as described above, a positive end-expiratory pressure of 4-5 cm H_2O was introduced; the rest of the ventilator settings was kept unchanged. Next, 0.15 ml of a natural bovine surfactant (200 mg/kg BW solved in 1:2 mixture of H_2O and saline) extracted in basically the same manner as described by others [10] or 0.15 ml solvent (1:2 mixture of H_2O and saline) was instilled into the lungs. A period of 10 min was allowed for stabilization and C_{tl} was registered again. After the second registration of C_{tl}, FRC was measured as described above.

After the experiment, the chest of each animal was opened and a cannula was inserted into the right ventricle. The pressure of 5 cm H_2O in the lungs was maintained while the lungs were perfused with formalin (4%) via the right ventricle. After perfusion, the lungs were removed and kept in formalin (10%) for at least 48 h. Paraffin sections were stained with hematoxylin and eosin and examined microscopically.

Statistical analysis and presentation of data

Statistical analysis of data was performed using the Mann-Whitney U test for analysis of intergroup differences or the Wilcoxon test for analysis of intragroup differences. Statistical significance was accepted at $p \leq 0.05$ (two-tailed). All data are expressed as mean ± SD.

RESULTS

From the third day after infection on, animals showed significant loss of BW (Table 1) and suffered from clinically manifest illness displaying ruffled furs, difficulties with moving and audible breathing difficulties.

C_{tl}/kg and FRC/kg (Table 1) were significantly decreased on the third and the fifth day after infection.

Surfactant replacement on the fifth day after infection (Table 2) significantly increased both C_{tl}/kg (compared to values obtained before instillation) and FRC/kg (compared to animals on the fifth day after infection, not receiving treatment with surfactant or solvent). Solvent instillation did not significantly change C_{tl}/kg (compared to values obtained before instillation). However, solvent instillation significantly reduced FRC/kg compared to animals on the fifth day after infection receiving no treatment, and surfactant treated animals (Table 2). Furthermore, both minimal and maximal surface tension of BAL fluid (Table 1) were significantly increased on the fifth day after infection compared to control values, indicating significant loss of surfactant activity. $Ratio_{w/d}$ was significantly increased on the third and fifth day after infection, compared to healthy controls (Table 1).

Histological examination of mouse lungs five days after infection with influenza A virus showed severe atelectasis and alveolar filling with leukocyte rich exudate. Histological examination of surfactant treated lungs shows clearly improved lung aeration compared to the solvent treated lungs (Figures 1A and 1B).

DISCUSSION

Influenza A virus pneumonia is characterized by permeability edema caused by disruption of the integrity of the alveolar-capillary membrane [11]. Pulmonary edema, in this study reflected by increased $Ratio_{w/d}$, was associated with decreased pulmonary compliance, decreased FRC and decreased surfactant activity in BAL fluid; findings which are confirmed by other studies on pneumonia-induced pulmonary edema [12-19].

Table 1.

	controls	Days after infection 1	3	5
Change of BW (%)		+5.5 (2.2)	-10.4 * (1.5)	-21.1 * (1.6)
$R_{w/d}$	4.28 (0.51)	4.21 (0.22)	4.83 * (0.28)	5.34 * (0.78)
FRC/kg (ml/kg)	20.8 (2.5)	20.0 (2.1)	14.1 * (2.4)	9.5 * (3.3)
C_{tl}/kg (ml/kg/cm H_2O)	0.57 (0.08)	0.56 (0.06)	0.46 * (0.06)	0.41 * (0.14)
Maximal surface tension (dynes/cm)	58.3 (3.7)	58.5 (4.2)	55.5 (6.6)	63.9 * (1.7)
Minimal surface tension (dynes/cm)	22.5 (1.7)	21.6 (1.4)	21.1 (3.3)	32.2 * (2.1)

Body weight (BW) changes, functional residual capacity (FRC/kg) at 5 cm H_2O, thorax-lung compliance (C_{tl}/kg) and lung wet/dry weight ratio ($R_{w/d}$) of control mice and mice infected with lethal dose influenza A virus. Mean (SD), n = 8/group. * = $p \leq 0.05$, Mann-Whitney U test.

Impaired lung function during pulmonary edema can be explained by impairment of the surfactant function by edema constituents. Constituents like albumin, fibrinogen and other plasma components are potent inhibitors of the surfactant system [20-25]. Impaired surfactant function will lead to decreased lung distensibility and collapse of alveoli and small airways leading to atelectasis, enlargement of the right-to-left shunt due to perfusion of non-ventilated alveoli, hypoxemia and acidosis [26-28]. Furthermore, the surfactant system itself is essential for stabilization of the fluid balance in the lung and for prevention of pulmonary edema [29-31]. In general, alveolar flooding will not occur as long as the suction force in the pulmonary interstitium exceeds the pressure gradient generated by surface tension in the alveolar air-liquid interface [32].

Table 2.

	Surfactant group		Solvent group	
	Before	After	Before	After
C_{tl}/kg (ml/kg/cm H_2O)	0.34 (0.15)	0.52 * (0.28)	0.35 (0.14)	0.32 (0.15)

	Healthy	Healthy corrected	Untreated day 5	Surfactant treated day 5	Solvent treated day 5
FRC/kg (ml/kg)	20.8 (0.04)	14.7 (2.2)	9.5 (3.3)	14.0 #,$ (2.3)	4.3 (1.9)

Influence of surfactant or solvent instillation on thorax-lung compliance (C_{tl}/kg) and postmortem functional residual capacity (FRC/kg) on the fifth day after infection with lethal dose influenza A virus. C_{tl}/kg at 25 cm H_2O, before and 10 min after instillation of 0.15 ml surfactant or solvent. * = $p < 0.05$, compared to values before treatment, Wilcoxon test. FRC/kg at 5 cm H_2O, after instillation of surfactant or solvent. # = $p \leq 0.05$ compared to solvent treated group, $ = $p \leq 0.05$ compared to untreated animals at the fifth day after infection, Mann-Whitney U test. n = 8/group.

Inhibition of the surfactant function by edema constituents therefore accelerates the formation of pulmonary edema and the development of respiratory failure, resulting in a vicious circle of surfactant inactivation and loss of pulmonary function. Another explanation for decreased surfactant function in this study is that influenza A virus infection leads to destruction of type II cells followed by a deficit of surfactant [3] which inevitably leads to the observed decreased compliance, FRC and pulmonary edema as described above and in other studies [4, 33].

From this study it cannot be concluded at which stage during the infection impairment of surfactant function significantly contributes to changes in pulmonary mechanics. Increased surface tension of BAL fluid on the fifth day after infection clearly indicates decreased surfactant function. This can be caused by loss of surfactant or by inhibition of surfactant. The fact that in vitro surface tension of BAL fluid was not changed on the

third day after infection, despite changes in lung mechanics, does not necessarily mean that impairment of surfactant function was not present in vivo.

It is conceivable that the inhibitory action of plasma proteins was diminished during measurement of surface tension with the Wilhelmy balance. Application of a small quantity of BAL fluid to the water phase of the Wilhelmy balance trough may have caused dissociation of the binding of inhibitory proteins to surfactant.

Surfactant substitution on the fifth day after infection restored C_{tl}/kg of infected animals almost to normal (from 60% of non-infected control values before substitution to 91% after surfactant substitution), which confirms preliminary data obtained in mice with influenza A pneumonia [33]. Instillation of solvent did not significantly affect C_{tl} (from 61% of non-infected control values before to 56% after instillation). Surfactant treatment also significantly improved FRC.

FRC/kg of infected animals on the fifth day after infection was 46% of non-infected control values. FRC/kg of the surfactant treated group was 67% of non-infected control values whereas FRC/kg in the solvent group was only 21% of non-infected control values. FRC was not completely restored to control values by surfactant.

One reason for this could be that instillation of 0.15 ml surfactant or solvent leads to filling of the FRC with fluid, resulting in a 0.15 ml decrease of FRC. Therefore, to take into account the effect of instillation of a certain amount of fluid 0.15 ml should be subtracted from control FRC values (or added in treated animals) to correct for the reduction of FRC by the volume of the instilled dose (assuming that no resorption of fluid takes place). If 0.15 ml is subtracted from control FRC/kg values (20.8 $\pm$ 0.04 ml), the corrected FRC/kg (FRC'/kg) in control animals becomes 14.7 $\pm$ 2.2 ml. FRC/kg of infected animals after surfactant or solvent instillation becomes then 95% and 29% of FRC'/kg, respectively.

The postmortem method for measurement of FRC as described in this study is highly reproducible and gives an accurate estimation of postmortem FRC, taking into account factors like body size, sex, age and, last but not least, the existence of lung disease.

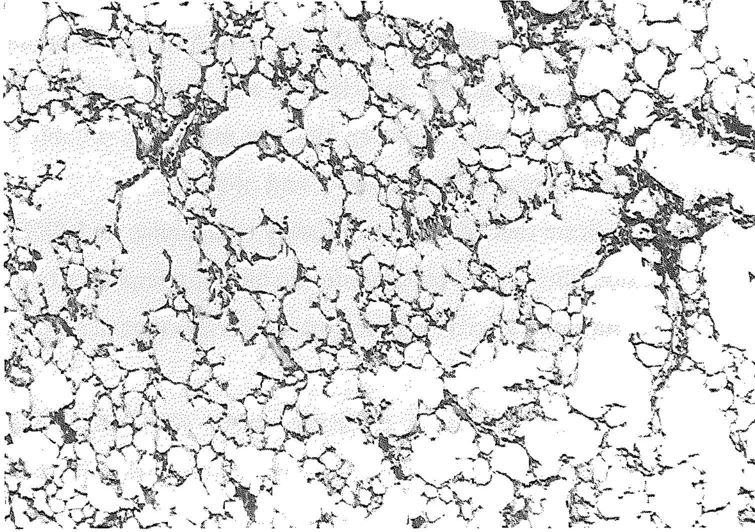

Figure 1 A. Mouse lung 5 days after infection with influenza A virus and after treatment with surfactant and artificial ventilation at 25/4. Before fixation, lungs were inflated with pure nitrogen at a pressure of 5 cm H_2O. Original magnification x 240, Hematoxylin/eosin staining.

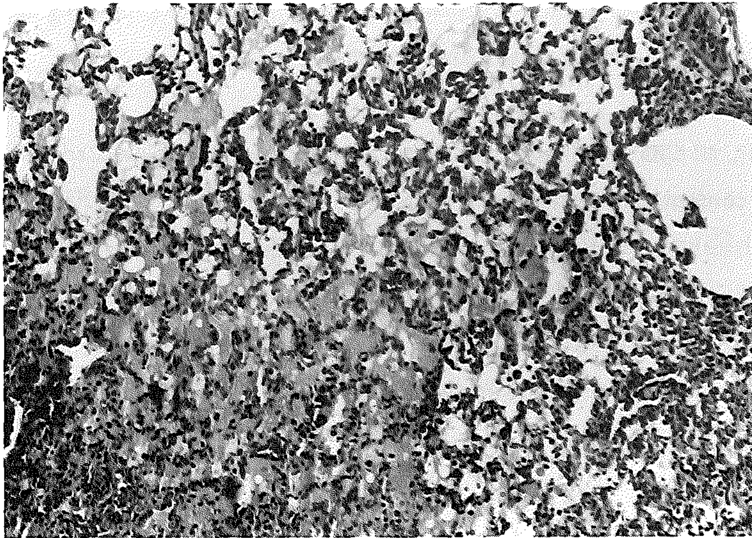

Figure 1 B. Mouse lung 5 days after infection with influenza A virus and after treatment with solvent and artificial ventilation at 25/4. Before fixation, lungs were inflated with pure nitrogen at a pressure of 5 cm H_2O. Original magnification x 240, Hematoxylin/eosin staining.

However, with this postmortem method, several factors that have profound influence on FRC in vivo, like posture, diaphragmatic muscle tone and blood content of the thorax are not taken into account. We opine, however, that the method is suitable for routine use in laboratory animals because of its simplicity and accuracy.

Findings in this study indicate that reduced pulmonary compliance and FRC caused by loss of surfactant function during viral pneumonia can almost completely be restored by surfactant replacement therapy, which is indirect proof for a shortage of surfactant during influenza A pneumonia.

In the winter of 1989/90 the influenza A epidemic caused 4100 excess deaths in the Netherlands (0.3 per 1000 population) whereas in the same period in the UK 7.700 patients died from pneumonia, of which 2440 were certified as being due to influenza [34, 35]. During the influenza epidemic of 1957 a close relationship between the degree of hypoxia and survival of severe influenzal pneumonia was observed [1, 2]. It is therefore desirable to further investigate the possibilities for restoring lung function during respiratory failure caused by viral or bacterial pulmonary infection. Based on findings in this study, we opine that surfactant replacement therapy seems a promising approach for the treatment of respiratory failure during viral pneumonia.

Summarizing, we conclude that influenzal pneumonia in mice is associated with increased lung water content and decreased pulmonary mechanics, including FRC, supposedly caused by disturbance of the integrity of the aveolar-capillary membrane and decreased function of the surfactant system. Surfactant replacement therapy can almost completely restore both C_{tl} and FRC, which will inevitably lead to improved gas exchange. These observations indicate that surfactant replacement therapy is a promising approach in the treatment of respiratory failure due to viral pneumonia.

APPENDIX

The formula for the calculation of FRC is based on Archimedes' principle that a body immersed in a fluid is subject to an upward force equal in magnitude to the weight of fluid it displaces.

The denotations in the equations are defined as follows:

FRC	the volume in the lungs filled with pure nitrogen at a pressure of 5 cm H_2O
W	the "dry" weight of the thorax
w	the presumed weight of the thorax as if it were made up of massive tissue, the FRC not containing air but tissue
F	the force exerted by the saline on the thorax, when immersed in saline at a preset depth
V	the volume of the thorax
D_{sal}	the density of saline (1.009)
D_{tiss}	the density of tissue (1.07) [36]

Two assumptions are made:

1) $F = V * D_{sal}$ and

2) $w = V * D_{tiss}$.

Subtraction of W from w gives the weight of the FRC as if it were made up from massive tissue:

3) $w - W = FRC * D_{tiss}$

Substitution of equation 2 into equation 3 gives:

4) $$V * D_{tiss} - W = FRC * D_{tiss},$$

which can be rearranged by inserting equation 1 to give:

5) $$F/D_{sal} * D_{tiss} - W = FRC * D_{tiss}.$$

Substituting $D_{sal} = 1.009$ and $D_{tiss} = 1.07$ gives:

6) $$F * 1.06 - W = FRC * 1.07,$$

which can be rearranged to give:

7) $$FRC = 0.99 * F - 0.94 * W.$$

Both F and W can easily be measured with a conventional balance.

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Ten

Summary and conclusions

CHAPTER X

SUMMARY AND CONCLUSIONS

PART I. ORAL IMMUNIZATION

Van der Heijden ended his thesis* on immunoglobulin production in the intestine of mice by concluding that *it must be possible to use the immune compartment of the intestine to induce protective immunity at the mucosae by means of safe vaccines containing non-replicating antigens*. This thesis and other studies on oral immunization (for overview see chapter 2 of this thesis) confirm his statement. In chapter 3 it was demonstrated that survival of mice after intranasal inoculation with S. pneumoniae could significantly be enhanced by previous oral administration of a polyvalent bacterial lysate. Although it is conceivable that increased sIgA production in the respiratory tract after oral immunization is involved in this protective effect, this is not proven by this study because sIgA was not measured. Furthermore, it is conceivable that enhanced nonspecific immune mechanisms are involved in the protection against respiratory pathogens after oral immunization. In chapter 4 it is reported that oral immunization not only reduces the pulmonary inflammatory reaction to infection with S. pneumoniae but also enhances T-cell activity, expressed as increased interferon-gamma concentrations in bronchoalveolar lavage fluid. The fact that enhancement of nonspecific immune mechanisms by oral immunization can indeed result in significant protection against respiratory pathogens is confirmed in chapter 5. This chapter reports that oral immunization with a polyvalent bacterial lysate results in effective protection against aerolized influenza A virus infection. It can be concluded from this study that T-cells and alveolar macrophages play an important role in the protection against influenza A virus, achieved by oral immunization. Summarizing, it is concluded from the first part of this

* Van der Heijden PJ. Quantitative aspects of immunoglobulin production in the intestine of mice. Thesis, Erasmus University Rotterdam 1990.

thesis that oral administration of a polyvalent bacterial lysate in mice can induce effective protection against bacterial and viral respiratory infection.

PART II. SURFACTANT REPLACEMENT THERAPY

In chapter 6 a short introduction to the pulmonary surfactant system and surfactant replacement therapy is given. To evaluate surfactant replacement therapy for the treatment of ARDS, experimental models are required that demonstrate as many characteristic features of ARDS as possible. Chapter 7 presents a new model of ARDS in rats, induced by Sendai virus infection. Preliminary results obtained in two animals indicate a positive effect of surfactant replacement therapy on arterial oxygenation. Subsequently, chapter 8 presents the results of a study on surfactant replacement in terminally-ill rats suffering from respiratory failure induced by Sendai virus infection. In the endstage of the pneumonia, gas exchange was severely impaired and arterial oxygenation was refractory to increased ventilatory pressures with PEEP. At this stage, however, exogenous surfactant induced a significant increase in arterial oxygen tension within five minutes after instillation, whereas no significant increase was observed in the control groups. In this study we did not investigate the effects of surfactant replacement therapy on pulmonary mechanics, because at that time no suitable technique for measurement of functional residual capacity (FRC) in small laboratory animals was available. Therefore, in chapter 9 a new technique, based on Archimedes' principle, is introduced for measurement of postmortem FRC in small laboratory animals. Chapter 9 reports that lethal influenza A virus infection results in a significant decrease in thorax-lung compliance, FRC and pulmonary surfactant paralell with a significant increase in lung water content. Further, it is demonstrated that surfactant replacement therapy at endstage pneumonia significantly improves thorax-lung compliance ánd FRC whereas in the control group thorax-lung compliance was not changed, but FRC was significantly decreased by instillation of solvent.
Summarizing, it is concluded from the second part of this thesis that experimental viral pneumonia may lead to severely decreased lung mechanics with impaired gas exchange.

Further, that in the terminal stage of viral pneumonia surfactant replacement therapy can almost completely restore lung mechanics and gas exchange and is, therefore, a promising approach for the treatment of respiratory failure caused by viral pneumonia.

SAMENVATTING EN CONCLUSIES

DEEL I. ORALE IMMUNISATIE

Van der Heijden besloot zijn proefschrift* over immunoglobulinen produktie in de darm van de muis met de konklusie dat *het in principe mogelijk moet zijn het immuunkompartiment van de darm te gebruiken om beschermende immuniteit aan de mucosae te induceren middels veilige vaccins die niet-replicerende antigenen bevatten.* Dit proefschrift en ander onderzoek naar orale immunisatie (voor overzicht zie hoofdstuk 2 van dit proefschrift) bevestigen deze stelling. In hoofdstuk 3 wordt aangetoond dat de overleving van muizen, die in de neus zijn geinfekteerd met S. pneumoniae, signifikant verbeterd kan worden door voorafgaande orale toediening van een polyvalent bakterielysaat. Alhoewel het redelijk is te veronderstellen dat een verhoogde sIgA produktie in de luchtwegen na orale immunisatie betrokken is bij het tot stand komen van dit beschermende effekt, wordt dit niet aangetoond in deze studie omdat sIgA niet is gemeten. Verder is het redelijk om aan te nemen dat versterking van niet-specifieke immuunmechanismen een rol spelen in de bescherming tegen respiratoire pathogenen waargenomen na orale immunisatie. In hoofdstuk 4 wordt beschreven dat orale immunisatie niet alleen de ontstekingsreaktie in de long na infektie met S. pneumoniae vermindert, maar ook de T-cel aktiviteit verhoogt, hetgeen tot uitdrukking komt in verhoogde interferon-gamma koncentraties in bronchoalveolaire lavagevloeistof. Dat door orale immunisatie versterkte niet-specifieke immuunmechanismen duidelijk bescherming bieden tegen respiratoire pathogenen wordt gedemonstreerd in hoofdstuk 5. In dit

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* Van der Heijden PJ. Quantitative aspects of immunoglobulin production in the intestine of mice. Proefschrift, Erasmus Universiteit Rotterdam 1990.

hoofdstuk wordt beschreven dat orale immunisatie met een polyvalent bakterielysaat effektief bescherming biedt tegen infektie met een influenza A aerosol. Uit deze studie kan gekonkludeerd worden dat T-cellen en makrofagen een aktieve rol spelen in de bescherming tegen influenza A virus, bereikt door orale immunisatie. Samenvattend wordt uit het eerste gedeelte van dit proefschrift gekonkludeerd dat orale toediening van een polyvalent bakterielysaat aan muizen resulteert in een effektieve bescherming tegen bakteriele en virale luchtweginfekties.

DEEL II. SURFACTANT VERVANGENDE BEHANDELING

In hoofdstuk 6 wordt een korte inleiding gegeven over het surfactantsysteem van de long en over behandeling met surfactant. Om surfactant vervangende behandeling voor ARDS te evalueren zijn proefdierenmodellen nodig die zoveel mogelijk karakteristieken van ARDS vertonen. In hoofdstuk 7 wordt een nieuw ARDS-model in ratten gepresenteerd, met Sendai virus als veroorzaker. Voorlopige resultaten verkregen van twee dieren laten een positief effekt zien van surfactanttoediening op de arteriele oxygenatie. Vervolgens worden in hoofdstuk 8 de resultaten getoond van een studie naar de effekten van surfactanttoediening bij ratten in het eindstadium van een respiratoire insufficientie, veroorzaakt door Sendai virus. In het eindstadium van de longontsteking was de gasuitwisseling ernstig verminderd en het zuurstofgehalte van het arteriele bloed kon niet verbeterd worden door verhoging van de beademingsdrukken met gelijktijdig gebruik van PEEP. In dit stadium echter resulteerde surfactant toediening in een signifikante stijging van de arteriele zuurstofspanning, binnen vijf minuten na toediening, terwijl geen signifikante verbetering optrad in de kontrolegroep. In deze studie werd niet het effekt van toediening van surfactant op de longmechanika onderzocht, omdat op dat moment geen goede techniek beschikbaar was in ons laboratorium voor het meten van de funktionele residuele kapaciteit (FRC) in kleine proefdieren. Daarom wordt in hoofdstuk 9 een nieuwe techniek geintroduceerd, gebaseerd op de Wet van Archimedes, voor het postmortem meten van de FRC in kleine proefdieren. In hoofdstuk 9 wordt getoond dat een lethale infektie met influenza A virus resulteert in een signifikante daling van de

thorax-long kompliantie, de FRC en het surfactant gehalte van de long, terwijl de hoeveelheid vocht in de long toeneemt. Verder wordt getoond dat behandeling met surfactant gedurende het eindstadium van de longontsteking resulteert in een signifikante verbetering van de thorax-long kompliantie én de FRC, terwijl in de kontrolegroep de thorax-long kompliantie niet verandert en dat de FRC zelfs signifikant verslechtert door toediening van het oplosmiddel.

Samenvattend wordt uit het tweede deel van dit proefschrift gekonkludeerd dat virale longontstekingen in proefdieren leiden tot een sterke verslechtering van de longmechanika en een verstoorde gasuitwisseling. Verder, dat behandeling met surfactant in het eindstadium van een virale longontsteking de longmechanika en de gasuitwisseling bijna volledig kan herstellen. Behandeling met surfactant lijkt daarom een veelbelovende manier om respiratoire insufficientie veroorzaakt door een virale longontsteking te behandelen.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 10 oktober 1960 te Amersfoort. In 1981 behaalde hij het diploma Gymnasium ß aan het Gymnasium Camphusianum te Gorinchem. Na het vervullen van de dienstplicht als reserve-officier der Artillerie begon hij in 1982 met de studie Geneeskunde aan de Erasmus Universiteit, waar hij in 1988 het doktoraalexamen behaalde. Gedurende zijn studie vervulde hij een student-assistentschap bij Prof Dr B Lachmann op de afdeling Experimentele Anesthesie van de Erasmus Universiteit. In 1988 trad hij als AIO in dienst van de Erasmus Universiteit alwaar tot eind 1990 op de afdeling Experimentele Anesthesie dit proefschrift werd bewerkt onder begeleiding van Prof Lachmann.

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