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ABSTRACT

Houses for intensive poultry production likely contain very high concentrations of airborne contaminants that may negatively affect human and animal health. However, very little is known of the relations between concentrations, size, nature and composition of airborne particles on animal health in intensive livestock housing. Also, mechanisms of responses of animals to unhygienic conditions such as airborne particles, and adaptation responses are unknown. It is likely that animals under high pressure for production such as broiler chickens may be affected severely by continuous antigenic stimulation. Accordingly, the aim of this thesis was to determine effects of airborne dust and its components, and particle size, respectively on the immune system of broilers, and consequently disease resistance and performance (in this case growth). The objectives were to address 1) dust concentrations and particle size distribution present in counts and in mass inside (and around) animal houses; 2) whether dust or its components (with emphasis on pathogen associated molecular patterns or PAMP) affect the immune competence and specific immune response of broilers after challenge via the respiratory tract at different ages; 3) whether broilers may adapt to respiratory challenge with dust and its different components, and particle size; 4) whether dust and its components including particle size affect growth (and heart parameters) of broilers; and finally 5) localization of 1 µm and 10 µm (fluorescent-labelled polystyrene) particles as a model for localization and transport of dust particles in the body of broilers after challenge via the respiratory route

In terms of mass, the dust concentration in poultry houses was generally higher than in pig houses, cattle houses, and mink houses. Mass concentrations of PM10 (particles with aerodynamic diameter smaller than 10 µm) was 0.83 to 4.60 mg m^{-3} in poultry houses, 0.13 to 1.62 mg m^{-3} in pigs farms, and 0.02 to 0.12 mg m^{-3} in cattle and mink farms. In counts, most particles (92%) inside were found smaller than 2.5 µm, whereas these particles only contributed for 2.6% to mass.

Fine dust and coarse dust collected from broiler houses also affected specific antibody responses to a model antigen (HuSA), either declining or enhancing, depending on age of challenge and isotype measured. Components known to be part of dust and with known or expected immunologically mediating features like lipopolysaccharide (LPS), β-glucan, lipoteichoic acid, chitin, NH$_3$, heat-dust, respectively, were used to intratracheally challenge broilers at 3 and 7 weeks of age. Especially LPS and β-glucan enhanced immune responses, but depressed body weight gain of the broilers after primary and secondary challenge. LPS

also enhanced antigen-specific responses at various ages, even when administered 4 weeks prior to the antigen. After intratracheal (and also cloacal) challenge, fluorescent-labelled polystyrene beads from two sizes (1 µm and 10 µm) were present in all tissues from the broiler studied during at least one week. Such beads might have been taken up by phagocytic cells or were transferred via the blood stream.

It was concluded that airborne particles in different sizes and with different components could alert the immune system of broilers as exemplified by enhanced primary responses in an antigen- nonspecific fashion. The absence of major effects of dust components on secondary immune responses on the other hand may indicate a regulating role of dust components on the immune system. Dust (components), however, had an important negative impact on body weight gain and heart parameters. It is concluded that there are relationships between hygienic conditions in broiler houses and immune mediated health, and as a consequence likely disease resistance and/or sensitivity to vaccination and other health management procedures. The current study urges further studies on the presence (and identification) and consequences of airborne constituents to protect health of poultry.

IN DEDICATION TO MY PARENTS, MY HUSBAND AND MY SON

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Poultry meat is high in protein and low in fat, is the second most widely eaten meat in the world, after pork, and accounts for 30% of world meat production (Raloff, 2003). In 2010, 81 million tons of poultry meat were delivered to the global market, with the US as leading producer, contributing 16.8 million tons (USDA, 2011). In the Netherlands, 103 million chickens were reared in 2010 on approximately 3000 chicken farms, which contributed 0.75 million tons of chicken meat to the market (CBS, 2011). Over the last decade intensive livestock production has contributed 2% to Dutch GDP; however, it contributed more than 15% to particulate matter (PM) emissions (Chardon and Van de Hoek, 2002). Intensive poultry production contributes over 50% to total PM emission from livestock production in the Europe (EMEP-CORINAIR., 2007).

Besides the high PM emission rates, one of the main problems in intensive poultry production in temperate areas is the bad air quality in poultry houses, which is caused by low ventilation rates, especially in winter time. Poultry houses have been found to have the highest dust concentrations of all livestock houses (Wathes et al., 1998). Dust production is especially high in floor systems with bedding. Animal welfare legislation has led to a ban on cages, thereby enabling the birds to use perches and to dust bathe in bedding material. These welfare demands probably affect air quality inside poultry houses. This aspect of animal welfare, which could seriously affect animal health, is often ignored or given little attention. Airborne dust in livestock houses may cause respiratory diseases in farmers and veterinarians (Andersen et al., 2004, Donham, 1993, Radon et al., 2001, Vogelzang et al., 1997). The dust in livestock houses has been characterized to some extent in terms of its concentration and its particle shape, particle size, density, and source (Cambra-Lopez et al., 2011a, Cambra-Lopez et al., 2011b). An airborne particle may contain many microorganisms: bacteria, fungi, and viruses. These particles can therefore spread animal and zoonotic diseases that may affect public health (Martin et al., 1996, Simecek et al., 1986, Seedorf et al., 1998). Furthermore, some components present in microorganisms can be very harmful for human and animal health. The main harmful components in airborne dust that have been described are endotoxins, lipoteichoic acid (LTA), β-glucans (BGL), chitin, and ammonia; it is probable that more harmful dust components will be identified in due course. Endotoxins or lipopolysaccharides (LPS) are components originating from the cell wall of gram-negative bacteria, LTA are components from gram-positive bacteria, BGL originate from fungi, chitin is derived from plants and arthropods, and ammonia from animal excreta. Eduard et al. (2009) have shown that intensive livestock farmers are at greater risk of respiratory morbidity and mortality. Aerial gaseous pollutants, e.g. ammonia and odorous compounds, are also found in

dust particles and these compounds can also affect human and animal health (Al Homidan and Robertson, 2003, Bolhuis et al., 2003).

Dust concentrations in animal houses are generally 10 to 100 times higher than concentrations in the outdoor environments (Zhang, 2004a). Concentrations of airborne dust have been measured in various studies (Cambra-López et al., 2009, Heber et al., 2006, Aarnink et al., 2004, Aarnink. et al., 2011). Regarding intensive poultry production in closed housing systems, a study by Takai et al. (1998) reported that mean dust concentrations in poultry houses were 0.45 mg m^{-3} for particles smaller than 5 µm and 3.6 mg m^{-3} for particles smaller than 100 µm.

Airborne dust from animal houses is mainly comprised of organic matter (up to 90%); the rest is inorganic (Aarnink et al., 1999, Seedorf and Hartung, 2001). Organic dust originates from feedstuff, manure, bedding, animal skin, feathers, and microorganisms (Aarnink et al., 1999, Martin et al., 1996). The contribution of each of these sources depends on several factors, such as housing system, type of bedding material, ventilation system, and animal activity, and it varies, depending on dust particle size (Heber et al., 1988b, Takai et al., 1998, Donham et al., 1986). In poultry houses, airborne dust primarily originates from feathers and manure in the litter (Cambra-Lopez et al., 2011b). Muller and Wieser (1987) reported that airborne dust in a floor layer system mainly originated from bedding material in the litter.

Endotoxin (LPS) is one of the major components of organic dust in animal houses that affects health. Endotoxins have proven to be harmful for farmers and their neighbours (Skorska et al., 2007, Muller et al., 2004, Schenker, 2004, Eduard et al., 2009). LPS is present in the outer membrane of gram-negative bacteria. In the Netherlands, the advised limit of the endotoxin level during an average 8-hour working day is 90 endotoxin units per m^3 (EU m^{-3}) ($\approx$ 9 ng m^{-3}) (The Health Council of the Netherlands, 2010). In Dutch poultry houses it has been found that the exposure levels are on average 10 to 20 times higher (Spaan et al., 2006). Wathes et al. (1997) measured LPS concentrations inside broiler and layer facilities in the UK and found that these varied between winter and summer, but were always much higher (by amounts ranging from 100 to 400 ng m^{-3}) than the recommended threshold. In a study on poultry houses, pig houses, and cattle houses Seedorf et al. (1998) found that the mean inhalable endotoxin concentrations (in particles <100 µm) were highest in poultry houses, where they ranged between 338.9 and 860.4 ng m^{-3}, while the respirable endotoxins (in particles < 5µm) in poultry houses ranged between 29.6 and 71.8 ng m^{-3}.

Another major component besides dust that determines the air quality inside poultry houses is ammonia (Hartung, 1995, Aarnink et al., 2006b). Ammonia is a colourless, highly irritant,

alkaline gas, which is mainly produced by enzymatic conversion of uric acid in the excreta of birds and to a lesser extent by decomposition of organic matter (Roumeliotis and Van Heyst, 2008) Ammonia often accumulates in high concentrations when poultry are confined to buildings that are artificially heated and ventilated. Results from a European study in which data were collected from ten typical poultry houses in England, The Netherlands, Denmark and Germany, with replicated measurements under summer and winter conditions, showed that mean 24-hour concentrations in poultry houses ranged from 6 – 30 ppm (Groot Koerkamp et al., 1998). Ammonia (NH_3) levels in animal houses can exceed 25 ppm when lower winter ventilation rates are used, and can reach 40 ppm in poorly ventilated buildings (Groot Koerkamp et al., 1998) or in the manure storage area of high-rise layer houses (Wathes et al., 1997). High NH_3 concentrations can cause pneumonia and other respiratory diseases and may decrease the growth rates of animals. Symptoms of high NH_3 concentrations shown by birds include watery eyes, closed eyelids, conjunctivitis, coughing, sneezing, and rubbing of eyes on the wing (Douwes et al., 2003).

The particle size distribution (PSD) of dust determines the potential impact on human and animal health (Mercer, 1978). The size of a dust particle affects its behavior in the air, as well as its deposition region in the human and animal respiratory tract when dust is inhaled. In general, the smaller particles travel deeper into the respiratory system and have a higher potential to cause lung disease (Collins and Algers., 1986). Because the dust deposition pattern in human and animal respiratory tracts is size-dependent, it is critical to know the size distribution of inhaled dust particles in order to evaluate its risk factor for human and animal health.

Particles are divided into three major categories: respirable particles (particles < 4 µm), which can penetrate into the gas exchange region of the lungs; thoracic particles (particles < 10 µm), being defined as particles that can penetrate into the head airway and the airway of the lungs; and inhalable or inspirable particles (particles < 100 µm) that can travel into the nose and mouth regions (ISO, 1995, CEN, 1993, ACGIH, 1999). When outside air quality is being determined, dust particles are often classified into two size classes: smaller than 10.0 µm (PM10), and smaller than 2.5 µm (PM2.5). Particles in the smaller size ranges generally contribute most to particle counts, while particles in the larger size ranges mostly contribute to particle mass. Heber et al. (1988a) found that about 80% of the particles in counts of aerial dust particles in pig houses were respirable. Maghirang et al. (1991) reported that 99% of the total number of particles in total dust in layer houses were in size ranges smaller than 10 µm.

The relationship between particle size or components of airborne dust and broiler health (including immune responses) is largely unknown. Neither are the effects of air quality on broiler's lung health and other diseases known. Heart morphology and physiology/functioning are frequently related with infection and ascites. In addition, little is known about the interactions between airborne particles and the immune system and their consequences for body weight gain, heart parameters, and disease resistance. Knowledge about these relations is useful in order to be able to determine whether and to what extent indoor air quality should be improved, and which components mainly affect the immune system and animal health. When air quality affects immunity, it may be useful to determine the efficiency of vaccinating chickens under certain housing conditions.

Muller and Wiese (1987) pointed out that dust can be biologically active, because it contains a variety of organic compounds, viruses, bacteria, fungi, endotoxins, parasites, and dust mites. It is becoming increasingly clear that the immune system recognizes microorganisms, e.g. fungi, endotoxin, parasites, and dust mites because they show Pathogen Associated Molecular patterns (PAMPs), which can bind to Toll like Receptors (TLR). The binding of PAMP to TLR on antigen-presenting cells has been shown to skew or modulate specific immune responses to specific antigens on these microorganisms, and also to induce immune responses to non-related antigens or microbes. It seems likely that the high level of microbes (either free or associated with dust particles, dead or alive) may communicate with the immune system of the chicken via PAMP–TLR interactions. Examples of the PAMPs probably present in airborne dust are (in addition to LPS), LTA, chitin and BGL. It should be kept in mind, however, that there are probably many PAMP that remain to be identified in poultry houses. The immune system of humans and animals is continuously triggered in the gut, but most probably also in the lungs by air constituents derived from microbiota present in manure, dust and planta. These PAMPs are probably present in high concentrations in airborne dust. High levels of LPS, LTA derived from gram-positive bacteria, BGL derived from fungi, and chitins derived from plants and arthropods are present in pig and poultry houses (Douwes et al., 2004). PAMP experimentally injected subcutaneously in layer chickens induced enhanced (LTA) or decreased (LPS) primary and secondary antibody responses to model antigens, whereas after PAMP treatment cellular immunity was affected for a prolonged period (Parmentier et al., 2004). PAMP also affected the non-antigen-specific humoral recall responses of poultry (Maldonado et al., 2005), confirming earlier results with mice (Berczi et al., 1998). When layers were intratracheally challenged with PAMP their

immune responses, including responses to obligatory vaccines, were also affected (Parmentier et al., 2006).

TLR are essential members of innate pattern recognition receptors (PRR) which identify microbe-specific and other danger signals related to groups of pathogens that fall under the name of PAMP (Medzhitov and Janeway, 1998). TLR play an important role as sentinel receptors of the innate immune system; moreover, through the regulation of co-stimulatory molecules they also facilitate the adaptive immune system (Medzhitov and Janeway, 2000). The chicken TLR family comprises 10 members, which detect specific surface markers of microorganisms such as LPS or BGL (Kannaki et al., 2010). For instance, TLR2B acts as a receptor for lipoprotein and also possibly recognizes LPS (Inoue et al., 2001). Different TLRs play vital roles in the activation of immune response to PAMPs. TLR1, TLR2, TLR4, TLR5, and TLR6 recognize bacterial components present on the outer surfaces, while TLR3, TLR7, TLR8, and TLR9 specifically identify viral nucleic acids. TLR respond to limited numbers of specific microbial ligands, but the TLR family can respond to a wide range of innate antigens associated with bacteria, virus, fungi and parasites, due to recombining TLR associations. Current understanding of the expression of TLR and their functions in activation of immune cells of chickens under various housing conditions is, however, still limited.

Repeated administration or crossover challenges with PAMP induced a refractory status in chickens with respect to immunity and growth, suggesting the chickens had adapted to airborne PAMP (Parmentier et al., 2004). High immune responsiveness to LPS was found to be related with enhanced mortality (Star et al., 2007), which suggests that chickens must adapt to high levels of environmental LPS to survive. In most experiments, PAMP and antigens were supplied at different locations, or at different moments, using fixed doses of PAMP during limited periods at limited ages. It is likely that different doses and combinations of PAMP the latter representing the complex interactions between different TLR (Nomura et al., 2000), and different forms of conjugated PAMP affect immune responses of broilers unpredictably (Kelly and Conway, 2005).

Animals should be able to adequately respond to challenges from the internal and external environments, in order to maintain internal equilibrium. The health status of an animal is reflected by its appropriate responsiveness through immune activity and performance. Thus, an appropriate specific immune response may be either T-helper 1 (T_h1) cell or T-helper 2 (T_h2) cell mediated, or no response at all, due to T-regulatory (Treg) cells. The binding of different innate antigens (PAMP) to different TLR on antigen-presenting cells (APC) results in different Th1 or Th2 activating cytokines being released by APC (Kapsenberg, 2003), so

the skewing of immunity via airborne PAMP in dust may have profound effects on health status. Th1 and Th2 cells are two major subpopulations of Cluster of Differentiation 4 (CD4+ T-helper cells). They are activated by antigens and various co-stimulators presented by different APC (Holen et al., 2001). In addition, the regulation of immune responses within limits (no hypersensitivity or immune suppression or auto-immunity) is an important form of adaptation and is probably controlled by Treg cells (Caramalho et al., 2003, Prescott and Dunstan, 2005) If animals cannot adapt, or if they respond inappropriately (e.g. due to their genetic disposition), life-threatening problems can occur. Chickens with high genetic potential require optimum conditions for best performance, but little is known about the effects of housing air quality conditions such as dust and dust components on their immune responsiveness, and about the mechanisms underlying these effects. It has been opined, however, that dust components are important in the modulation of immune reactivity. It is not known, however, how the housing environment (fine dust concentration and composition, microbial components) affects levels of innate and levels and type of specific immune responses, and consequently neither specific disease resistance and/or disease susceptibility are known.

Measuring immune responses and production parameters under various indoor animal housing conditions (dust, airborne endotoxins) will give information on the negative or positive correlation between the immune system and dynamic environmental conditions. Information is also required on the parameters resulting from the reaction of an adapted chicken to simultaneous exposure to several unfavorable environmental conditions (such as different internal air quality). Investigating the current situations and finding ways to improve the indoor air quality in animal houses will help to prevent animals and the people who work in livestock houses succumbing to disease. Furthermore, our knowledge about the effects of airborne dust and its components on production parameters such as body weight gain and other physical parameters of broilers still needs to be extended. And because it has profound financial consequences, it is important to determine the effects of poor air quality on animal performance.

OBJECTIVES AND OUTLINE OF THE THESIS

The research described in this thesis aimed to investigate dust concentrations, particle size distribution, and primary and secondary systemic antibody responses as indicators of immune competence in broilers. The study was done on broilers of various ages challenged with dust of different particle sizes and with different constituents. To address these aims, the following objectives were defined:

- To make an inventory of the present situation of dust concentrations and particle size distribution in counts and in mass inside and around animal houses.

- To evaluate the effects of dust and its constituents on the immune competence and specific immune response of broilers after challenge via the respiratory tract at different ages.

- To detect the adaptation of broilers when they are repeatedly respiratorily challenged with dust of different particle sizes and components.

- To assess the effectiveness of dust of different particle sizes and its components on the growth and heart parameters of broilers at different ages.

- To identify the localization of small (1 µm) and larger (10 µm) fluorescent- labelled polystyrene particles as a model of the dust particles in the body of broilers after challenge via the respiratory and cloaca routes.

The outline of the thesis is as follows:

Chapter 2 describes the concentration and size distribution of airborne particles inside and outside 13 different livestock housing systems, including poultry (broilers in floor housing with litter, layers in floor housing with litter, layers in aviary housing with litter, broiler breeders in floor housing with litter, turkeys in floor housing with litter), pigs (piglets on partially slatted floors, growing finishing pigs in low emission houses with dry feed and wet feed, sows in group and individual housing), dairy cattle (free stall barns), and mink (cages). We focused on particle counts and mass distribution in 30 different size ranges.

In chapter 3 the systemic total antibody and isotype-specific IgM and IgG antibody responses in broilers under challenge of different dust components at 3 weeks and 7 weeks of age are described. In addition, in this chapter the effects of dust components on body weight gain and heart parameters are reported.

The study described in chapter 4 reports the effects of repeated LPS challenge concurrently with or before immunizations with specific antigen human serum albumin (HuSA) and rabbit gamma globulin (RGG) on primary and secondary systemic antibody responses and isotype IgM and IgG responses on broilers at 3 and 7 weeks of age. The main purpose of this study was to determine the immunologic adaptation of the birds after challenge with an important dust component (LPS) at different ages.

Chapter 5 presents a study of the influences of fine dust (diameter smaller than 2.5 µm) and coarse dust (diameter between 2.5 and 10 µm), collected from a broiler house, on systemic antibody and isotype-specific response of broilers at early age (3 weeks of age) and later age (7 weeks of age) to simultaneously administered HuSA. The body weight gain of broilers after primary and secondary challenge was determined, as well as the effect of two size classes of airborne dust particles on heart morphology and heart weight.

In the study presented in chapter 6 we investigated the localization in chickens of two different sizes of non-immunogenic particles applied simultaneously via the respiratory tract and the cloaca as a model for entry of dust particles in chicken houses. We did so using red (TRITC) fluorescein-labelled polystyrene beads and green (FITC) fluorescein-labelled polystyrene beads. The role of phagocytic cells as transport vehicle within the chicken body is also discussed.

Finally, in the general discussion (chapter 7), the main findings of the project presented in this thesis are discussed and compared with previous studies, and recent insights. In addition, in this chapter the main conclusions from this thesis are summarized and recommendations are given for future research.

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CHAPTER 2

SIZE DISTRIBUTION OF AIRBORNE PARTICLES IN ANIMAL HOUSES

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ABSTRACT. Concentration and size distribution of airborne particles were measured inside and outside typical animal houses for broilers, broiler breeder (both floor housing with litter); layers (floor housing system and aviary housing system); turkeys (floor housing with litter), pigs: fattening pigs (traditional houses, low emission houses with dry feed, and low emission houses with wet feed), piglets, sows (in individual and in group housing); cattle (cubicle house), and mink (cages). Particles were counted and classified into 30 size ranges with an aerosol spectrometer (total range: 0.25 – 32 µm). Particles were measured on two days in two of each species/housing combination during 30 minutes inside and outside the animal house. Outside temperature and relative humidity were measured, as well. Particle counts in the different size ranges were generally higher in poultry houses than in pig houses, and counts in pig houses were generally higher than those in cattle and mink houses. The particle counts in animal houses were highest (on average 87%) in the size ranges < 1.0 µm, while particle mass was highest in size ranges > 2.5 µm (on average 97%). Most particles outside were in the size range < 1.0 µm (99% in counts). Mean count median diameter of particles inside the animal houses ranged from 0.32 to 0.59 µm, while mean mass median diameter ranged from 3.54 to 12.4 µm. Particle counts in different size fractions were highly correlated, with correlation coefficients varying from 0.69 to 0.98; higher coefficients were found when size ranges were closer. This study shows that although large variations occur in particle counts in different size ranges and in CMD and MMD, most variation, except for PM1, could be accounted for by species/housing combination and outside temperature and relative humidity.

Keywords: Particle size distribution, animal houses, CMD, MMD, temperature, relative humidity

INTRODUCTION

In animal houses, especially those for pigs and poultry, air quality can be seriously impaired by high dust concentrations (Wathes et al. 1997; Takai et al. 1998). These cause health problems for humans working in this environment (Donham et al. 1995; Pope et al. 2002; Andersen et al. 2004; Herr et al. 1999), and probably also for the animals living in these houses (Al Homidan and Robertson 2003). In addition, animal houses contribute significantly to particle concentrations in the ambient air through emission of particles with the exhausted air (Takai et al. 1998; Seedorf and Hartung 2000).

The main characteristics of dust from animal houses are: 1) it is biologically active – the dust contains a variety of organic compounds, from the animals themselves (skin, hair, feathers), from feed, faeces and bedding material) (Cambra-López et al. 2010; Aarnink et al. 1999; Aarnink et al. 2004; Takai et al. 1998; Welch 1986) and from microbes (viruses, bacteria, fungi, parasites, dust mites); 2) it is highly concentrated in the air – typically ten or even one hundred times more concentrated in the air of animal houses than in other buildings such as offices (Muller and Wieser 1987); 3) it spans a wide spectrum of particle sizes and shapes – from less than one µm (one millionth of a meter) to a hundred µm in diameter (Cambra-López et al. 2009).

One of the most important characteristic of dust is the size of the airborne particles, because this influences the behaviour and transport of the particles in the air (Wang et al. 2005) and the choice of control technology (Zhang 2004). Particle size determines the impact of dust on human and animal health too (Mercer 1978). Particles are often classified into three size classes: smaller than 10 µm (PM10), smaller than 2.5 µm (PM2.5) and smaller than 1.0 µm (PM1). Particles in these size ranges are mainly responsible for health problems because they can travel into the respiratory system (Collins and Algers 1986). Generally, the smaller the particles are, the deeper they can penetrate into the respiratory system and the greater their impact is on animal and human health.

Some studies have investigated the particle size and size distribution in animal houses, but only for certain animal houses, e.g. pig buildings (Maghirang et al. 1997; Lee et al. 2008); and cattle feedlots (Sweeten et al. 1998) and layers (Cao et al. 2009). Lee et al. (2006) investigated the effect of different farm activities on personal exposure of dust in different size ranges on pig, poultry, and dairy farms. Particle size distribution (PSD) has not been investigated yet in a comparative way with the same instrument in a wide range of species/housing combinations. Because of variations in space and time in dust concentrations

(Maghirang et al. 1997) sampling was done twice, in spring and summer, in two animal houses of each species/housing combination. The objective of this study was to determine the particle size distribution, in terms of counts and mass, in different commercial animal houses in the Netherlands.

MATERIAL AND METHODS

Animal houses

PM$_{10}$ mass and particle size distribution (PSD) were determined in houses of 13 different combinations of animal species/housing types, located in the Netherlands. Each species/housing combination was measured at two farms (replicates) at two time points in spring and summer 2009. The following animal species/housing combinations were studied: broilers, layers housed in floor system (layer_floor), layers in aviary system (layer_aviary), broiler breeders, turkeys, piglets, fattening pigs in traditional houses (fat_pig_trad), fattening pigs in modern low-emission housing with dry feed (fat_pig_mod_dry), fattening pigs in modern low-emission housing with wet feed (fat_pig_mod_wet), sows in individual housing (sow_individual), sows in group housing (sow_group)), dairy cattle (cattle), and mink. The housing systems and conditions of the different animal species are shown in Table 1.

Dust sampling

PM$_{10}$ mass concentrations and PSD in counts were both measured using aerosol spectrometers based on the light-scattering principle. With these instruments each particle is individually detected by scattered light photometry inside an optical measuring cell. The intensity of the scattered light signal is a measure of the size of the particle. Calibration with standardized dust allows comparisons between measurements where the source or type of dust is predominantly the same.

PM$_{10}$ mass concentrations were measured with a DustTrak aerosol monitor model 8520 (TSI inc., 500 Cardigan road Shoreview, MN 55126-3996, USA), which consisted of a portable, battery-operated, laser-photometer (spectrometer). The DustTrak provided real-time measurement based on 90° light scattering. This monitor can be used to measure aerosol mass concentrations in the range from 0.0001 – 100 mg m^{-3}. The sampling air flow rate was 1.7 L min^{-1}. The monitor was factory calibrated to the respirable fraction of standard ISO 12103-1, A1 test dust (formerly Arizona Test Dust). This allows comparison between measurements.

Particle size distribution in counts was measured with a Grimm instrument model number 1.109 (Grimm Aerosol Technik GmbH & Co., Ainring, Germany). This portable aerosol

spectrometer determined particle counts for 31 size ranges (optical latex equivalent diameter) with lower limits (in μm) of 0.25, 0.28, 0.30, 0.35, 0.40, 0.45, 0.50, 0.58, 0.65, 0.70, 0.80, 1.0, 1.3, 1.6, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.5, 7.5, 8.5, 10.0, 12.5, 15.0, 17.5, 20, 25, 30 and 32. The upper limit of the biggest particle size range (>32 μm) is not well defined and therefore this size range was left out of the analyses. The sampling airflow rate was 1.2 L min^{-1}. The sampling interval was 1 min. Mean values per location and measuring day for the different size ranges were used in the analysis.

Air samples with Grimm were taken during short periods to prevent contamination of the monitor in environments with high dust concentrations. Samples with Grimm and DustTrak were taken inside and outside each animal house. Inside the house, the samplers were placed at a height of approximately 1.5 m from the floor and as close as possible to the air outlet, but at least 1.5 m from ventilators. This location was chosen to obtain representative samples of the exhaust air and to avoid the high air velocities near to the exhaust fans, which would have affected the sampling efficiency (Hinds, 1999). For naturally ventilated buildings, with the air outlet in the ridge, the distance to the air outlet was larger (5 to 8 m). When sampling outside, samplers were placed upwind from the animal house. Sampling inside the animal house started directly after installation for 60 minutes. However, only the last 30 minutes of each measurement were used to skip possible effects of human disturbance. All measurements were done in daytime between 10:00 and 15:00 h. In Figure 1 the time periods in which the samplings were done are given in relation to the diurnal pattern of PM_{10} concentrations for the various animal categories (Winkel et al., 2011). These diurnal patterns were not determined at the same days as the measurements in this study. Outside sampling started directly after inside sampling was finished.

Table 1. Characteristic of the animal houses (n=26) in this study

Animal type	Animal category	Farm	Number of animals	Animal density (No./m2)1	Production Cycle (weeks of age)	Sampling moments (weeks of age)	Inside/outside conditions (T, RH)	Housing	Feed	Ventilation
Poultry	Broiler	1	52 000	20-24	0 – 6/7	4	18.2°C, 52.5%/14.5°C, 47.7%	Floor with bedding	Automatically dispensed crumbs and pellets	Side inlet, ventilators in end wall
						5	24.0°C, 67.0%/22.5°C, 57.1%			
		2	2675			3	20.2°C, 41.1%/18.9°C, 44.4%			
						4	23.2°C, 55.1%/23.5°C, 45.3%			
	Layer_floor	1	3 850	8.8-9	18 – ±75	52	14.8°C, 75.0%/11.4°C, 88.9%	Floor with bedding, slattedhopper, laying nests	Automatically dispensed crumbs and pellets	Side inlet, ventilators in end wall
						71	18.8°C, 71.7%/15.5°C, 70.8%			
		2	16 500			22	15.5°C, 82.4%/12.5°C, 54.0%			
						31	21.9°C, 52.5%/22.8°C, 39.5%			
	Layer_aviary	1	25 650	17-18	18 – ±75	52	13.6°C, 72.7%/11.4°C, 88.9%	Floor with bedding, slattedhopper, laying nests	Automatically dispensed crumbs and pellets	Side inlet, ventilators in end wall
						71	18.2 oC, 69.5%/15.5 oC, 70.8%			
		2	33 500			50	17.1°C, 68.9%/12.5°C, 54.0%			
						59	24.0°C, 45.6%/22.8°C, 39.5%			
	Broiler_breeder	1	3 698	7.7 - 8.5	20 – 60	29	20.4°C, 47.8%/18.6°C, 28.2%	Floor with bedding, slattedhopper, laying nests	Automatically dispensed crumbs and pellets	Side inlet, ventilators in end wall
						39	24.2°C, 57.2%/22.8°C, 56.2%			
		2	7 430			27	24.8°C, 40.4%/21.8°C, 40.9%			
						37	24.0°C, 62.6%/20.3°C, 58.9%			
	Turkey	1	4 750	3.0 – 3.4	4/5 – 21	12	16.8°C, 55.6%/14.9°C, 46.1%	Floor with bedding,	Automatically dispensed crumbs and pellets	Natural ventilation with open ridge and side inlets
						20	19.4°C, 70.5%/15.6°C, 50.9%			
		2	3 800			10	17.1°C, 56.0%/16.6°C, 25.9%			
						17	20.9°C, 50.3%/22.0°C, 42.3%			

Table 1. Characteristic of the animal houses (n=26) in this study (Continued)

Animal type	Animal category	Farm	Number of animals	Animal density (No./m2)1	Production Cycle (weeks of age)	Sampling moments (weeks of age)	Inside/outside conditions (T, RH)	Housing	Feed	Ventilation
	Piglet	1	75	2.9 – 3.3	4 – 10/11	4	21.6°C, 83.5%/14.8°C, 33.8%	Partially slatted	Automatically dispensed crumbs and pellets	Ceiling inlet, ventilator in ceiling
						9	23.5°C, 53.4%/19.9°C, 55.2%			
		2	125			6	22.4°C, 83.2%/15.9°C, 67.7%	Fully slatted		Door inlet, ventilator in ceiling
						8	24.2°C, 66.2%/19.9°C, 53.8%			
	Fat_pig_trad	1	60	1.0 – 1.3	10/11 – 25/27	17	20.8°C, 54.4%/14.8°C, 33.5%	Partially slatted	Automatically dispensed crumbs and pellets	Ceiling inlet, ventilator in ceiling
						20	21.4°C, 68.9%/18.0°C, 55.5%			
		2	120			18	21.8°C, 76.8%/15.9°C, 67.7%			Door inlet, ventilator in ceiling
						20	24.1°C, 66.2%/19.9°C, 53.8%			
Pig	Fat_pig_mod_dry	1	132	1.0 – 1.3	10/11 – 25/27	22	22.7°C, 53.2%/19.9°C, 48.1%	Partially slatted	Automatically dispensed crumbs and pellets	Floor inlet, ventilator in ceiling
						16	24.3°C, 55.2%/25.7°C, 43.9%			
		2	144			20	22.1°C, 50.4%/18.2°C, 42.2%			Door inlet, ventilator in ceiling
						24	26.2°C, 49.1%/26.5°C, 39.5%			
	Fat_pig_mod_wet	1	144			22	25.4°C, 64.2%/18.4°C, 64.9%	Partially slatted	Liquid feeding	Floor inlet, ventilator in ceiling
						19	22.7°C, 66.9%/18.4°C, 80.5%			
		2	120			19	24.1°C, 51.4%/18.2°C, 42.2%			
						24	26.5°C, 62.1%/26.5°C, 39.5%			
	Sow_individual	1	32		Diverse	Diverse	21.2°C, 60.3%/19.8°C, 57.3%	In crates, partially slatted	Automatically dispensed pellets	Door inlet, ventilator in ceiling
							22.6°C, 58.8%/20.5°C, 68.0%			
		2	145		Diverse	Diverse	24.4°C, 42.8%/20.3°C, 41.4%			
							22.4°C, 71.9%/22.3°C, 58.6%			

Table 1. Characteristic of the animal houses (n=26) in this study (Continued)

Animal type	Animal category	Farm	Number of animals	Animal density (No./m2)1)	Production Cycle (weeks of age)	Sampling moments (weeks of age)	Inside/outside conditions (T, RH)	Housing	Feed	Ventilation
	Sow_group	1	46	0.4	Diverse	Diverse	19.7°C, 56.6%/22.2°C, 31.2% 25.7°C, 61.9%/21.6°C, 64.9%	Partially slatted with feeding crates	Automatically dispensed crumbs and pellets	Ceiling inlet, ventilator in ceiling
		2	34		Diverse	Diverse	19.4°C, 82.1%/14.2°C, 33.8% 23.6°C, 54.4%/19.9°C, 55.2%			Valves inlet, ventilator in ceiling
Cattle	Cattle	1	51	0.3 - 0.4	Diverse	Diverse	19.8°C, 51.2%/19.9°C, 56.6% 18.9°C, 64.6%/20.8°C, 56.1%	Cubicle house	Roughage (maize and grass silage) two times /day	Naturally ventilated With side curtains and ridge
		2	150		Diverse	Diverse	18.8°C, 46.8%/17.5°C, 45.0% 27.9°C, 60.7%/25.4°C, 66.4%			
Mink	Mink	1	9 015	4-5	48 - 52	7 17	18.9°C, 71.7%/17.9°C, 72.9% 22.0°C, 71.3%/22.3°C, 68.6%	Cages	Feeding wet day feed 3 times/	Naturally ventilated with side curtains and ridge
		2	5 086		48 - 52	6 20	22.9°C, 54.1%/22.5°C, 51.8% 18.7°C, 68.7%/19.2°C, 67.2%			

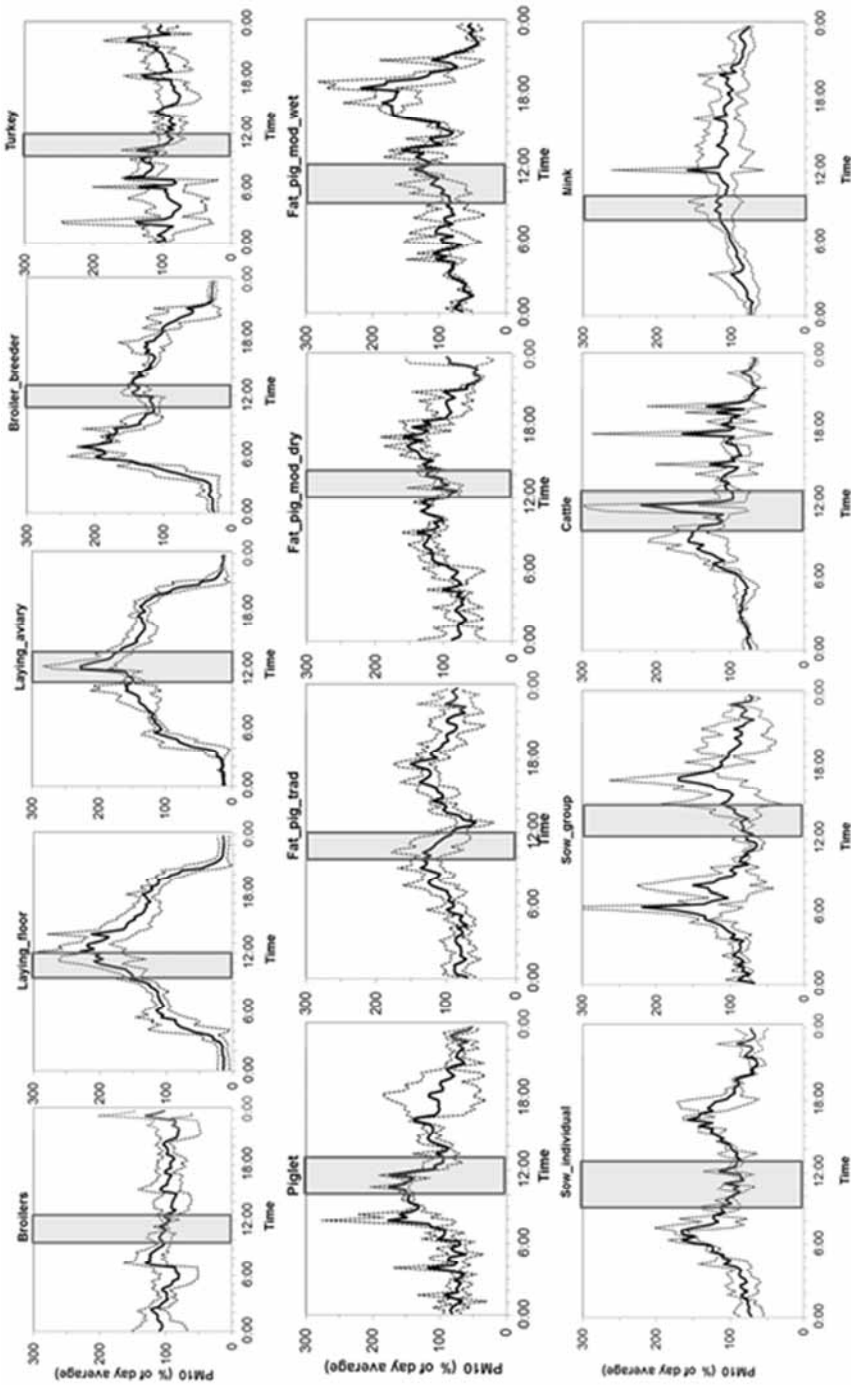1) per m^2 basic floor space, so excluding floor space at tiers.

Figure 1. Time periods in which the samplings were done, given in relation to the diurnal pattern of PM10 concentrations for the various animal categories (Winkel et al., 2011).

Environmental parameters

Temperature and relative humidity inside and outside the animal house were recorded during each sampling, using temperature and relative humidity sensors (Escort ilog data logger, Askey Leiderdorp, The Netherlands). The data were averaged over the measuring interval per measuring day for inside and outside the animal house and are given in Table 1.

Data analyses

Mass of particles in the different size ranges were calculated as follows:

$$M_i = \frac{1}{6}\pi\left(d_i 10^{-3}\right)^3 \rho_i F_i \tag{1}$$

where:

M_i = mass of particles in size range i, mg m^{-3}

d_i = midpoint diameter (mean diameter between upper and lower limits) of particles in size range i, μm

ρ_i = density of particles in size range i, mg mm^{-3}

F_i = number of particles in size range i per unit of volume, m^{-3}

Within the calculation of equation 1 it was assumed that particles in all size ranges had a spherical shape with unit density. The particle counts and mass of the 30 measured size ranges were pooled to form four classes of particulate matter concentration in 0.25 - 1.0 μm (PM$_1$), 1.0 - 2.5 μm (PM$_{1-2.5}$), 2.5 - 10 μm (PM$_{2.5-10}$), and 10 – 32 μm (PM$_{10-32}$). After log$_e$-transformation, the data in these size ranges were analysed with the ANOVA statistical procedure, to determine the effect of animal category on counts and mass. Multiple comparisons were made with Bonferoni's two-tailed t-test. Differences with P-values less than 0.05 were considered to be statistically significant. Furthermore, correlation coefficients between particle counts in different size ranges were calculated and the effects of outside climate (T, RH) on particle counts in different size ranges, CMD, and MMD (after log$_e$-transformation) were estimated with multiple linear regression with groups (species/housing combination). Within the multiple linear regression analysis parallel lines were calculated because the model was not significantly improved by including interactions in the model (P>0.05). The data were analysed using Genstat software (Genstat, 2008).

Particle sizes and PSD can be reported in different ways and characterized using different equations for particle numbers and mass. We used the equations given by Zhang (2004a) for

standardizing the measured values. The following equations / formulas were used to describe particle size distribution:

- *Count median diameter (CMD, µm)*

Though most particle size distributions are skewed, with a long tail to the right, the median is often used. The CMD of particles is defined as the diameter for which half of the particles in the sample are smaller and the other half are larger. Equation 2 was used to calculate the CMD.

$$CMD = \exp\left[\frac{\sum F_i \ln d_i}{N}\right] \tag{2}$$

where:

F_i = number of particles in size range i, m^{-3}

d_i = midpoint diameter of particles in size range i, µm

N = total number of particles (sum of all size ranges) , m^{-3}

- *Standardized number fraction distribution (Δf$_i$, µm^{-1})*

The different size ranges for which the number of particles is counted with the particle-sizing instrument are generally not of the same width. These size ranges (Δd_i) can vary by magnitudes. It is especially important to present standardized fractions in line graphs. The standardized number fraction can be calculated using equation 3.

$$\Delta f_i = \frac{F_i / \Delta d_i}{N} \tag{3}$$

where:

F_i = number of particles in size range i, m^{-3}

Δd_i = difference between upper and lower limits of size range i, µm

N = total number of particles (sum of all size ranges) , m^{-3}

- *Mass median diameter (MMD, µm)*

Similar to the CMD, the MMD is the particle diameter below which half of the mass of the particles in the sample is in particles with smaller diameters and the remaining half is in particles that have larger diameters. MMD can be calculated using equation 4.

$$MMD = \exp\left(\frac{\sum F_i d_i^3 \ln d_i}{\sum F_i d_i^3}\right) \tag{4}$$

where:

F_i = number of particles in size range i, m^{-3}

d_i = midpoint diameter of particles in size range i, μm

- Standardized mass fraction distribution (Δf_{mi}, μm^{-1})

The standardized mass fraction is defined in a similar way as the standardized count fraction. It can be calculated using equation 5.

$$\Delta f_{im} = \frac{m_i F_i / \Delta d_i}{M} \tag{5}$$

where:

m_i = midsize particle mass of size range i, mg

F_i = number of particles in size range i, m^{-3}

Δd_i = difference between upper and lower limits of size range i, μm

M = total mass of the particle population, mg m^{-3}

RESULTS

PM10 mass concentration

Mean PM$_{10}$ mass concentrations were highest on poultry farms (0.83 – 4.60 mg m^{-3}), followed by pig farms (0.13 – 1.62 mg m^{-3}), cattle farms (0.02 – 0.12 mg m^{-3}) and mink farms (0.04 – 0.12 mg m^{-3}). Figure 2 shows the mean PM$_{10}$ mass concentrations for the different animal species/housing combinations. PM$_{10}$ mass concentrations were highest in layer_floor (3.78 mg m^{-3}) followed by layer_aviary (2.81 mg m^{-3}), turkey (1.87 mg m^{-3}), broiler (1.42 mg m^{-3}), piglet (1.15 mg m^{-3}), broiler_breeder (0.89 mg m^{-3}), fat_pig_trad (0.87 mg m^{-3}), fat_pig_mod_dry (0.65 mg m^{-3}), fat_pig_mod_wet (0.47 mg m^{-3}), sow_group (0.30 mg m^{-3}), sow_individual (0.18 mg m^{-3}), mink (0.07 mg m^{-3}) and cattle (0.07 mg m^{-3}). Outside PM$_{10}$ concentrations were on average 0.08 mg m^{-3} (range 0.01 to 0.25 mg m^{-3}).

Particle size distribution

Number distribution

Most particles inside the animal houses were found in PM_1: on average, 86.8% of total particles; 5.5% in $PM_{1-2.5}$; 7.4% in $PM_{2.5-10}$ and 0.22% in PM_{10-32}. In the outside air, 99.2% of the particles were found in PM_1; 0.7% in $PM_{1-2.5}$; 0.1% in $PM_{2.5-10}$ and 0.005% in PM_{10-32}. On average, compared with inside air, outside air contained less particles, especially in the larger size ranges. The number of particles in the outside air were 52% of the number of particles in the inside air in PM_1; this was 5.6% for $PM_{1-2.5}$; 0.7% for $PM_{2.5-10}$, and 1.1% for PM_{10-32}. The counts of particles in the different size ranges for the different animal species/housing combinations and outside are given in Table 2.
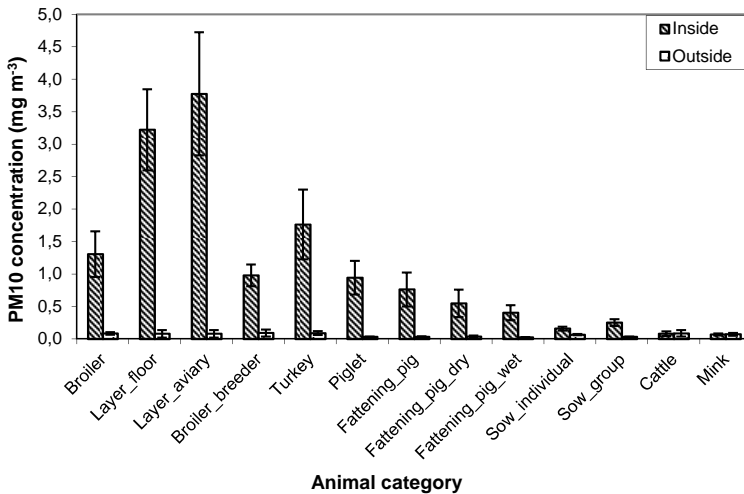

Figure 2. Estimated means (bars) and standard errors (given as lines on top of the bars) of PM10 dust concentrations for 5 species/housing combinations for poultry, 6 for pigs, 1 for cattle and 1 for mink.

Table 2 shows that in all particle size ranges, the average numbers of particles were higher in poultry houses than in pig, cattle and mink houses, with one exception: broiler_breeder houses had similar particle counts as pig houses in all size ranges. On average, particle counts in pig houses were higher than in cattle and mink houses for all particle size ranges, except for PM_1. The number of particles in PM_1 in pigs, cattle and mink houses did not differ much from the number of particles in PM_1 measured outside.

Table 2. Estimated mean particle counts (particles cm^{-3}) in the different size ranges and count median diameter for the different animal species/housing combinations. Standard errors of means are given between brackets [1]

	Animal category	0.25-1.0 μm	1.0-2.5 μm	2.5-10 μm	10-32 μm	CMD[2] (μm)
1	Broiler	416^{ab} (143)	19.8^{bcde} (12.8)	34.7^{def} (19.8)	1.5^{de} (0.6)	0.44^{abcde} (0.04)
2	Layer_floor	683^{b} (164)	69.7^{de} (15.9)	102.4^{ef} (23.7)	2.1^{e} (0.7)	0.59^{e} (0.03)
3	Layer_aviary	763^{b} (107)	83.4^{e} (19.9)	111.7^{f} (27.1)	1.6^{de} (0.2)	0.57^{de} (0.03)
4	Broiler_breeder	128^{a} (16)	12.4^{bcde} (2.1)	17.3^{def} (2.9)	0.6^{cde} (0.1)	0.54^{cde} (0.03)
5	Turkey	395^{ab} (85)	41.6^{cde} (11.2)	33.4^{def} (10.7)	1.1^{de} (0.3)	0.48^{bcde} (0.04)
6	Piglet	207^{ab} (58)	14.9^{bcd} (6.0)	24.1^{def} (8.4)	0.9^{de} (0.3)	0.49^{bcde} (0.03)
7	Fat_pig_trad	234^{ab} (30)	11.4^{bc} (4.5)	16.1^{cde} (6.1)	1.0^{cde} (0.4)	0.43^{abcd} (0.03)
8	Fat_pig_mod_dry	239^{ab} (54)	5.2^{ab} (1.8)	9.0^{cd} (3.6)	0.8^{cde} (0.3)	0.38^{ab} (0.04)
9	Fat_pig_mod_wet	208^{ab} (68)	4.7^{ab} (0.8)	7.4^{cd} (1.6)	0.5^{cde} (0.1)	0.39^{abc} (0.04)
10	Sow_individual	232^{ab} (65)	2.1^{ab} (0.4)	1.9^{bc} (0.3)	0.1^{bc} (0.02)	0.33^{a} (0.009)
11	Sow_group	194^{ab} (46)	4.4^{ab} (1.0)	4.9^{cd} (1.2)	0.3^{cd} (0.06)	0.36^{ab} (0.01)
12	Cattle	177^{a} (103)	1.5^{a} (0.7)	1.1^{ab} (0.9)	0.04^{b} (0.02)	0.32^{a} (0.006)
13	Mink	380^{ab} (75)	0.9^{a} (0.1)	0.13^{a} (0.03)	0.004^{a} (0.001)	0.32^{a} (0.008)
1-13	Overall mean (in counts)	327 (78)	20.9 (6.0)	28.0 (8.2)	0.80 (0.24)	0.43 (0.03)
1-13	Overall mean (% of total counts)	86.8	5.5	7.4	0.22	
14	Outside	179 (24)	1.2 (0.3)	0.19 (0.02)	0.009 (0.001)	0.32 (0.03)

[1]Means within a column lacking a common superscript letter are significantly different (P<0.05).

[2]CMD = count median diameter (see equation 2).

The CMD of particles in this study (Table 2) averaged from 0.32 μm to 0.59 μm. The mean CMD of particles was 0.53 μm in poultry houses, 0.40 μm in swine houses, 0.32 μm in cattle houses, 0.32 μm in mink houses and 0.32 μm in outside air. The CMDs of particles in layer_floor- and layer_aviary houses were significantly higher than those in most pig categories and higher than those in cattle and mink farms.

There were significant correlations between the number of particles in the different size fractions (P<0.001). Correlation coefficients were 0.82 between PM$_1$ and PM$_{1-2.5}$, 0.82 between PM$_1$ and PM$_{2.5-10}$, and 0.69 between PM$_1$ and PM$_{10-32}$. The correlation coefficient

between $PM_{1-2.5}$ and $PM_{2.5-10}$ was 0.98, between $PM_{1-2.5}$ and PM_{10-32} it was 0.80, and between $PM_{2.5-10}$ and PM_{10-32} it was 0.84

Figure 3 shows the standardized number fraction of particles in poultry, pig, cattle, and mink houses. The standardized number fraction for outdoor particles are given in each sub-figure, for comparison. For all animal house categories and also for outside samples, the highest fraction of particles was in the size range 0.25 – 0.30 μm. Number fractions decreased sharply with increasing particle size. For pig and poultry houses, two small peaks were observed: one between 0.65 to 0.70 μm, and one between 2.5 to 3.7 μm. It is obvious from Figure 3 that within the animal houses, especially those for poultry and pigs, the number fractions of the larger particles were much higher than outside.

Mass distribution

As shown in Table 3, particle size distribution in mass is dominated by particles in the size range > 2.5 µm. On average, 0.5% of particle mass was found in PM_1, 2.1% in $PM_{1-2.5}$, 52.6% in $PM_{2.5-10}$, and 44.8% in PM_{10-32}. For outside air, 11.0% of particle mass was found in PM_1, 5.9% in $PM_{1-2.5}$ µm, 17.1% in $PM_{2.5-10}$, and 66.0% in PM_{10-32}. On average, compared with inside air, outside air contained less particle mass in the different size ranges. The mass of particles in the outside air was 30.8% of the mass of particles in the inside air in PM_1; this was 3.9% for $PM_{1-2.5}$; 0.45% for $PM_{2.5-10}$, and 2.0% for PM_{10-32}.

Table 3. Mean mass distribution (mg m^{-3}) of particles in the different size ranges and mass median diameter for the different animal species/housing combinations. Standard errors of means are given between brackets [1]

	Animal category	0.25-1.0 µm	1.0-2.5 µm	2.5-10 µm	10-32 µm	MMD[2] (µm)
1	Broiler	0.019^{bcd} (0.009)	0.064^{defg} (0.04)	2.10^{de} (1.09)	2.36^{d} (0.96)	10.11^{b} (0.43)
2	Layer_floor	0.042^{cd} (0.01)	0.210^{fg} (0.05)	5.72^{e} (1.63)	2.99^{d} (0.79)	7.66^{b} (0.03)
3	Layer_aviary	0.048^{d} (0.008)	0.246^{g} (0.06)	5.63^{e} (1.21)	2.51^{d} (0.38)	7.32^{b} (0.18)
4	Broiler_breeder	0.007^{ab} (0.001)	0.038^{cdefg} (0.007)	0.96^{cde} (0.18)	1.07^{cd} (0.19)	9.94^{b} (0.19)
5	Turkey	0.019^{bcd} (0.004)	0.117^{efg} (0.033)	1.51^{de} (0.46)	2.24^{d} (0.45)	10.53^{b} (0.57)
6	Piglet	0.01^{abc} (0.003)	0.044^{cdef} (0.017)	1.59^{de} (0,53)	1.33^{cd} (0.43)	9.29^{b} (0.67)
7	Fat_pig_trad	0.009^{abc} (0.003)	0.034^{cde} (0.013)	1.07^{cde} (0.41)	1.62^{cd} (0.65)	10.26^{b} (0.69)
8	Fat_pig_mod_dry	0.007^{ab} (0.002)	0.016^{bcd} (0.006)	0.68^{cd} (0.27)	1.44^{cd} (0.61)	12.39^{b} (0.47)
9	Fat_pig_mod_wet	0.006^{ab} (0.0009)	0.014^{bcd} (0.003)	0.52^{cd} (0.11)	0.92^{cd} (0.22)	11.47^{b} (0.46)
10	Sow_individual	0.005^{ab} (0.001)	0.006^{abc} (0.0009)	0.12^{bc} (0.02)	0.21^{bc} (0.03)	10.67^{b} (0.37)
11	Sow_group	0.005^{ab} (0.001)	0.012^{abcd} (0.003)	0.29^{cd} (0.07)	0.49^{cd} (0.12)	10.86^{b} (0.75)
12	Cattle	0.004^{a} (0.002)	0.004^{ab} (0.002)	0.058^{ab} (0.04)	0.094^{b} (0.05)	10.96^{b} (2.03)
13	Mink	0.009^{abc} (0.002)	0.002^{a} (0.0003)	0.005^{a} (0.001)	0.008^{a} (0.003)	3.54^{a} (1.23)
1-13	Overall means (mass)	0.015 (0.004)	0.062 (0.018)	1.56 (0.52)	1.33 (0.37)	9.62 (0.61)
1-13	Overall means (% of total mass)	0.5	2.1	52.6	44.8	
14	Outside	0.0045 (0.0009)	0.0024 (0.0005)	0.0070 (0.0006)	0.027 (0.004)	9.15 (0.63)

[1]Means within a column lacking a common superscript letter are significantly different (P<0.05).

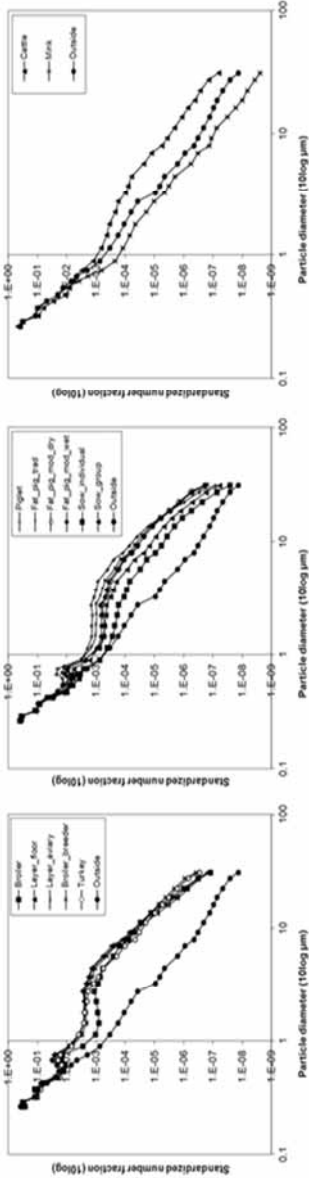[2]MMD = mass median diameter (see equation 4).

Figure 3. Standardized number fraction (at log$_{10}$-scale) of particles in the different size ranges (at log$_{10}$-scale) in 5 species/housing combination for poultry (left), 6 for pigs (middle) and for cattle and mink (right).
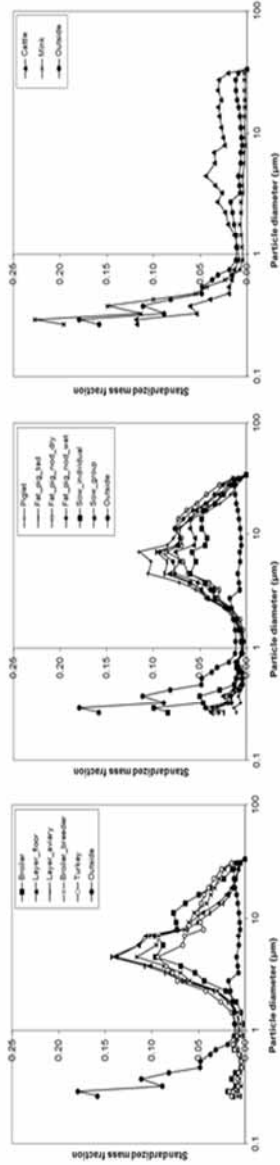

Figure 4. Standardized mass fraction of particles in the different size ranges (at log$_{10}$-scale) in 5 species/housing combination for poultry (left), 6 for pigs (middle) and for cattle and mink (right).

The standardized mass distribution for the different animal species/housing combinations are shown in Figure 4. From these figures it is clear that the standardized mass distribution is totally different from the standardized count distribution. Contrary to the standardized count distribution, the standardized mass distribution of particles inside had a very different pattern than the pattern outside. Because of the relatively high numbers of small particles and very few big particles outside, the contribution of the small particles to mass was relatively large, while the mass of inside particles was dominated by the bigger particles. The standardized mass fraction was especially high in the size range from $2.5 - 10$ µm. Peaks in standardized mass fractions occurred in the size range $4.0 - 6.5$ µm, except for mink. The standardized mass distributions of particles inside cattle and mink houses were very similar to those outside.

The MMD inside animal houses averaged from 3.54 μm to 12.4 μm for the different animal species/housing combinations; outside, the MMD was 9.15 μm. The MMDs in poultry (9.11 μm), pig (10.8 μm) and cattle (11.0 μm) houses were significantly higher than the MMD in mink houses (3.54 μm) ($P<0.05$).

Effect of outside climate on particle size distribution inside animal houses

In Table 4 the results of the multiple regression analyses are given. This model accounted for 85 to 91% of the variations in counts in $PM_{1-2.5}$, $PM_{2.5-10}$, and PM_{10-32}; for 36% of the variation in counts of PM_1, for 81% of the variation in CMD, and for 62% of the variation in MMD.

Particle numbers in PM_1 were not influenced by outside temperature; particle numbers in $PM_{1-2.5}$, $PM_{2.5-10}$, and PM_{10-32}, however, were significantly influenced by outside temperature. Higher outside temperatures gave lower particle counts in these size ranges. Outside relative humidity did not have a significant effect on particle counts in all size ranges. Count median diameter was significantly influenced by outside temperature and relative humidity. At higher outside temperature and humidity levels CMD became smaller. Mass median diameters were not affected by outside temperature and relative humidity.

Table 4. Linear effects (regression coefficient: rc) of outside climate (T, RH) on particle counts in different size ranges and on count (CMD) and mass (MMD) median diameter (after $\log_e$-transformations) inside the animal house. The standard errors of rc and the probability that rc is not different from 0 are given, as well.

Size range, CMD, MMD	T outside			RH outside			$R^{2\,[2]}$
	rc	s.e.	P[1]	rc	s.e.	P[1]	
0.25-1.0 µm	0.009	0.028	0.75	0.006	0.007	0.40	0.36
1.0-2.5 µm	-0.108	0.028	<0.001	-0.012	0.007	0.10	0.85
2.5-10 µm	-0.110	0.029	<0.001	-0.012	0.007	0.10	0.91
10-32 µm	-0.098	0.030	0.003	-0.011	0.008	0.17	0.89
CMD	-0.021	0.005	<0.001	-0.004	0.001	0.01	0.81
MMD	0.001	0.013	0.94	-0.004	0.003	0.23	0.62

[1] A P-value < 0.05 is considered to be statistically significant, meaning there is a significant linear effect of T, RH on $\log_e$ (particle count, CMD, MMD).

[2] R^2 is the variance accounted for with the multiple regression model with outside T and RH as variables and species/housing combinations as groups.

DISCUSSION

Results of PM_{10} mass concentrations and particle counts in the different size ranges show highest concentrations in poultry houses, followed by pig houses, and were lowest in cattle and mink houses. Takai et al. (1998) found the same order for concentrations in poultry, pig, and cattle houses in different livestock buildings in Northern Europe. The high dust concentrations in poultry houses were most probably related to the presence and use of litter. By scratching, dust-bathing and other activities, dust particles are formed, especially from manure and feathers (Cambra-Lopez et al., 2011), and suspended in the air. In layer houses with battery cages, where no litter is present, and where there is no contact between animals and their manure, a lot lower dust concentrations were reported (Takai et al., 1998). The low dust concentrations in cattle and mink houses are probably the result of a low dust production in combination with a high ventilation rate in the open naturally ventilated buildings.

The results showed that the number of particles smaller than 1.0 µm in pigs, cattle and mink houses did not differ much from the number of particles in this size range measured outside. This corroborates the hypothesis that the small particles in animal houses mainly come from outside (Zhang et al., 1998). The particle counts in mink and cattle houses were

more or less similar and not very different from the particle counts outside for all particle size ranges.

The most striking result from this study is the totally different particle size distribution for counts and mass, especially in poultry and pig houses. Inside the animal houses, most particles in counts were found in PM_1, on average 87%, while this was only 0.5% in mass. On average, only 7.6% of the number of particles inside the animal houses were > 2.5 µm, while this was 97% in mass. In the outside air 99% of the number of particles were found in PM_1, while these particles accounted for only 11% of the mass. The very small contribution of the number of particles in PM_{10-32} (0.005%) to total number of particles in the outside air, contributed to a large extend to particle mass (66%). It should be noted that when calculating particle mass distribution from particle count distribution we assumed that the density of the particles was 1 mg mm^{-3} and that the particles had a spherical shape. Both can vary, depending on the source of dust (Cambra-Lopez et al., 2011) and probably also depending on the way the dust is generated. While we do not know the contribution of each dust source to the particles in the different size ranges, we made these simple assumptions for density and shape. The density of the particles and the shape factor are both generally higher than 1. McCrone (1992) reported densities of 1.2 g cm^{-3} for feathers, 2.6 g cm^{-3} for feed, 1.3 g cm^{-3} for hair, 1.5 g cm^{-3} for manure and wood shavings, 1.4 g cm^{-3} for skin, and 2.1 g cm^{-3} for outside particles. Zhang (2004b) reported shape factors of 1.06 for feathers and wood shavings, 1.08 for feed and outside, 1.15 for poultry manure, 1.36 for pig manure, and 1.88 for skin. When calculating the mass of a particle, the volume of an assumed sphere particle should be multiplied by the density and be divided by the shape factor, so these factors will to some extend compensate each other.

The results showed the highest CMD for poultry (0.53 µm), followed by pigs (0.40 µm), while the CMD of particles inside cattle and mink houses were similar as the CMD of particles in the outside air (0.32 µm). The MMD's for particles inside poultry, pig and cattle houses were very similar (9.11 – 11.0 µm), while the MMD for particles in mink houses were clearly lower (3.54 µm). The relatively high MMD for outside particles (9.15 µm) is probably caused by some small particles in the highest size ranges, causing a big increase in the MMD. In the outside air there are a lot of very small particles (< 1.0 µm), but few in the higher size ranges. Therefore a few extra particles in the largest size ranges have a big effect on MMD. The MMD of particles is very much depending on the maximum size ranges of the particles that are collected. Within this study the upper limit was 32 µm. In a study of Jerez et al. (2009) particles up to a diameter of 600 µm were analysed. They found an average MMD of

particles leaving a building for growing-finishing pigs of 26.8 μm, so a lot higher than found in this study (10.3 – 12.4 μm). Also Lee et al. (2008) generally found higher MMD's, varying from 9 to 25 μm, for particles in different pig houses. Their maximum measurable particle size varied from 600 to 1200 μm. Maghiran et al. (1997) found a mean MMD for piglets of 13 μm, measured with an eight-stage cascade impactor with a maximum measurable size range of > 21.3 μm (so not exactly defined). In our study we determined a mean MMD for particles in piglet houses of 9.3 μm. Sweeten et al. (1998) found MMD's for cattle feedlot dust of 9.5 μm for total dust and of 6.9 μm for PM_{10} dust. In our study in cattle houses we found a mean MMD of 11.0 μm. The relatively low values found in the study of Sweeten et al. (1998) can have different causes, of which one of the main reasons might be the relatively high amounts of manure that is probably dustified in the feedlot system.

Within our study the particle size distribution in different animal houses during daytime at 'normal' activity levels of the animals was determined. Normal activity means that animals were not disturbed by human activities or other disturbing effects from outside. However, animals have their own activity pattern, as well. Ideally, particle size distribution should be determined during 24-h periods, at different locations inside the animal house, at different locations with similar species/housing combinations, and during different seasons of the year. This study was limited with respect to estimation of variations during the day, only half-hour samples were taken, with respect to different measuring spots inside the animal house, only one spot was sampled, and with respect to different seasons, measurements were only done during the spring/summer period. Within this study we focused on doing comparable measurements at different locations with various species/housing combinations during a similar time period of the day.

From Figure 1 it can be seen during what PM10 concentration levels our measurements were done. In broilers, turkey, fattening pigs, sows and mink measurements were done at approximately average PM10 concentrations during the day. In broiler breeders and cattle, PM10 concentrations were somewhat raised during our measurements and in layers and piglets PM10 concentrations were largely raised during our measurements (1.5 – 2.0 times higher than average). These differences should be considered when comparing data between different species/housing combinations. However, as can be seen from Table 2 and 3, differences between species/housing combinations are a lot higher than the diurnal variations within species/housing combinations.

High variations in particle concentrations occurred not only between animal species/housing combinations, but also between farms of the same category and within farms

(two measurements at different moments), as shown by the relatively high standard error of means (s.e.m., Table 2). This agrees with the findings of Martin et al. (1996) who also reported high variations in dust particle concentrations between animal houses. They suggested that this is caused by the fact that each animal farm has its own control and managing practices and its own details in housing design. Another reason for the variations within farms of the same category in our study was the fact that farms were sampled on different days and at different moments in the production cycle. These factors can have a large effect on the ventilation rate and thereby on the dilution of particles with fresh air.

We only measured dust concentrations at one spot within an animal house. Maghirang et al. (1997) found significant higher total dust concentrations (< 100 µm) above the pens than above the alley, however, the respirable dust fraction (particles < 4.0 µm) did not show any significant spatial variability. Jerez et al. (Jerez et al., 2009) concluded from their study in a swine building that larger particles re-entrained in the air by animal activity, but settled again before they reached the ventilation outlet. In our study we measured dust concentrations close to the ventilation outlet to get a kind of average sample of the dust concentration within the total space and a sample that indicates the particles that are emitted to the outside air.

We only did measurements during the spring and summer period. As was shown in different studies dust concentrations are generally higher during the winter than during the summer period, especially in pig and poultry houses (Roumeliotis and Van Heyst, 2007, Takai et al., 1998, Lee et al., 2008). The variations in temperature and relative humidity, however, enabled us to estimate temperature and humidity effects on particle counts and count and mass median diameter of particles inside the animal house. These calculations showed that effects of outside temperature were very similar for particle counts in the different size ranges > 1.0 µm. Counts in these size ranges decreased by approximately 10% for every 1°C increase in outside temperature. This can be explained by the higher ventilation rates at higher temperatures, causing a dilution of particle concentrations inside the animal house. Number of particles < 1.0 µm were not affected by outside temperature. This seems logical while concentrations of these particles were not significantly different between outside and inside the animal house. The CMD of inside particles was significantly affected by the outside temperature. This is a logical result of the former mentioned effects of outside temperature on particle counts in size ranges < 1.0 µm (no effect) and on particle counts in size ranges > 1.0 µm (negative effect). Particles counts in the different size ranges inside the animal house were not significantly affected by outside relative humidity, although there was a tendency for lower counts at higher humidity levels for $PM_{1.0-2.5}$ and for $PM_{2.5-10}$ (P=0.10).

The decrease of CMD of particles inside the animal house with increasing outside relative humidity might be caused by a faster deposition of big particles at higher humidity levels. The MMD was not affected by outside temperature or humidity. Also Cao et al. (2009) found no significant effect of season (fall, winter, spring) on MMD for total dust particles in a high-rise barn for layers.

We found significant correlations between the number of particles in the different size fractions. Correlation coefficients varied from 0.69 to 0.98 between PM_1, $PM_{1-2.5}$, $PM_{2.5-10}$, and PM_{10-32} , with higher coefficients for size ranges that were close in size.

This study shows that although large variations occur in particle counts in different size ranges and in CMD and MMD, most variation, except for PM_1, could be accounted for by species/housing combination and outside temperature and relative humidity.

CONCLUSIONS

From this study the following can be concluded:

- Large variations in particle counts and mass in the different size ranges exist between and within animal species/housing combinations.

- In terms of counts and mass, the dust concentrations in the different particle size ranges are generally higher in poultry houses than in pig houses, and are generally higher in pig houses than in cattle houses and mink houses.

- Particle counts and mass in mink and cattle houses are more or less similar to the particle counts and mass in outside air for all particle size ranges.

- Particle counts in animal houses are highest in the size range < 1.0 μm (on average 87%), while particle mass is highest in size ranges > 2.5 μm (on average 97%). Most particles outside are in the size range < 1.0 μm (99% in counts).

- Count median diameter of particles in animal houses ranged from 0.32 to 0.59 μm, and was 0.32 μm for outside particles. Mass median diameter of particles in animal houses ranged from 3.54 to 12.4 μm, compared with 9.15 μm for outside particles.

- Particle counts in different size fractions are highly correlated, with correlation coefficients varying from 0.69 to 0.98; higher coefficients were found when size ranges are closer.

- Although large variations occur in particle counts in different size ranges and in CMD and MMD, most variation, except for PM_1, could be accounted for by species/housing combination and outside temperature and relative humidity.

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CHAPTER 3

EFFECTS OF DUST AND AIRBORNE-DUST-COMPONENTS ON ANTIBODY RESPONSES, BODY WEIGHT GAIN, AND HEART MORPHOLOGY OF BROILERS

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ABSTRACT. Pathogen-associated molecular patterns (**PAMP**) such as lipopolysaccharide (**LPS**), lipoteichoic acid (**LTA**), β-glucans (**BGL**), and possibly many others are important parts of (fine) dust in animal houses. When intratracheally (**i.t.**) administered, PAMP affected specific primary and secondary humoral immune responses to concurrently i.t. or systemically administered antigens and body weight gain (**BWG**) of layer chickens. In the present study we evaluated the effects of i.t. challenge with various PAMP known to be present in dust: LPS, LTA, Zymosan-A (containing 1,3 β-glucan (BGL), next to heat-inactivated dust particles as a representative of mechanical stress, a combination of the former components, and NH_3 as a chemical component of dust on primary and secondary (total) systemic antibody (**Ab**) responses and (isotype) IgM and IgG responses to concurrently i.t. administered human serum albumin (**HuSA**) in broilers. Birds were challenged via the trachea for two consecutive days at 3 and 7 weeks of age, respectively. All treatments affected immune responses at several moments, BWG and heart morphology. β-Glucans and LPS affected the birds most pronounced and for a prolonged period. Intratracheally administered LPS and BGL significantly enhanced primary and secondary total Ab, IgM Ab, and IgG Ab responses to HuSA. All birds that were challenged with dust, PAMP or NH_3 concurrently with HuSA showed a decreased BWG especially after primary, but also after secondary challenge. Weight, width and length of hearts were enhanced in dust and PAMP-treated birds as well when these birds were challenged with HuSA. The present results indicated that components of dust such as PAMP when i.t. administered affect humoral immune responsiveness of broilers which may lead to an enhanced status of immune reactivity. Furthermore, our results suggest that the hygienic status of the environment influences BWG and may affect heart morphology, and as a consequence physiology in broilers.

The consequences of our findings with respect to dust, (airborne) PAMP, hygienic conditions in the barn, and immune responsiveness of broilers are discussed.

Keywords: *airborne, broiler chickens, dust, modulation, PAMP, intratracheal*

INTRODUCTION

Airborne (fine) dust is thought to be a carrier for gases, microorganisms, endotoxins and various other substances (Aarnink and Wagemans, 1997; Aarnink et al., 2003; Gustafsson, 1999; Takai et al., 1998; Tielen et al., 1978, Collins and Algers, 1986). Organic dust may evolve from animals themselves: skin, hair and feathers (collagen), arthropods (chitin), and from feed, faeces and bedding material (Aarnink et al., 2004, 2005, Aarnink and Ellen, 2007). The highest dust concentrations were found in poultry houses followed by pig and cattle stables. Also the highest emission rate for airborne microbes and dust was found in poultry houses (3.5 x 109 cfu/h for bacteria, and 3.6 x 107 cfu/h for fungi), whereas inhaled and respiratory dust levels were estimated on 3 and 0.5 g/h, respectively, from which inhaled endotoxin levels were calculated at 1 µg/h (Seedorf et al., 1998). Amongst many factors such as stock density, age of animals, ventilation, dust plays an important role in the level of bacteria and their products in the air of animal houses (Collins and Algers, 1986; Murch, 2001). Muller and Wieser (1987) demonstrated the main differences of dust originating from animal houses and dust from other sources. First, dust from animal houses is more biologically active, containing a variety of organic compounds, viruses, bacteria, fungi, endotoxins, parasites and dust mites. Second, the concentration of dust from animal houses is typically 10 or even 100 times higher than dust in other buildings such as an office. Third, it spans a wide spectrum of particle sizes and shapes: dust particles from a animal house are composed of dander, hair, feed dust and fecal materials, and range in size from less than 1 to 100 µm in diameter. The size of a dust particle affects its behavior in the air. Respiratory particles (smaller than 10 µm in diameter; that is, similar to tobacco smoke) are responsible for the health problems in humans because particles of that size can travel deep into the lungs (Collins and Algers, 1986; Wathes, 1995).

An important part of dust is formed by microbes (Collins and Algers, 1986) and Pathogen-associated molecular patterns (**PAMP**) derived from microbes. Pathogen-associated molecular patterns can bind to specific innate (toll-like) receptors expressed by antigen presenting cells (APC), which in mammals, and likely also in poultry, via the release of cytokines direct specific immune responses towards T-helper (**TH**)2-mediated Ab, or TH1-mediated cellular inflammatory responses (De Jong et al., 2002, Kapsenberg, 2003), or to the more recently described TH17 responses (Betelli et al., 2007). High levels of PAMP such as lipopolysaccharide (**LPS**), found on gram negative bacteria, lipoteichoic acid (**LTA**) derived from gram-positive bacteria, β-glucans (**BGL**), found on yeast and many others are present in

chicken houses either airborne or as part of dust. Chickens inhale (approximately 1 m^3 air/24 h) PAMP, obtain them via the cloaca, contact them through the eyes, or take them up orally. Concentrations of airborne endotoxins (LPS) and BGL ranging from 240 to 13,400 endotoxin units/m3 (1 endotoxin units/m^3 ≈ 0.1 ng/m^3) were found in chicken farms (Douwes, 1998; Douwes et al., 2004, Anonymous, 1998), but levels as high as 63 μg/m^3 were also reported (Pomorska et al., 2007).

In a dose-dependent fashion, PAMP modulated primary and secondary immune responses of layers and BWG (Ploegaert et al. 2007; Parmentier et al., 2008), whereas signs of cannibalistic behavior were found in PAMP-sensitized layers (Parmentier et al., 2009). In broilers, PAMP such as LPS increased pulmonary arterial pressure after i.v. exposure (Chapman et al., 2005; Wideman et al., 2004), decreased respiratory capacity and caused death (Rocksen et al., 2004)..

In the present study, we evaluated the effect of concurrent intratracheal (i.t.) challenges with either LPS, LTA, BGL, chitin, heat-inactivated dust, various combinations thereof, respectively, and human serum albumin (**HuSA**) as a model to mimic possible interactions of dust components such as airborne PAMP (innate immunity) in broiler houses and protein antigen (T-cell dependent specific immunity) via the respiratory route on systemic antibody (**Ab**) responses to HuSA, and BWG of broilers. Earlier we reported interactions between i.t. administered HuSA and concurrently i.t. administered LPS with respect to BWG and humoral immune responses in layer birds (Parmentier et al., 2008). The purpose of the present study was 4-fold. First, we studied whether broilers, like layers, are prone to i.t. immunization with HuSA, and respond with primary and secondary humoral immune responses to HuSA. Second, we studied if dust, and components of dust such as PAMP may affect specific Ab responses in broilers after (repeated) i.t. challenge. Third, we studied whether an immune reaction towards a major dust component such as LPS is found in broilers as in layers, and affected by the treatments. Fourth, we studied possible consequences of (repeated) concurrent challenge with dust and dust components (e.g. PAMP) and HuSA on BWG and heart parameters. The consequences of our findings are discussed.

MATERIALS AND METHODS

Birds and husbandry

The experiment was conducted with commercial 101 slow growing (Hubbard ISA) Hubbard JA 957 female broilers (cock M99 x hen JA57), 3 weeks of age. The birds were

housed in saw dusted floor pens from hatch and during the entire experimental period. Two barns were divided into nine smaller pens of 2.5 m × 3 m × 2.5 m with 12 birds (Groups 1 to 8) or 5 birds (Group 9) each. The light regimen was 14 light and 10 h dark, and temperatures varied between 18 and 24°C during the complete observation period. The birds were feed ad lib with standard broiler diet (204 g/kg of CP, 2,859 kcal of ME/kg). Water was provided ad lib via drinking nipples. Chickens were vaccinated with (all live) vaccines for Newcastle disease, Infectious Bursal disease (Gumboro), and Infectious Bronchitis at hatch.

The experiment was approved by the Animal Welfare Committee of Wageningen University according to Dutch law.

Reagents

Human serum albumin (lot H3383), *Escherichia coli* derived LPS (lot L2880-017K4097), LTA from *Staphylococcus aureus* (lot L2515-105K4061). Chitin from crab shells (lot C7170-065K7026), and BGL derived from *Saccharomyces cerevisiae*, (Zymosan A, Z-4250, lot Z4250-084K1220) were from Sigma Chemical Co.(St. Louis, MO). NH_3 was from Merck KGaA (Darmstadt, Germany)

Experimental design

At 3 weeks of age (i.e., day 0 of the experiment), all birds from Groups 1 to 8 were challenged intratracheally (i.t.) with 0.5 mg HuSA in 0.5 ml PBS at two consecutive days, therefore receiving 1 mg HuSA in total. Challenges were performed by placing a 1.2 × 60 mm blunted anal canule (InstruVet, Cuijk, The Netherlands) on a 1-ml syringe gently in the trachea of the bird. Group 1 received only HuSA in PBS. Group 2 was challenged similarly on 2 consecutive days with 0.5 mg HuSA and 0.5 mg chitin. Group 3 received 0.5 mg HuSA and 0.5 mg LTA on 2 consecutive days. Group 4 received 0.5 mg HuSA and 0.5 mg heat-inactivated dust on 2 consecutive days. Group 5 received 0.5 mg HuSA and 0.5 mg LPS on 2 consecutive days. Group 6 received 05 mg HuSA and 0.5 mg BGL on 2 consecutive days, Group 7 received 0.5 mg HuSA and 0.25 mg LPS + 0.25 mg LTA + 0.25 mg chitin + 0.25 mg BGL on 2 consecutive days. Group 8 received 0.5 mg HuSA and 100 ppm NH_3. Group 9 received PBS i.t. only. On days 0, 3, 7, 10, 14, 21, and 28 after primary i.t. challenge 0.5 ml of heparinized blood was collected from the wing vein from all birds. On day 28 (7 weeks of age) all groups received the identical treatment to measure secondary responses to concurrently i.t. administered HuSA and PAMP. On days 0, 3, 7, 10, 14, and 21 after secondary i.t. challenge 0.5 ml of blood was collected. Plasma was stored at -20° C until use.

Body weight was measured at day 0 before to and day 2 after primary and secondary challenge. At 10 weeks of age, birds were killed, and weight, length and width or the heart were measured.

Humoral immune response to HuSA and LPS

Total Ab titers to HuSA and LPS in plasma from all birds were determined by ELISA at days 0, 3, 7, 10, 14, 21, and 28 after primary challenge with airborne dust components and HuSA, and at days 0, 3, 7, 10, 14, 21 after secondary challenge, respectively. Briefly, 96-well plates were coated with 100 µl containing either 4 µg/mL of HuSA, or 4 µg/mL of LPS, respectively. After subsequent washing with PBS containing 0.05% Tween, the plates were incubated for 60 min at room temperature with serial four-step double dilutions of plasma in PBS containing 1% horse serum and 0.05% Tween. Binding of total antibodies to HuSA, or LPS antigen was detected after 1 h of incubation at room temperature with 1:20,000 in PBS (containing 1% horse serum and 0.05 Tween) diluted rabbit anti-chicken IgG_{H+L} couple to peroxidase (RACh/IgG_{H+L}/PO, Nordic, Tilburg, The Netherlands). Immunoglobulin M and IgG Ab binding to HuSA or LPS were determined at all days as well. After incubation with serial dilution of plasma and subsequent washing, bound isotype-specific antibodies to HuSA or LPS were detected using 1:20,000 diluted goat anti-chicken IgM coupled to PO (GACh/IgM/PO) directed to the µ heavy chain of IgM (Bethyl, Montgomery, TX), 1:20,000 diluted goat anti-chicken IgG_{Fc} coupled to PO (Bethyl), respectively. After washing, tetramethylbenzidine and 0.05% H_2O_2 were added and incubated for 10 min at room temperature. The reaction was stopped with 50 µL H_2SO_4. Extinctions were measured with Multiscan (Labsystems, Helsinki, Finland) at wavelength of 450 nm. A pool of plasma taken from all birds at day 7 was used as positive standards. Titers were expressed as $\log_2$ values of the dilutions that gave an extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of a standard positive (pooled) plasma present on every microtiter plate.

Statistical analysis

Primary and secondary Ab titers (total, IgM and IgG) to HuSA, and LPS (total) were analyzed by 3-way ANOVA for the effect of dust component (PAMP), time and their interactions using the repeated measurement procedure with a bird nested within PAMP treatment. A 1-way ANOVA was done to determine differences in BWG after primary and secondary challenges with dust components, and characteristics of the heart (weight, length, width and relative heart weight) at slaughter. Body weight at 3 weeks of age was tested as a

covariate for effects of intratracheal treatments on BW gain after treatments and during the complete experimental period, body weight at 10 weeks of age was tested as a covariate for heart characteristics at the conclusion of the experiment. All analyses were according to SAS institute GLM procedures (SAS, 1990).

RESULTS

The kinetics of primary and secondary total Ab, IgM, and IgG titers to HuSA in plasma from birds immunized i.t. with various dust components (PAMP) and HuSA are shown in Figures 1, 2, and 3, respectively. Least square means of mean total (IgT) and isotype (IgM, IgG)-specific Ab titers to HuSA during 4 weeks after primary and 3 weeks after secondary immunization with different airborne dust component and HuSA are shown in Table 1.

Primary Total Antibody Responses to HuSA

Total primary Ab titers to HuSA were significantly affected by a Treatment × Time interaction (Table 1; $P < 0.0001$). Titers were highest 10 days after primary i.t. immunization for the BGL-treated birds (Group 6) and the NH_3-treated birds (Group 8), whereas titers were highest at 7 days after i.t. immunization in all other treatment groups (Figure 1). Titers to HuSA were low in the nontreated birds (Group 9) and therefore left out of the analyses. A significantly higher total Ab titers to HuSA were found in birds challenged with BGL (Group 6) or the cocktail containing LPS, LTA, BGL, and chitin (Group 7). At day 7 post-primary challenge, all dust component treatments induced significantly higher total Ab titers to HuSA compared with the birds solely challenged with HuSA (Group 1). At days 10, 14, 21, and 28, significantly higher total Ab titers to HuSA were found in the BGL-challenged birds (Group 6; Figure 1).
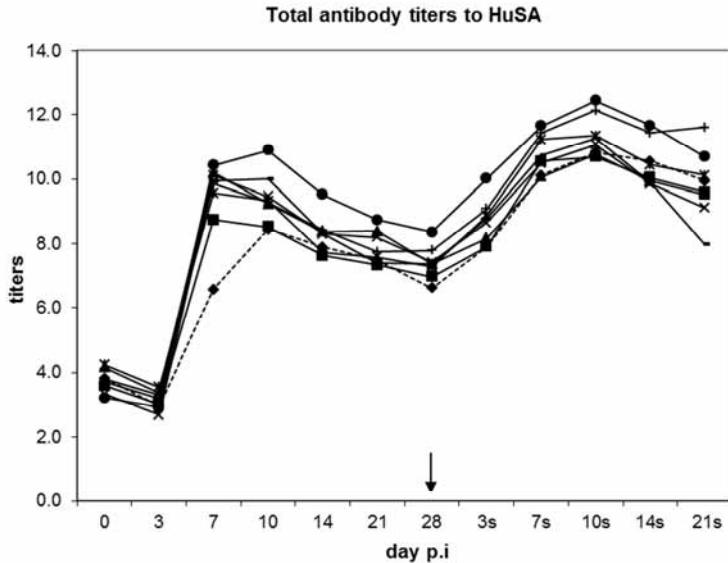
Figure 1. **The time course of the primary and secondary total antibody titers to HuSA of birds immunized intratracheally** (i.t.) with HuSA (--◆--) and concurrently with either Chitin (■), or LTA (▲), or Dust (×), or LPS (∗), or BGL (●), or Cocktail (+), NH3(−), respectively, at 3 weeks of age, and similarly after secondary i.t.. immunization 4 weeks later (arrow) with HuSA concurrently with chitin, LTA, LPS, dust, BGL, or cocktail, respectively. Data represent mean antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization with HUSA, and d 0, 3, 7, 10, 14 and 21 after secondary immunization with HuSA, as estimated by ELISA of serial dilutions of sera from 12 birds per treatment group and using 1 : 20,000 diluted IgG_{H+L} couple to peroxidase. P.i = post infection

Isotype-Specific Primary Antibody titers to HuSA

Immunoglobulin M isotype-specific primary Ab responses to HuSA during the observation period were affected by a Treatment × Time interaction ($P < 0.0001$; Table 1) and affected by treatment ($P < 0.001$). Titers of IgM binding HuSA were highest at days 7 (Group 2, chictin), group 3 (LTA), Group 4 (dust), Group 6 (BGL), and Group 7 (cocktail), but highest at day 10 for the LPS-treated birds (Group 5) and the nontreated birds (Group 1; Figure 2). Primary IgM Ab titers were significantly enhanced in the chitin-challenged birds (group 2), the LPS-challenged birds (group 5), the BGL-challenged birds (Group 6), and the birds challenged with the cocktail (group 7; $P < 0.05$; Table 1). Primary IgM Ab titers to HuSA were significantly enhanced at days 3 (chitin, LPS, BGL, cocktail treatment), day 7 (all treatments), day 10 (LPS, BGL treatment), day 14 (BGL, cocktail treatment), day 21 (LPS, BGL

treatment), and day 28 (BGL treatment) versus solely with HuSA challenged birds (Group 1; Figure 2).
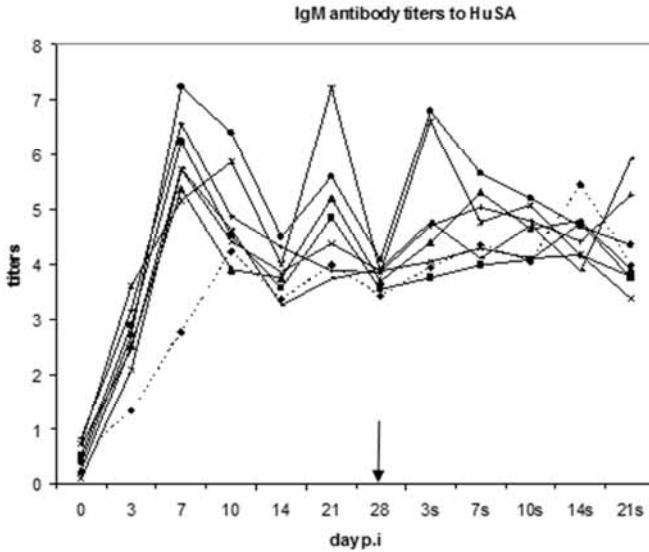

Figure 2. **The time course of the primary and secondary IgM antibody titers to HuSA of birds immunized intratracheally (i.t.)** with HuSA (--◆--) and concurrently with either Chitin (■), or LTA (▲), or Dust (×), or LPS (✶), or BGL (●), or Cocktail (+), NH3(–),respectively, at 3 weeks of age, and similarly after secondary i.t.. immunization 4 weeks later (arrow) with HuSA concurrently with chitin, LTA, LPS, dust, BGL, or cocktail, respectively. Data represent mean antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization with HUSA, and d 0, 3, 7, 10, 14 and 21 after secondary immunization with HuSA, as estimated by ELISA of serial dilutions of sera from 12 birds per Treatment group and using 1 : 20,000 diluted goat anti-chicken IgM coupled to peroxidase, p.i = post infection.

Immunoglobulin G isotype-specific primary Ab responses to HuSA during the observation period were affected by a treatment × time interaction (P < 0.0001; Table 1), but not affected by treatment (P > 0.05). Birds challenged with BGL (Group 6) showed significantly higher primary IgG Ab directed to HuSA than solely HuSA-challenged birds (Group 1; Table 1). Primary IgG Ab titers to HuSA were significantly enhanced at days 3 (heat-inactivated dust, LPS treatment), day 7 (BGL, cocktail treatment), day 10 (BGL treatment), day 14 (BGL treatment), day 21 (BGL, cocktail treatment), and day 28 (heat-inactivated dust, LTA treatment) versus solely with HuSA challenged birds (Group 1; Figure 3).
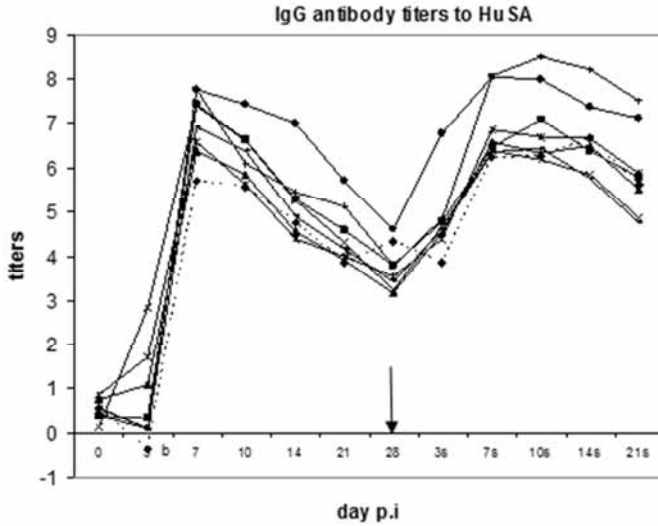
Figure 3. **The time course of the primary and secondary IgG antibody titers to HuSA of birds immunized intratracheally (i.t.)** with HuSA (--◆--) and concurrently with either Chitin (■), or LTA (▲), or Dust (×), or LPS (∗), or BGL (●), or Cocktail (+), NH3(−),respectively, at 3 weeks of age, and similarly after secondary i.t.. immunization 4 weeks later (arrow) with HuSA concurrently with chitin, LTA, LPS, dust, BGL, or cocktail, respectively. Data represent mean antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization with HUSA, and d 0, 3, 7, 10, 14 and 21 after secondary immunization with HuSA, as estimated by ELISA of serial dilutions of sera from 12 birds per Treatment group and using 1 : 20,000 diluted goat anti-chicken IgG_{Fc} coupled to peroxidase, p.i = post infection.

Secondary Total Specific Antibody Responses to HuSA

Total primary Ab titers to HuSA were significantly affected by a Treatment × Time interaction (Table 1; $P < 0.001$), and Treatment ($P < 0.05$; Table 1). Titers were highest 10 days after secondary i.t. immunization in all treatment groups (Figure 1). Significantly higher total Ab titers to HuSA were found in birds challenged with BGL (group 6) or the cocktail containing LPS, LTA, BGL, and chitin (Group 7; Table 1). Significantly higher secondary total Ab titers to HuSA post secondary challenge were found at day 3 (BGL treatment), day 7 (BGL treatment), day 10 (BGL treatment), day 14 and day 21 (BGL treatment) as opposed to the birds solely challenged with HuSA (Group 1; Figure 1).

Table 1. Total (IgT) and isotype (IgM, IgG) plasma antibody titers to Human serum albumin (HuSA) during 4 weeks after primary intratracheal immunization with HuSA at 3 weeks of age and during 3 weeks after secondary immunization with HuSA at 7 weeks of age in the presence of several dust components (DC)[1].

Group	Challenge (twice)		Primary			Secondary		
	DC (mg)	HuSA (mg)	IgT-I	IgM-I	IgG-I	IgT-II	IgM-II	IgG-II
1. HuSA	-	1	6.24^b	2.85^b	3.47^b	9.30^b	4.16^b	5.34^b
2. Chitin	1	1	7.21^{ab}	3.75^a	4.01^{ab}	9.44^b	4.15^b	5.70^{ab}
3. LTA	1	1	6.86^{ab}	3.49^b	3.76^{ab}	9.56^b	4.42^b	5.46^{ab}
4. Dust	1	1	7.15^{ab}	3.50^b	4.28^{ab}	9.29^b	3.96^b	5.09^b
5. LPS	1	1	7.21^{ab}	4.38^a	3.97^{ab}	9.98^{ab}	4.67^{ab}	5.83^b
6. β-glucan	1	1	7.67^a	4.42^a	4.67^a	10.75^a	5.10^a	6.88^a
7. Cocktail	1	1	7.26^a	3.97^a	4.14^{ab}	10.75^a	4.75^a	6.95^a
8. NH₃	1 ppm	1	7.08^{ab}	3.50^b	3.73^{ab}	9.38^b	4.50^b	5.32^b
S.E.			0.35	0.24	0.41	0.42	0.20	0.45
Treatment[2]			NS	**	NS	*	**	*
Time[2]			***	***	***	***	***	***
Time×Treatment[2]			***	***	***	**	***	***

[a,b]Means with different treatments differ significantly (P<0.05)

[1]Least squares mean ± S.E.M. of the complete observation period (day 0, 3, 7, 10, 14, 21, and 28 after primary (I) immunization with HuSA, and day 0, 3, 7, 10, 14, 21 after secondary (II) immunization with HuSA), after analysis by a repeated measures procedure. Titers are $\log_2$ of the reciprocal of the antibody dilution.

[2]Treatment = treatment (intratracheal HuSA, LPS, LTA, Chitin, β-glucans, NH₃, Cocktail, dust) effect; Time = time effect; Time × Treatment = time by treatment interaction.

*P<0.05; **P<0.01; ***P<0.001

Isotype-specific secondary antibody titers to HuSA

Immunoglobulin M isotype-specific secondary Ab responses to HuSA during the observation period were affected by a Treatment × Time interaction (P < 0.0001; Table 1) and affected by treatment (P < 0.001). Secondary IgM Ab titers were significantly enhanced in the BGL-challenged birds (Group 6), and the birds challenged with the cocktail (Group 7; P < 0.05, Table 1). Secondary IgM Ab titers to HuSA were significantly enhanced at days 3 (BGL, cocktail treatment), day 7 (BGL treatment), day 10 (LPS, BGL treatment), day 14 (LPS, BGL, cocktail treatment), but significantly decreased at day 21 (dust, LPS, BGL, cocktail treatment) versus solely with HuSA challenged birds (Group 1; Figure 2).

Immunoglobulin G isotype-specific secondary Ab responses to HuSA during the observation period were affected by a treatment × time interaction (P < 0.0001; Table 1) and affected by treatment (P < 0.05). Birds challenged with BGL (group 6) or the cocktail (Group

7) showed significantly higher secondary IgG Ab directed to HuSA than solely HuSA-challenged birds (group 1; Table 1). Secondary IgG Ab titers to HuSA were significantly enhanced at days 3 (BGL treatment), day 7 (BGL, cocktail treatment), day 10 (BGL, cocktail treatment), day 14 (cocktail treatment), day 21 (BGL, cocktail treatment) versus solely with HuSA challenged birds (Group 1; Figure 3).

Total Antibody Titers to LPS

The kinetics of primary and secondary total Ab titers to LPS in plasma from birds immunized i.t. with various dust components (PAMP) and HuSA are shown in Figure 4. Least square means of mean total Ab titers to LPS during 4 weeks after primary and 3 weeks secondary immunization with different airborne dust components and HuSA are shown in Table 2.

Table 2. Total (IgT) plasma antibody titers directed to LPS during 4 weeks after primary immunization with HuSA at 3 weeks of age, and 3 weeks after secondary immunization with HuSA at 7 weeks of age in the presence of several dust components (DC)[1].

Treatments	DC (mg)	HuSA(mg)	IgT-I	IgT-II
1. HuSA	-	1	1.53^b	2.35^b
2. Chitin	1	1	1.93^c	2.65^b
3. LTA	1	1	2.10^b	2.39^b
4. Dust	1	1	2.26^b	2.62^b
5. LPS	1	1	2.95^a	3.14^a
6. β-glucan	1	1	2.34^b	2.69^{ab}
7. Cocktail	1	1	2.4^b	2.99^a
8. NH$_3$	1 ppm	1	1.98^c	2.93^a
S.E.			0.20	0.18
Treatment[2]			**	**
Time[2]			***	***
Time×Treatment[2]			***	*

[a-c]Means with different treatments differ significantly (P<0.05)

[1]Least squares mean ± S.E.M. of the complete observation period (day 0, 3, 7, 10, 14, 21, and 28 after primary (I) immunization with HuSA, and day 0, 3, 7, 10, 14, 21 after secondary (II) immunization with HuSA), after analysis by a repeated measures procedure. Titers are log$_2$ of the reciprocal of the antibody dilution.

[2]Treatment = treatment (intratracheal HuSA, LPS, LTA, Chitin, β-glucans, NH$_3$, Cocktail, dust) effect; Time = time effect; Time × Treatment = time by treatment interaction.

*P<0.05; **P<0.01; ***P<0.001

Total Primary Antibody Titers to LPS

Total primary titers to LPS were affected by a Treatment × Time interaction (P < 0.0001; Table 2). Titers were highest at 10 days after primary i.t. challenge in all Treatment groups (Figure 4). Primary Ab titers to LPS were significantly higher in birds challenged with LPS (Group 5) than all other groups, whereas birds challenged with LTA (Group 3), dust (Group 4), BGL (Group 6), and cocktail (Group 7) also had significantly higher primary Ab tiers to LPS than birds solely challenged with HuSA (Group 1) or HuSA and NH$_3$ (Group 8; Table 2). Primary total Ab titers to LPS were significantly enhanced at days 3 (LPS, cocktail treatment), day 7 (LPS, BGL, cocktail, NH$_3$ treatment), day 10 (LTA, dust, LPS, cocktail treatment), day 14 (BGL treatment), day 21 (LPS, dust and BGL treatment) and day 28 (LPS, NH$_3$, and cocktail treatment) versus solely with HuSA challenged birds (Group 1; Figure 4).

Total Secondary Ab Titers to LPS

Total secondary titers to LPS were affected by a treatment × time interaction (P < 0.05; Table 2) and affected by treatment (P < 0.05). Titers were highest at 3 and 7 days after secondary i.t. challenge in all treatment groups (Figure 4). Secondary Ab titers to LPS were significantly higher in birds challenged with LPS (Group 5), cocktail (Group 7) and NH$_3$ (Group 8) than all other groups (Table 2). Secondary total Ab titers to LPS were significantly enhanced at days 3 (LPS, cocktail, NH$_3$ treatment), day 7 (LPS, and cocktail treatment), and day 10 (LPS) versus solely with HuSA challenged birds (Group 1; Figure 4).
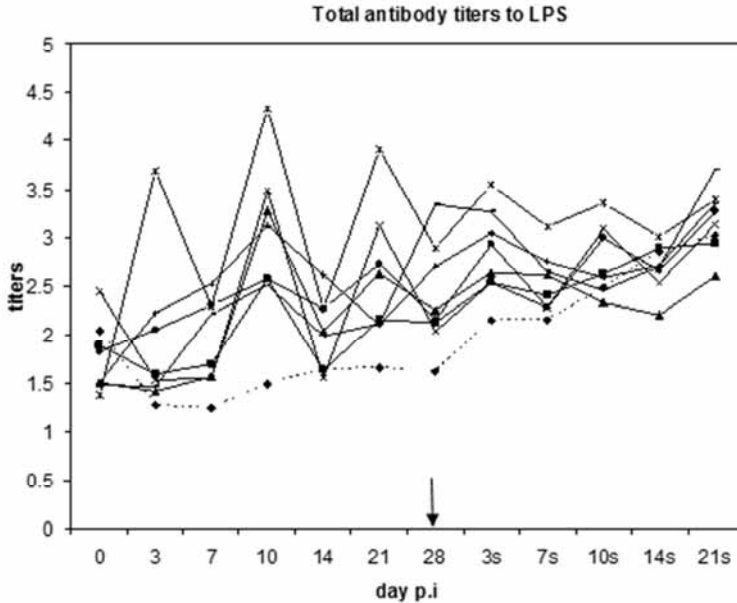
Figure 4. **The time course of the primary and secondary total antibody titers to LPS of birds immunized intratracheally (i.t.)** with HuSA (--♦--) and concurrently with either Chitin (■), or LTA (▲), or Dust (×), or LPS (∗), or BGL (●), or Cocktail (+), NH₃(−),respectively, at 3 weeks of age, and similarly after secondary i.t.. immunization 4 weeks later (arrow) with HuSA concurrently with chitin, LTA, LPS, dust, BGL, or cocktail, respectively. Data represent mean antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization with HUSA, and d 0, 3, 7, 10, 14 and 21 after secondary immunization with HuSA, as estimated by ELISA of serial dilutions of sera from 12 birds per Treatment group and using 1 : 20,000 diluted rabbit anti-chicken IgG_{H+L} couple to peroxidase, p.i = post infection.

Effects of Repeated Challenge with Dust Components on BWG

Body weight gain at 2 days after primary challenge at 3 weeks of age with dust components and HuSA was significantly affected by treatment (Table 3). Significantly lower BWG at 2 days after primary challenge was found in LTA-, LPS-, BGL-, dust-, cocktail- and NH₃-treated birds as opposed to solely HuSA- and chitin-treated birds. When compared to solely PBS-treated birds (i.e. birds that did not receive a PAMP treatment nor a HuSA challenge), the PBS-treated birds had lower BWG at 2 days after primary challenge at 3 weeks of age than birds treated with dust, LTA or PBS, respectively, concurrently with a HuSA challenge. When BW at 3 weeks of age was used as a covariate in the model a significant negative relation was found between BW at 3 weeks of age on the one hand and treatment with LPS (Group 5) or cocktail (Group 7) at the other hand, whereas BW at 3 weeks

of age was positively related with all other treatments. BWG at 2 days after secondary challenge at 7 weeks of age was also significantly affected by treatment; however, only cocktail- and BGL-treated birds showed significantly reduced BWG (Table 3). Again, a significant negative relation with BW at 3 weeks of age was estimated. These data suggest that LPS and cocktail treatments affected heavier birds at 3 and 7 weeks of age more severely than slower growing birds. Total BW at 10 weeks of age was highest in the chitin-treated group and lowest in the cocktail-treated group (Table 3). Total BWG during a period of 10 weeks was significantly higher in birds challenged twice with chitin (Group 2) and dust-treated birds (Group 4), but lowest in solely HuSA treated birds (Group 1). Body weight at 10 weeks of age and BWG during a period 10 weeks were significantly positively correlated with BW at 3 weeks of age (data not shown).

Table 3. Body weight gain (BWG) 2 days after primary (BWG-I) at 3 weeks, and secondary immunization (BWG-II) at 7 weeks of age with human serum albumin (HuSA) and dust components (DC), BW at slaughter at 10 weeks of age, and BWG during 10 weeks (BWG-T)[1].

Treatment	DC (mg)	HuSA (mg)	BWG-I	BWG-II	BW	BWG-T
1. HuSA	-	1	101.96[a]	132.21[a]	3086.4[ab]	2243.3[b]
2. Chitin	1	1	91.05[a]	145.48[a]	3214.6[ab]	2659.0[a]
3. LTA	1	1	78.33[b]	107.69[a]	3040.4[ab]	2488.0[ab]
4. Dust	1	1	83.06[b]	114.98[ab]	3259.8[a]	2666.6[a]
5. LPS	1	1	61.62[c]	103.89[ab]	3159.5[ab]	2579.0[ab]
6. β-glucans	1	1	69.08[bc]	102.93[b]	3189.8[ab]	2609.6[ab]
7. Cocktail	1	1	58.67[c]	65.86[c]	2982.3[b]	2403.4[ab]
8. NH$_3$	1 ppm	1	72.88[bc]	118.23[ab]	2967.0[ab]	2557.2[ab]
9. PBS	-	-	63.00[c]	106.00[ab]	3117.9[ab]	2360.0[ab]
SE			4.30	10.38	93.5	134.8
Treatment[2]			***	***	NS	NS
BW3[3]			**	*	***	*

[a-c]Means with different treatments differ significantly (P<0.05).

[1]Least squares mean ± S.E.M.

[2]Treatment = treatment (intratracheal HuSA, lipopolisaccharide (LPS), lipoteichoic acid (LTA), Chitin, β-glucans, NH$_3$, Cocktail, dust) effect.

[3]BW week3 = body weight at 3 weeks of age as covariate.

* P<0.05; **P<0.01; ***P<0.001

Effects of Repeated Challenge with Dust Components on Heart Morphology

Measurement of weight, length, and width of the hearts, and relative heart weights (heart weight : BW) after slaughter at 10 weeks of age revealed that significantly heavier hearts were found in birds that underwent repeated challenges with dust, LPS, BGL or NH_3. The length of hearts was significantly higher in birds that underwent repeated challenges with chitin, dust, LPS, or BGL as compared to solely HuSA-treated birds, whereas the solely PBS control birds were in-between. The width of the hearts were significantly higher in birds that underwent repeated challenge with chitin, dust, LPS, BGL, and cocktail, respectively than birds solely challenged with HuSA (Table 4). Relative heart weight was significantly higher in birds treated twice with dust (Group 4), LPS (Group 5), cocktail (Group 7) and NH_3 (Group 8), respectively as compared to the solely HuSA-treated birds (Group 1), whereas the birds which only received PBS (Group 9) were in-between (Table 4). Weight and length of heart were significantly correlated with BW at 10 weeks of age (data not shown).

Table 4. Heart characteristics and relative heart weight (RHW) at slaughter at 10 weeks of age after concurrent intratracheal immunization with human serum albumin (HuSA) and dust components (DC) at 3 and 7 weeks of age[1].

Treatment	DC (mg)	HuSA (mg)	Weight (g)	Length (cm)	Width (cm)	RHW[2]
1. HuSA	-	1	11.94[b]	4.50[b]	3.07[b]	0.00383[b]
2. Chitin	1	1	12.55[ab]	5.11[a]	3.66[a]	0.00404[b]
3. LTA	1	1	12.86[ab]	4.76[ab]	3.22[ab]	0.00413[ab]
4. Dust	1	1	13.65[a]	4.99[a]	3.46[a]	0.00440[a]
5. LPS	1	1	13.87[a]	5.19[a]	3.45[a]	0.00443[a]
6. β-glucans	1	1	13.50[a]	5.17[a]	3.54[a]	0.00432[ab]
7. Cocktail	1	1	13.53[ab]	4.85[ab]	3.45[a]	0.00435[a]
8. NH_3	1 ppm	1	13.87[ab]	4.78[ab]	3.26[ab]	0.00446[a]
9. PBS	-	-	13.86[ab]	4.60[ab]	3.53[ab]	0.00444[ab]
SE			0.62	0.16	0.12	0.00016
Treatment[3]			NS	NS	*	NS
BW10[4]			***	***	NS	NS

[a,b]Means with different treatments differ significantly (P<0.05).

[1]Least squares mean ± S.E.M.

[2]RHW = heart weight / body weight at 10 weeks of age.

[3]Treatment = treatment (intratracheal HuSA, lipoposaccharide (LPS), lipoteichoic acid (LTA), Chitin, β-glucans, NH_3, cocktail, dust) effect.

[4]BW10 = body weight at 10 weeks of age as covariate.

*P<0.05; ***P<0.001.

DISCUSSION

We evaluated modulatory features of airborne dust (and its most important components) on specific primary and secondary Ab responses to HuSA and to LPS, cachectin responses in the form of BWG after primary and secondary challenges, and effects of these challenges on heart morphology of broilers. Intratracheal challenge of growing broilers with dust and components known to be important parts of dust was used as a model for possible modulation of immune responses and growth of broilers via the respiratory tract, which may represent hygienic conditions of a chicken house. Lipoteichoic acid, LPS, BGL and chitin were chosen as important and representative PAMP from gram-negative bacteria (LPS), gram-positive bacteria (LTA), yeast (BGL), and arthropods (chitin), respectively, from which it is known that they may severely affect the respiratory tract of chickens. For instance, i.t. LPS administration induced pulmonary hypertension in poultry (Lorenzoni and Wideman, 2008). In addition, water soluble dust and NH_3 were chosen as mechanic, physic or chemical challenge as they are known to affect the respiratory tract of animals as well, or predispose the respiratory tract for infection (NH_3). Deposition of components in the lower airways of poultry has been found to be independent of size, but especially at young age (< 4 weeks) increase of particle size results in lower deposition in the lungs, but increasing deposition in the air sacs (Corbanie et al., 2006). Only one concentration of all components was used, although it is likely that different doses may have resulted in different responses. Human serum albumin was chosen as model antigen to prevent possible interference with obligatory vaccinations of the birds because HuSA is no part of vaccine additives. Antibody responses were measured since they provide information on kinetics of the immune system. Birds were exposed twice for 2 consecutive days to ascertain i.t. challenge instead of esophageal challenge. The challenge doses for LPS and BGL were 500 times higher per day than the amount that is normally inhaled by a healthy chicken per day (approximately 1 µg) kept under routine husbandry (floor) conditions. At 7 weeks of age, however, levels of airborne dust and PAMP in the pens might have affected immune responsiveness of the birds differently as compared to 3 weeks of age, apart from the experimental treatments.

Four questions were addressed. First, we studied whether broiler similar to layers (Parmentier et al., 2008) respond to i.t. challenges with the soluble T-cell dependent antigen HuSA. Second, we studied whether i.t. -administered dust and components of dust such as LPS, LTA, chitin and BGL can modulate primary and secondary Ab responses to HuSA, both total and isotype-specific. Third, we studied whether Ab responses to dust components, such

as LPS can be found in broilers and whether these responses are affected by i.t. challenges. Fourth, we studied whether dust and the various components of dust after i.t. challenge affect BWG of broilers. In addition, morphological parameters of the heart were estimated.

With respect to the first question, primary and secondary Ab responses to HuSA were found in broilers after primary and secondary i.t. challenges showing that the respiratory tract of broilers is a well-accessible location for airborne immune (natural and vaccination) challenges as was earlier found for layers (Parmentier et al., 2008). With respect to the second objective, dust, all dust components and NH_3 enhanced primary (total) Ab responses to concurrently i.t. immunized HuSA, especially early (day 7) after challenge, showing 1) that modulation by various PAMP seems to be most pronounced during primary challenges, and 2) an early non-specific enhancing effect of the dust components on Ab responses. β-glucan and LPS induced more pronounced and prolonged effects during the complete observation period, which suggests that either these components have a more stronger effect than the others, or environmental background levels interacted with the current experiment. Further studies on concentrations of dust, LTA, chitin and NH_3 may be required to find more pronounced and prolonged effects of these components as well. Several mechanisms, which are not mutually exclusive may underlie the anti-HuSA Ab-enhancing features of the dust components. Macroscopical and microscopical (data not shown) signs of inflammation in the bronchi and gas exchange surface were found at 24 h after the second i.t. administration of dust components. Inflammation may have facilitated uptake and subsequent immune processing of HuSA. Second, toll-like receptors (**TLR**) present on antigen presenting cells (APC) were identified as the signal-transducing receptor for PAMP. In chickens, TLR's 2, 3, 4, 6 and 7 have been identified on monocytes (He et al., 2006), which produced NO (nitric oxide) after induction by LPS. Furthermore, in chickens TLR2 and TLR4 mediated the LPS – stimulated oxidative burst in heterophils (Farnell et al., 2003ab). The current study suggests that in broilers dust components applied via the respiratory route can also enhance TH2-mediated primary and secondary immune responses. Secondary i.t. challenges of layers were characterized by less-pronounced effects, suggesting adaptation of the birds (Parmentier et al., 2008). On the whole in the present study with broilers, total primary Ab responses were enhanced by BGL, and the cocktail treatment containing BGL. Treatments not only differed with respect to the moments of effect, but also with respect to the affected Ab isotype. Primary IgM responses to HuSA were affected by chitin, LPS or BGL, whereas primary IgG responses to HuSA were affected by BGL, LPS, dust and LTA, albeit various treatment effects were restricted to moments after challenge. These results suggest a complex

mechanism of interactions between environmental stimuli (dose and moment) and the immune system (type of response). With respect to the latter, effects of secondary challenges with dust and dust-related PAMP and secondary challenges with HuSA also revealed a pronounced enhancing effect of BGL on secondary total, IgM, and IgG responses to HuSA, but similar to primary responses, on several moments after challenge, significant enhancing effects of LPS treatments were found as well. Repeated i.v. and i.t. LPS injections caused birds to become refractory to LPS (Parmentier et al., 2006; Korver et al., 1998). Earlier, we found immune enhancing effects of i.t. administered LPS on concurrently i.t. administered HuSA, however, in a dose dependent fashion in layers (Parmentier et al., 2008). The present results suggest that also broilers are prone to immune modulation via the respiratory tract, but as is true for layers, dose and possibly also broiler breed and age, and moment after challenge may determine the effects of i.t. administered dust and dust components. Finally, size of the dust components may determine the site of effect. Birds have a well-developed bronchus–associated lymphoid tissue (Reese et al., 2006), but small particles penetrate the lung tubuli and eventually the air sacs much further then large (> 1 μm) particles (Corbanie et al., 2006).

With respect to the third objective, enhanced Ab responses directed to LPS were found in birds challenged with LPS. In addition, challenge of the birds with NH_3, LTA, BGL, and dust also enhanced Ab responses to LPS, most likely birds were stimulated by these components to airborne LPS present in the environment of chicken. It is tempting to speculate that airborne challenges with dust or PAMP may enhance translocation of (other) PAMP such as LPS across intestinal or respiratory tract mucosa with possible consequences for the immune status. Again, modulation of secondary Ab responses to LPS was less pronounced after secondary challenge with dust and dust components than after primary challenge.

Finally, with respect to the fourth objective, all birds treated i.t. with dust and dust components concurrently with a HuSA challenge showed a reduced BWG early after primary challenge as compared to the solely HuSA challenged birds, but solely HuSA treated birds had higher 48-h BWG after primary challenge than PBS control birds. Although the latter group may appear the most appropriate control group, it is important to note that a HuSA challenge may positively interact with a concurrent PAMP challenge as published before (Parmentier et al., 2008); thus, solely HuSA-challenged birds were regarded as more appropriate control for the dust (component) treatments. After secondary challenge only the cocktail-treated group was still affected. Body weight at 3 weeks of age was related with the effects of dust component treatments at 3 and 7 weeks of age, however, there were much severe effects of LPS and cocktail treatments on heavier birds, as opposed to the other

treatments, suggesting that fast growing birds are more affected by hygienic conditions than slower growing birds. Total BWG after 10 weeks of age was not significantly affected by the experimental treatments but did depend on the BW at 3 weeks of age. These data suggested specific adaptation of the birds to challenge with dust components and final recovery of the birds to i.t. challenges. The morphology of the heart at 10 weeks of age was affected by the i.t. treatments with dust components, although we cannot distinguish between primary and secondary challenges. Weight, length and width of the heart of the broilers i.t. treated with either BGL, LPS or NH_3 concurrently with HuSA were enhanced as compared to the solely HuSA treated birds, but were not significantly different from the solely PBS treated birds. Whether HuSA challenge interacts in a positive fashion with negative effects of airborne PAMP on heart morphology, as discussed above for BWG, remains to be established. Higher heart weight and heart lengths were found in heavier birds at 10 weeks of age, but contrast analyses revealed significant treatment effects. Changes in heart morphology of broilers (enhanced right ventricle mass : total ventricle mass) have been associated with ascites and infectious diseases (Nijdam et al., 2006). The present data suggest that environmental immune-enhancing stimuli may either directly or indirectly (lung or air sac inflammation) have caused increased heart activity. No signs of ascites were found in the present experiment.

We conclude that components commonly present in dust can modulate specific Ab responses (HuSA) in broilers as well as Ab responses to environmentally present components (LPS) similar as in layers. Our data suggest that environmental hygienic conditions may not only affect immune responsiveness and therefore responses to vaccination but may also affect BWG (albeit temporarily) and functioning of the heart. Finally, effects of various dust components require further studies on concentrations, repeated challenges, environmental background levels, and age of application. Further studies are in progress to address these issues.

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CHAPTER 4

EFFECTS OF REPEATED INTRATRACHEALLY ADMINISTERED LIPOPOLYSACCHARIDE ON PRIMARY AND SECONDARY SPECIFIC ANTIBODY RESPONSES, AND BODY WEIGHT GAIN OF BROILERS

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ABSTRACT. Earlier we reported that pathogen-associated molecular patterns (PAMP) such as lipopolysaccharide (LPS), when intratracheally (i.t.) administered, affected specific primary and secondary specific antibody (Ab) responses to antigens administered concurrently, either i.t. or systemically, and also affected body weight gain (BWG) of layers and broilers. In the present study we evaluated the effects of repeated i.t. challenge with LPS concurrently with, or before i.t. immunizations with the specific antigens human serum albumin (HuSA) and rabbit gamma globulin (RGG) on primary (HuSA, RGG) and secondary (HuSA) systemic Ab responses and (isotype) IgM and IgG responses at two different ages. Broilers were challenged via the trachea at 3 and 7 weeks of age with various combinations of LPS, HuSA and RGG. All treatments affected immune responses at several time points and also affected BWG, albeit temporarily for the latter. Lipopolysaccharide enhanced primary antibody responses to HuSA and to RGG, when challenged concurrently, but birds challenged solely with LPS at 3 weeks of age also showed enhanced primary Ab responses to HuSA and RGG given at 7 weeks of age. This was true for IgM as well as IgG isotype responses. Lipopolysaccharide challenge negatively affected BWG at three weeks of age, whereas the negative effects of LPS after secondary LPS challenge at 7 weeks of age were most pronounced in the birds challenged with LPS at 3 weeks of age. The present results indicated that LPS when i.t. administered at a young age may affect specific humoral immune responsiveness to antigens administered simultaneously and to BWG of broilers, but also when challenged 4 weeks later with specific antigens, suggesting an enhanced status of immune reactivity or sensitivity. The hygienic status of broiler houses at a young age may thus influence BWG, immune responsiveness, and, consequently, the vaccine efficacy and disease resistance in broilers at later ages. The consequences of our findings are discussed.

Keywords: airborne, broiler chickens, lipopolysaccharide, modulation, pathogen-associated molecular patterns, intratracheal.

INTRODUCTION

An important part of fine dust found in poultry (and other animal) houses is formed by microbes, skin (Collins and Algers, 1986) and Pathogen-associated molecular patterns (**PAMP**) either airborne or derived from microbes in feed or feces. Pathogen-associated molecular patterns can bind to "specific" innate Toll-like (**TLR**) receptors expressed by antigen presenting cells (APC), which, in mammals and likely also in poultry via the release of cytokines, direct specific immune responses towards T helper (**TH**)2-mediated Ab, TH1-mediated cellular inflammatory responses (De Jong et al., 2002, Kapsenberg, 2003), or to TH17 responses (Betelli et al., 2007). High levels of PAMP such as lipopolysaccharide (**LPS**), found on gram negative bacteria, lipoteichoic acid (**LTA**) derived from gram-positive bacteria, β-glucans (BGL), found on yeast and many others are present in chicken houses either airborne or as part of dust. Chickens may inhale (approximately 1 m^3 air/24 h) PAMP, obtain them via the cloaca or the eyes, or take them up orally. Concentrations of airborne endotoxins (LPS) and β-glucans ranging from 240 to 13,400 endotoxin units/m^3 (1 endotoxin units/$m^3 \approx 0.1$ ng/m^3) (Douwes, 1998; Douwes et al., 2004, Anonymous, 1998) up to 63 μg/m^3 (Pomorska et al., 2007) have been found in chicken houses.

Various PAMP such as LPS, when administered intratracheally (i.t.) modulated primary and secondary immune responses and body weight gain (BWG) of layers (Ploegaert et al. 2007; Parmentier et al., 2008, 2004), and broilers (Lai et al., 2009) in a dose-dependent fashion. Next, various effects such as increased pulmonary arterial pressure (Chapman et al., 2005; Wideman et al., 2004), decreased respiratory capacity and death (Rocksen et al., 2004), and reduced BWG early after exposure (Lai et al., 2009) were found in LPS-challenged broilers. In both layers and broilers the negative effect of secondary or repeated LPS challenge on BWG was less severe, suggesting a refractive response of poultry to LPS (Korver et al., 1998; Parmentier et al., 2006), as a form of "memory" or adaptation of the birds to LPS by mechanisms yet to be defined.

Antigen-presenting cells express TLR, and may respond to various types of PAMP via the release of various types of cytokines (Lee and Iwasaki, 2007; Prescott and Dustan, 2005). However, immune responses to or initiated by PAMP are probably not restricted to components of the innate immune system. Mammalian $CD4^+$ T regulator cells (**Tregs**) (Caramalho et al., 2003; Xu et al., 2004), $CD8^+$ Tregs (Prescott and Dustan, 2005), and B cells (Quintana et al., 2008; Bernasconi et al., 2003), all being part of the acquired or specific immune system, also expresses various TLR and have been shown to respond to PAMP such

as LPS. The "improved hygiene" hypothesis states that the ability to regulate proper balances of immune responses by Tregs rests on appropriate microbial exposure during early life. Early life exposure of Tregs to PAMP may accelerate maturation of regulatory pathways (Caramalho et al., 2003) in mammals, whereas TH1- and TH2-mediated hypersensitivity and allergy may rest on a lack of (CD8$^+$) Treg maturation by early PAMP exposure (Prescott and Dustan, 2005). Recognition of specific proteins leads to antigen-specific memory, but (nonspecific) recognition of danger signals by the innate immune system may determine the size, nature, and longevity of a specific response. Recognition of danger might have long-lasting effects on CD8 memory T cells, specifically enhancing early cytokine release and altering the nature of subsequent immune responses (Noble, 2009). Finally, PAMP activation of TLR on B cells might be involved in the maintenance of antigen-specific and-nonspecific humoral memory (Bernasconi et al., 2003). It is unknown whether PAMP such as LPS affect T and B cells of poultry and if so, the consequences thereof.

In the present study, we evaluated 1) the effect of early exposure (at 3 wk of age) to LPS in the presence or absence of a concurrent immunization with a specific antigen (HuSA) to measure the effect of LPS on primary T-cell dependent specific Ab responses to HuSA, 2) the effect of repeated exposure to LPS at 7 wk of age in the presence or absence of HuSA to measure LPS effects on secondary Ab responses to HuSA, 3) the effect of concurrent LPS and rabbit gamma globulin (**RGG**) challenge at 7 wk of age on primary Ab responses to RGG, and 4) the effect of the early 3 wk LPS exposure on specific Ab to primary HuSA or RGG responses 4 weeks later. We hypothesized that LPS may enhance specific T-cell dependent Ab responses at 3 and 7 wk of age, whereas a lack of enhancement of secondary Ab responses at 7 wk of age might reflect regulation. Whether early exposure to LPS (3 wk of age) might also modulate T-cell dependent Ab responses, either primary as well as secondary in a non-antigen-specific fashion later in life (7 wk of age) was also subject of this study. In addition, isotype-specific (IgM, IgG) Ab responses were measured as an indication of T-cell involvement (levels of IgG isotype Ab). The consequences of our findings are discussed.

MATERIAL AND METHODS

Birds and husbandry

The experiment was conducted with 132 commercially obtained slow-growing (Hubbard ISA) Hubbard JA 957 male broilers (paternal M99 x maternal JA57 lines). The birds were housed in 11 saw dusted 2.5 m × 3 m × 2.5 m floor pens with 12 birds per Group (1-11)

during the entire experimental period. The light regimen was 14 h light and 10 h dark, and temperatures varied between 18 and 24 ^{0}C during the complete observation period. The birds were fed ad libitum with standard broiler diet (204 g/kg crude protein, 2,859 kcal/kg metabolizable energy). Water was provided ad libitum via drinking nipples. Chicks were vaccinated with (all live) vaccines for Newcastle disease, Infectious Bursal disease, and Infectious Bronchitis at hatch.

The experiment was approved by the Animal Welfare Committee of Wageningen University according to Dutch law.

Reagents

Human serum albumin (lot H3383), and *Escherichia coli* derived lipopolysaccharide (lot L2880-017K4097) were from Sigma Chemical Co. (St. Louis, MO). Rabbit gamma globulin (lot 68007) was from ICN (Aurora, OH).

Experimental design

The experimental design is shown in Table 1. Treatments at 3 weeks of age were designated as Challenge-1 (1-4). The 11 combinations of treatments at 3 weeks of age (Challenge-1) and 7 weeks of age (Challenge-2) were designated as Group (1-11). At 3 weeks of age (i.e. Day 0 of the experiment), all birds (12 birds/group) from Groups 1, 2, and 10 were challenged intratracheally (i.t.) with 0.5 ml PBS (Challenge 1-1). Groups 3, 5, and 8 were challenged i.t. with 0.5 mg LPS and 0.1 mg HuSA in 0.5 ml PBS (Challenge-1,2). Groups 4, 6, and 11 were challenged i.t. with 0.5 mg LPS (Challenge 1-3), and Groups 7, and 9 were challenged with 0.1 mg HuSA in 0.5 ml PBS (Challenge 1-4) by gently placing a 1.2 × 60 mm blunted anal canule (InstruVet, Cuijk, The Netherlands) on a 1-ml syringe gently in the trachea of the bird. At 7 wk of age, all birds from Groups 1, 3, 4, and 7 were i.t. challenged with 0.1 mg HuSA and 0.1 mg RGG and 0.5 mg LPS in 0.5 ml PBS (Challenge 2-1), Groups 2, 5, and 6 were challenged with 0.1 mg RGG and 0.5 mg LPS in 0.5 ml PBS (Challenge 2-2), and Groups 8, 9, 10, and 11 were challenged i.t. with 0.1 mg HuSA and 0.1 mg RGG in 0.5 ml PBS (Challenge 2-3). At days 0, 3, 7, 10, 14, 21, 28 after primary i.t. challenge, and at days 0, 3, 7, 10, 14, and 21 after secondary i.t. challenge 0.5 ml of heparinized blood was collected from the wing vein from all birds. Plasma was stored at -20° C until use. Body weight was measured at the day of challenge-1 or -2 prior to and 24 h after at 3 and 7 wk of age. At 10 wk of age, all birds were euthanized, and BW was measured.

Table 1. Experimental set up and treatments at 3 and 7 weeks of age.

Challenge-1[1]	Challenge-2[2]	Group
1. PBS	1. HuSA+LPS+RGG	1
2. HuSA+LPS	1. HuSA+LPS+RGG	3
3. LPS	1. HuSA+LPS+RGG	4
4. HuSA	1. HuSA+LPS+RGG	7
1. PBS	2. LPS+RGG	2
2. HuSA+LPS	2. LPS+RGG	5
3. LPS	2. LPS+RGG	6
2. HuSA+LPS	3. HuSA+RGG	8
4. HuSA	3. HuSA+RGG	9
1. PBS	3. HuSA+RGG	10
3. LPS	3. HuSA+RGG	11

[1]Challenge 1-1= 0.5 ml PBS intratracheally (i.t.); Challenge 1-2 = 0.1 mg of human serum albumin (HuSA) and 0.5 mg of lipopolysaccharide (LPS) in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg of LPS in 0.5 ml PBS i.t.; Challenge 1-4: 0.1 mg of HuSA in 0.5 ml PBS i.t.

[2]Challenge 2-1 = 0.1 mg of HuSA+0.5 mg of LPS+ 0.1 mg of rabbit gamma globulin (RGG) i.t.; Challenge 2-2 = 0.5 mg of LPS+0.1 mg of RGG; Challenge 2-3 = 0.1 mg of HuSA+0.1 mg of RGG i.t.

Humoral immune response to HuSA, RGG, and LPS

   Total Ab titers to HuSA, RGG, and LPS in plasma from all birds were determined by ELISA at days 0, 3, 7, 10, 14, 21, and 28 after Challenge 1 with PBS, LPS, HuSA, or their combination, and at days 0, 3, 7, 10, 14, 21 after Challenge 2 with HuSA, RGG, LPS, or their combination. Briefly, 96-well plates were coated with 4 µg/ml of HuSA, 4 µg/ml of LPS, or 4 µg/ml of RGG. After subsequent washing with H_2O containing 0.05% Tween, the plates were incubated for 60 min at room temperature with serial four-step double dilutions of plasma in PBS containing 1% horse serum and 0.05% Tween. Binding of total antibodies to HuSA, RGG, or LPS antigen was detected after 1 h of incubation at room temperature with 1:20,000 in PBS (containing 1% horse serum and 0.05 Tween) diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase (RACh/IgG_{H+L}/PO, Nordic, Tilburg, The Netherlands). Immunoglobulin M, and IgG antibodies binding to HuSA, LPS, or RGG were determined at all days as well. After incubation with serial dilutions of plasma and subsequent washing, bound isotype-specific antibodies to HuSA, LPS, or RGG were detected using 1:20,000 diluted goat anti-chicken IgM coupled to PO (GACh/IgM/PO) directed to the µ heavy chain of IgM (Bethyl, Montgomery, TX), or 1:20,000 diluted goat anti-chicken IgG_{Fc} coupled to PO (GACh/IgG_{Fc}/PO; Bethyl), respectively. After washing, tetramethylbenzidine and 0.05% H_2O_2 were added and incubated for 10 min at room temperature. The reaction was stopped with 50 µl H_2SO_4. Extinctions were measured with Multiscan (Labsystems, Helsinki, Finland) at wavelength of 450 nm. A pool of plasma taken from all birds at day 7 after challenge at 7

wk of age was used as positive standard for RGG, and a pooled known positive sample for HuSA and LPS was used for HuSA or LPS, respectively. Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of a standard positive (pooled) plasma present on every microtiter plate.

Statistical analysis

Antibody titers (total immunoglobulin, IgM and IgG) to HuSA, LPS, or RGG) at 3 wk of age were analyzed by 2-way ANOVA for the effect of Challenge 1 (PBS, HuSA + LPS, LPS, or HuSA), time, and their interactions using the repeated measurement procedure with a bird nested within Challenge 1. Antibody titers (total immunoglobulin, IgM and IgG) to HuSA, LPS, or RGG at 7 wk of age were analyzed by 2-way ANOVA for the effect of the combined Challenge 1 and Challenge 2 treatments (11 Groups), time, and their interactions using the repeated measurement procedure with a bird nested within Group. At every point after Challenge 1 at 3 wk of age or Challenge 2 at 7 wk of age, a one-way ANOVA was done for the effect of Challenge 1 (3 wk) or Group (7 wk), respectively on Ab titers to HuSA, LPS, and RGG. A one-way ANOVA was done to determine differences in BWG after challenges at 3 and 7 wk of age. All analyses were according to GLM procedures (SAS institute, 1990). Multiple mean differences between Challenge 1 treatments at 3 wk of age or Groups at 7 wk of age in the repeated measurement procedure and the one-way ANOVA at every time point were tested with Bonferroni's test.

RESULTS

Antibody Titers to HuSA

The kinetics of primary and secondary total antibodies, isotype-specific IgM, and IgG titers to HuSA in plasma from birds concurrently immunized i.t. with combinations of LPS and HuSA at 3 wk of age (Challenge 1), and combinations of LPS, HuSA and RGG at 7 wk of age (Groups) are shown in Figures 1 and 2, respectively. Least square means of mean total (IgT) and isotype (IgM, IgG)-specific Ab titers to HuSA for 4 weeks after Challenge 1 at 3 wk of age, and for 3 wk after Challenge 2 at 7 wk of age (Group) with different combinations of LPS, HuSA and RGG are shown in Table 2 (3 wk) and Table 3 (7 wk), respectively.

Table 2. Total (IgT) and isotype (IgM, IgG) plasma antibody titers to Human serum albumin (HuSA) for 4 weeks after primary intratracheal immunization with HuSA at 3 weeks of age in the presence of PBS, HuSA, and lipopolysaccharide (LPS)[1].

Groups	Challenge 1[2]	IgT	IgM	IgG
1,2,10	1. PBS	2.67[b]	4.36	2.17[b]
3,5,8	2. HuSA+LPS	4.23[a]	4.69	3.68[a]
4,6,11	3. LPS	2.79[b]	4.41	2.29[b]
7,9	4. HuSA	3.46[ab]	4.36	3.11[a]
SE		0.30	0.12	0.27
Challenge-1[3]		***	NS	***
		2≥4≥1,3		2,4>1,3
Time		***	***	***
Challenge-1×Time		***	***	***

[a,b]Means within a column with defferent superscripts denote significant differences between Challenge 1 treatments.

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, and 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG.

[2]Challenge 1-1 = 0.5 ml PBS intratracheally (i.t); Challenge 1-2 = 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-4 = 0.1 mg HuSA in 0.5 ml PBS i.t.

[3]Main effects: Challenge 1 = Challenge 1 treatment effect, Time = Time effect, Challenge 1 × Time = Challenge 1 treatment × Time interaction.

*** $P < 0.0001$

Total (Primary) Antibody Responses to HuSA at 3 wk of Age

Titers of Ab binding HuSA were highest at 7 d (Challenge 1-4) and 7 to14 days (Challenge 1-2) after i.t. challenge for the birds that received HuSA (Figure 1A). Titers to HuSA were low in birds that received solely LPS or PBS (Challenge 1-1 and 1-3). The highest titers were found in birds receiving HuSA + LPS (Challenge 1-2; Figure 1A). Total (primary) Ab titers to HuSA were significantly affected by a Challenge 1 × Time interaction (Table 2; $P < 0.05$), and a Challenge 1 effect. Significantly higher total Ab titers to HuSA were found in birds challenged with either LPS and HuSA (Challenge 1-2) or HuSA alone (Challenge 1-4) as compared to the birds treated with PBS (Challenge 1-1), or LPS (Challenge 1-3). At d 14, birds sensitized to LPS + HuSA (Challenge 1-2) showed significantly higher total Ab titers to HuSA than did birds challenged solely with HuSA (Challenge 1-4; Figure 1A).
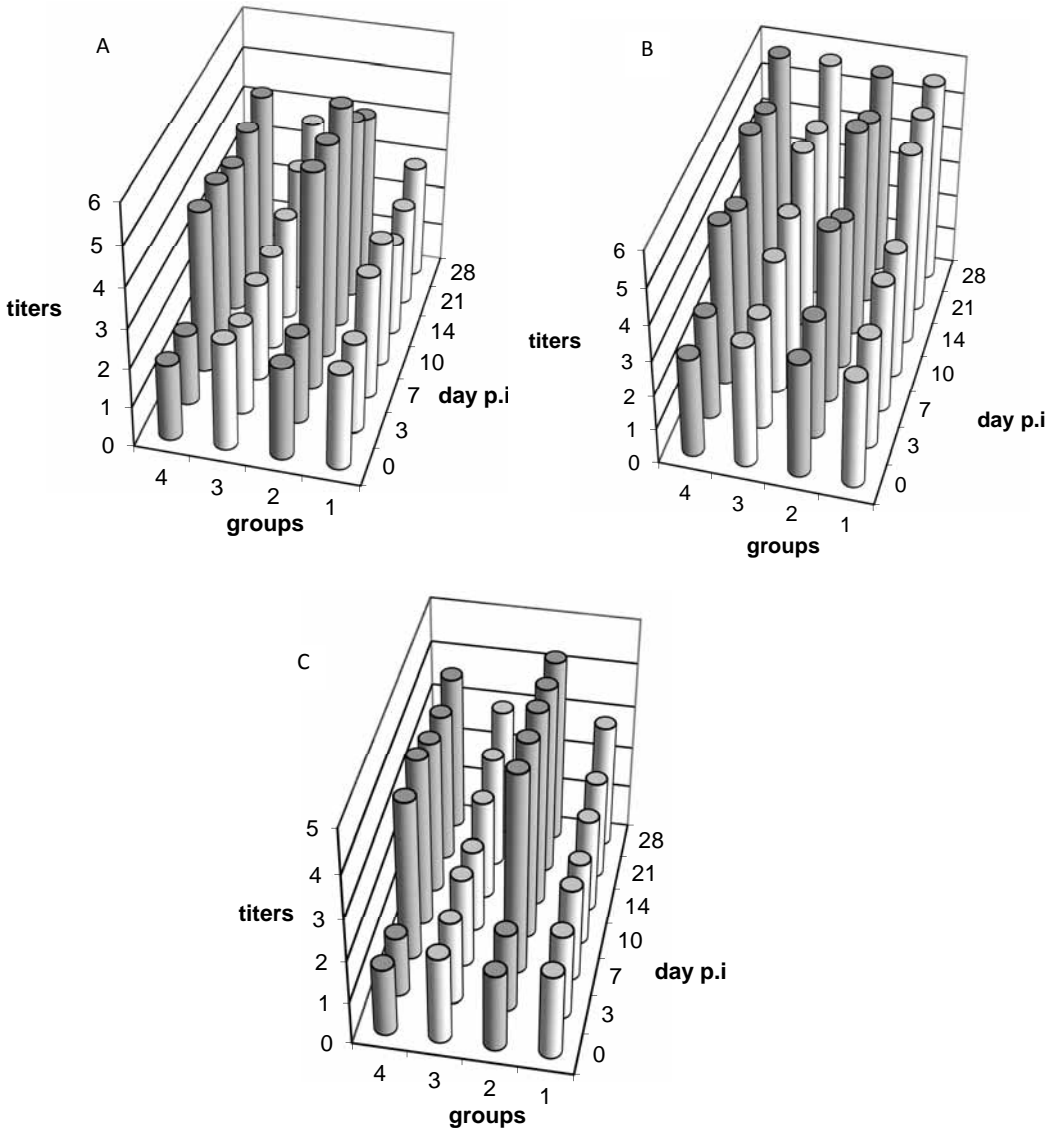
Figure 1. **The time course of the primary total (A), IgM (B) and IgG (C) antibody titers (y axis) to HuSA of birds immunized intratracheally (i.t.)** with PBS (Challenge 1-1: bar 1); or HuSA + LPS (Challenge 1-2: bar 2); or LPS (Challenge 1-3: bar 3), or HuSA (Challenge 1-4: bar 4) x axis, respectively, at 3 weeks of age. Data represent mean antibody titers at d 0, 3, 7, 10, 14, 21 and 28 (z axis) after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase ($RACh/IgG_{H+L}/PO$; Nordic, Tilburg, The Netherlands) for total antibodies, 1:20,000 diluted goat anti-chicken IgM coupled to PO (GACh/IgM/PO; Bethyl, Montgomery, TX) for IgM, or 1:20,000 diluted goat anti-chicken IgG_{Fc} coupled to PO (GACh/IgG_{Fc}/PO; Bethyl) for IgG. Pi = post immunization.

Isotype-Specific (Primary) Antibody titers to HuSA at 3 wk of Age

Titers of IgM-binding HuSA gradually increased over time and were highest in the groups that received HuSA and LPS concurrently (Challenge 1-2; Figure 1B). Immuloglobulin M isotype-specific (primary) Ab responses to HuSA during the observation period were affected by a Challenge 1 treatment × Time interaction ($P < 0.05$; Table 2), but were not affected by Challenge 1 treatment ($P > 0.05$). On day 14, birds sensitized to LPS + HuSA (Challenge 1-2) showed significantly higher IgM Ab titers to HuSA than did birds challenged solely with HuSA (Challenge 1-4; Figure 1B).

Titers of IgG-binding HuSA were, in general, highest at day 10 for the birds that received HuSA (Challenge 1-4), or HuSA + LPS (Challenge 1-2). Of these groups highest IgG titers were found birds receiving HuSA + LPS, and lower IgG titers were found in birds receiving HuSA alone (Figure 1C). Immunoglobulin G isotype-specific primary Ab responses to HuSA during the observation period were affected by a Challenge 1 treatment × Time interaction ($P < 0.05$; Table 2), and a Challenge 1 treatment effect. Significantly higher total Ab titers to HuSA were found in birds challenged with LPS + HuSA (Challenge 1-2) or with HuSA alone (Challenge 1-4) as compared with birds treated with PBS (Challenge 1-1), or with LPS alone (Challenge 1-3). At specific time points, there were no significant differences in IgG Ab titers binding HuSA between birds challenged solely with HuSA (Challenge 1-4) and birds challenged with LPS + HuSA (Challenge 1-2).

Total Specific Antibody Responses to HuSA at 7 wk of Age

Total Ab titers to HuSA peaked at 10 days after challenge at 7 wk of age in all Groups challenged with HuSA (Groups 1, 3, 4, 7, 8, 9, 10, and 11), whereas no or low responses were found in the Groups that did not receive HuSA at 7 wk of age (Groups 2, 5, and 6; Figure 2A). Total Ab titers to HuSA at 7 wk of age were significantly affected by a Group × Time interaction (Table 3; $P < 0.05$), and by Group ($P < 0.05$; Table 3). The highest titers were found in Group 4 (that received LPS alone at 3 wk of age), followed by Group 7 (HuSA at 3 wk and HuSA + LPS at 7 wk of age), Group 8 (HuSA + LPS at 3 wk and HuSA at 7 wk of age), and Group 1 (PBS at 3 wk and HuSA at 7 wk of age). Titers to HuSA in these Groups were all significantly higher than titers to HuSA in Group 3 (HuSA + LPS at 3 wk and 7 wk of age), and in all other Groups (Table 3). Significant differences were found between Group 4 (LPS at 3 wk) and Group 1 (PBS at 3 wk) and Group 3 (HuSA + LPS at 3 wk); Group 8 (HuSA + LPS at 3 wk) versus Group 9 (HuSA at 3 wk), Group 10 (PBS at 3 wk) and Group 11 (LPS at 3 wk; Table 3). At all time points highest total Ab titers to HuSA were found in birds that received LPS alone at 3 wk (Group 4). At 10 and 14 d, except for titers of Group 4, birds that received HuSA + LPS (+ RGG) at 7 wk (Groups 1, 7), and birds that received LPS + HuSA at 3 wk (Group 8) had significantly higher titers to HuSA than did birds in the other groups (Figure 2A).

Table 3. Total (IgT) and isotype (IgM, IgG) plasma antibody titers to Human serum albumin (HuSA) for 3 weeks after secondary immunization with HuSA at 7 weeks of age in the presence of HuSA, lipopolysaccharide (LPS), and rabbit gamma globulin (RGG)[1].

Group	Challenge 1[2]	Challenge 2[3]	IgT	IgM	IgG
1	PBS	1. HuSA+LPS+RGG	$^x6.64^b$	$^x7.03^{ab}$	$^x6.41^{ab}$
3	HuSA+LPS	1. HuSA+LPS+RGG	$^y5.75^c$	6.47^b	$^y5.38^b$
4	LPS	1. HuSA+LPS+RGG	$^x8.63^a$	$^x7.40^a$	$^x7.85^a$
7	HuSA	1. HuSA+LPS+RGG	$^x7.18^{ab}$	6.39^b	$^x6.76^{ab}$
2	PBS	2. LPS+RGG	3.75	5.92	3.71
5	HuSA+LPS	2. LPS+RGG	4.25	5.95	4.26
6	LPS	2. LPS+RGG	3.16	6.02	2.97
8	HuSA+LPS	3. HuSA+RGG	$^x7.20^a$	6.39	$^x7.34^a$
9	HuSA	3. HuSA+RGG	$^y4.59^b$	6.07	$^y4.54^b$
10	PBS	3. HuSA+RGG	$^y3.71^b$	$^y6.47$	$^y3.64^b$
11	LPS	3. HuSA+RGG	$^y4.91^b$	$^y6.01$	$^y4.34^b$
SE			0.70	0.27	0.64
Group[3]			***	**	***
			4≥7,8≥1>3, 11≥2,5,6,9,10	4≥1≥2,3,5, 6,7,8,9,10,11	4,8≥1,7≥3≥ 2,5,6,9,10,11
Time			***	***	***
Group×Time			***	***	***

$^{a-c}$Means within a colum with different superscripts denote significant differences within Challenge 1 treatments.

$^{x-y}$Means within a colum with different superscripts denote significant differences between Challenge 2 treatments receiving HuSA.

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, and 21 after secondary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, and 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG.

[2]Challenge 1-1 = 0.5 ml PBS intratracheally (i.t); Challenge 1-2 = 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-4 = 0.1 mg HuSA in 0.5 ml PBS i.t.

[3]Challenge 2-1 = 0.1 mg HuSA+0.5 mg LPS+ 0.1 mg RGG i.t.; Challenge 2-2 = 0.5 mg LPS+0.1 mg RGG; Challenge 2-3 = 0.1 mg HuSA+0.1 mg RGG i.t.

[3]Main effects: Group = Group effect, Time = Time effect, Group × Time = Group × Time interaction.
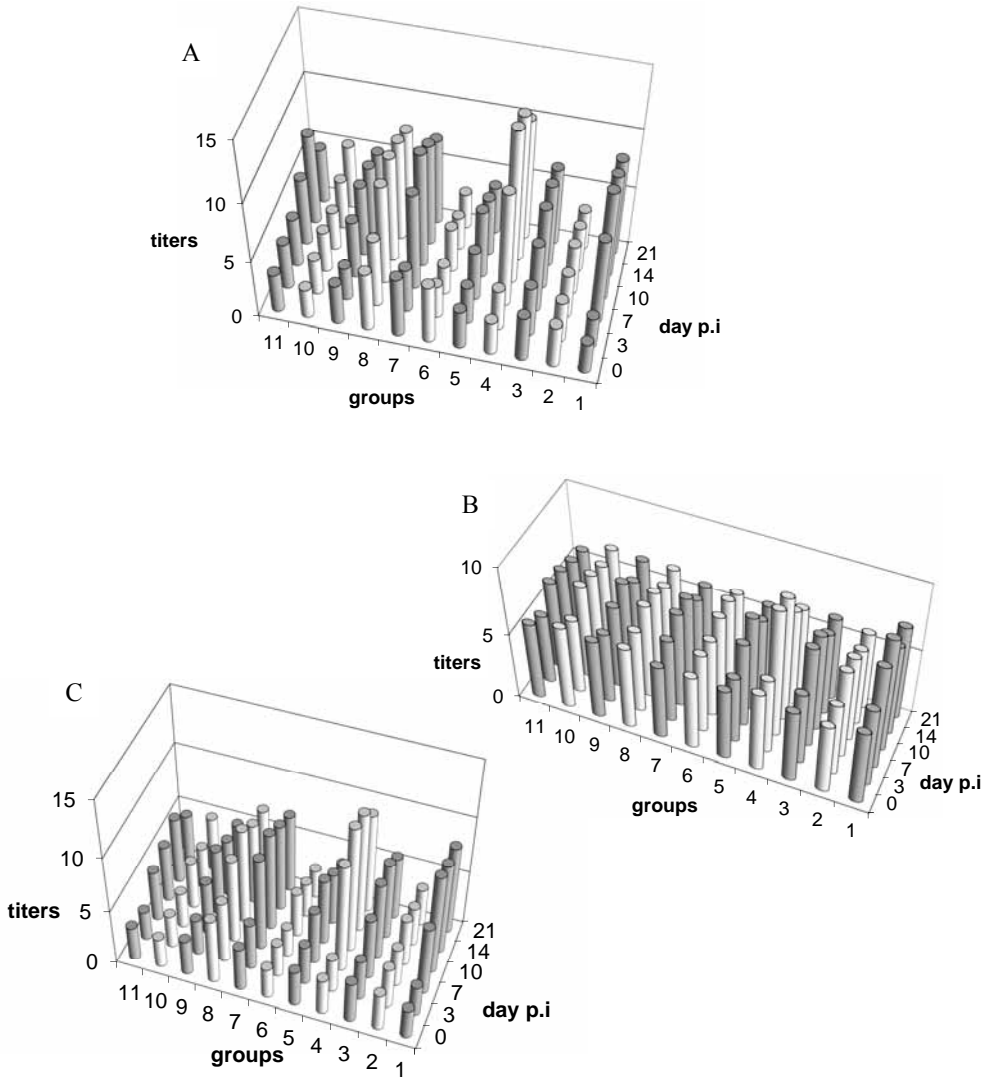
**P < 0.01, *** P < 0.0001.

Figure 2. **The time course of the secondary total (A), IgM (B) and IgG (C) antibody titers (y axis) to HuSA of birds immunized intratracheally (i.t.) with HuSA + LPS + RGG (Groups 1, 3, 4, 7), or LPS + RGG (Groups 2, 5, 6), or HuSA + RGG (Groups 8, 9, 10, 11) x axis, respectively, at 7 weeks of age**. Data represent mean antibody titers at d 0, 3, 7, 10, 14, and 21 after secondary immunization (z axis), as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase ($RACh/IgG_{H+L}/PO$; Nordic, Tilburg, The Netherlands) for total antibodies, 1:20,000 diluted goat anti-chicken IgM coupled to PO (GACh/IgM/PO; Bethyl, Montgomery, TX) for IgM, or 1:20,000 diluted goat anti-chicken IgG_{Fc} coupled to PO (GACh/IgG_{Fc}/PO; Bethyl) for IgG. Pi = post immunization.

Isotype-Specific Antibody Responses to HuSA at 7 wk of Age

Immunoglobulin M-isotype specific Ab titers to HuSA peaked at 7 (Groups 3 and 4) or 10 (Groups 1, 7, 8, 9, 10, 11) days after challenge at 7 wk of age in all Groups challenged with HuSA, whereas lower responses were found in the Groups that did not receive HuSA at 7 wk of age (Groups 2, 5, and 6; Figure 2B). Immunoglobulin M isotype-specific Ab titers to HuSA at 7 wk of age were significantly affected by a Group $\times$ Time interaction (Table 3; $P < 0.05$), and Group ($P < 0.05$; Table 3). The highest titers, as estimated by repeated measurement procedures, were found in Group 4 (that received LPS alone at 3 wk of age), followed by Group 1 (PBS at 3 wk and HuSA + LPS at 7 wk of age), and then by all other Groups (Table 3). Immunoglobulin M titers binding HuSA were highest in Group 4 (d 7, 10, 14), in birds that received LPS alone at 3 wk and LPS + HuSA + RGG at 7 wk, and in Group 1, (d 7, 10, 14, 21) that received HuSA + LPS + RGG at 7 wk (Figure 2B).

Immunoglobulin G isotype-specific Ab titers to HuSA peaked at d 7 (Group 7), d 10 (Groups 3, 4, 8, 9, 10) or d 14 (Group 11) after challenge at 7 wk of age in all Groups challenged with HuSA, whereas lower responses were found in the Groups that did not receive HuSA at 7 wk of age (Groups 2, 5, and 6; Figure 2C). Immunoglobulin G isotype-specific Ab titers to HuSA at 7 wk of age were significantly affected by a Group $\times$ Time interaction (Table 3; $P < 0.05$), and by Group ($P < 0.05$; Table 3). The highest titers were found in Group 4 (which received LPS alone at 3 wk and HuSA + LPS at 7 wk of age) and Group 8 (which received HuSA + LPS at 3 wk and HuSA at 7 wk of age), followed by Group 1 (which received PBS at 3 wk of age and HuSA + LPS at 7 wk of age) and Group 7 (which received HuSA at 3 wk and HUSA + LPS at 7 wk of age), and then all other Groups (Table 3). At d 7, 10, 14, and 21, significantly higher IgG Ab titers to HuSA were found in Group 4 birds, which received solely LPS at 3 wk, and Groups 1 and 7 birds, which received LPS + HuSA + RGG at 7 wk; and Group 8 which received HuSA + LPS at 3 wk (Figure 2C).

Antibody Titers to LPS

Least square means of mean total (IgT) and isotype (IgM, IgG)-specific Ab titers to LPS for 4 weeks after i.t. challenge at 3 wk (Challenge 1), and for 3 wk after i.t. challenge (Challenge 2) at 7 wk of age with different combinations of LPS, HuSA and RGG are shown in Table 4 (3 wk) and Table 5 (7 wk).

Total and isotype-specific Primary Antibody Titers to LPS at 3 wk of Age

Total and isotype-specific IgG titers to LPS, but not isotype-specific IgM titers to LPS were affected by a Challenge 1 treatment $\times$ Time interaction ($P < 0.05$; Table 3). Sensitization with LPS (Challenge 1-2 and 1-3) at 3 wk of age enhanced the IgG titers to LPS as compared to the Groups that received HuSA alone (Challenge 1-4) at 3 wk of age.

Table 4. Total (IgT) and isotype (IgM, IgG) plasma antibody titers to Lipopolysaccharide (LPS) for 4 weeks after primary intratracheal immunization with LPS at 3 weeks of age in the presence of PBS, human serum albumin (HuSA), and LPS[1].

Groups	Challenge 1[2]	IgT	IgM	IgG
1,2,10	1. PBS	1.57	3.58	1.21[ab]
3,5,8	2. HuSA+LPS	1.55	3.36	1.37[a]
4,6,11	3. LPS	1.84	3.45	1.32[ab]
7,9	4. HuSA	1.59	3.41	1.04[b]
SE		0.10	0.11	0.10
Challenge-1[3]		NS	NS	NS
Time		***	***	***
Challenge-1×Time		***	NS	***

[a,b]Means within a colum with different superscripts denote significant differences between Challenge 1 treatments.

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, and 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG.

[2]Challenge 1-1: 0.5 ml PBS intratracheally (i.t), Challenge 1-2: 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t., Challenge 1-3: 0.5 mg LPS in 0.5 ml PBS i.t., Challenge 1-4: 0.1 mg HuSA in 0.5 ml PBS i.t.

[3]Main effects: Challenge 1 = Challenge 1 treatment effect, Time = Time effect, Challenge 1 × Time = Challenge 1 treatment × Time interaction.

*** $P < 0.0001$

Total and Isotype-Specific Antibody Titers to LPS at 7 wk of Age

Total Ab titers to LPS at 7 wk of age were affected by a Group × Time interaction and by Group ($P < 0.05$; Table 5). The highest titers were found in Group 6 birds (LPS at 3 wk and LPS + RGG at 7 wk of age), followed by Group 5 (LPS + HuSA at 3 wk and LPS + RGG at 7 wk of age), Group 7 (HuSA at 3 wk and LPS + HuSA + RGG at 7 wk of age), Group 8 (LPS + HuSA at 3 wk and HuSA + RGG at 7 wk), and Group 9 (HuSA at 3 wk and HuSA + RGG at 7 wk of age), and then by the other Groups. Isotype-specific IgM and IgG titers to LPS were affected by a Group × Time interaction, but there was no significant Group effect (Table 5). A significant difference was found between Group 6 (LPS alone at 3 wk of age) and Group 2 (PBS at 3 wk of age).

Table 5. Total (IgT) and isotype (IgM, IgG) plasma antibody titers to Lipopolysaccharide (LPS) during 3 weeks after secondary immunization with LPS at 7 weeks of age in the presence of human serum albumin (HuSA), LPS, and rabbit gamma globulin (RGG)[1].

Group	Challenge 1[2]	Challenge 2[3]	IgT	IgM	IgG
1	PBS	1. HuSA+LPS+RGG	1.85	6.37	1.92
3	HuSA+LPS	1. HuSA+LPS+RGG	1.89	6.16	1.76
4	LPS	1. HuSA+LPS+RGG	2.17	6.21	1.97
7	HuSA	1. HuSA+LPS+RGG	2.68	5.83	2.28
2	PBS	2. LPS+RGG	2.29[b]	6.17	2.37
5	HuSA+LPS	2. LPS+RGG	2.32[ab]	6.07	2.35
6	LPS	2. LPS+RGG	2.95[a]	6.36	2.55
8	HuSA+LPS	3. HuSA+RGG	2.44	6.35	1.87
9	HuSA	3. HuSA+RGG	2.92	6.18	1.82
10	PBS	3. HuSA+RGG	2.28	6.41	1.83
11	LPS	3. HuSA+RGG	1.76	5.84	1.44
SE			0.22	0.27	0.26
Group[4]			**	NS	NS
			6≥5,7,8,9≥1,2		
			,3,4,10,11		
Time			***	***	***
Group×Time			***	***	***

[a,b]Means within a colum with different superscripts denote significant differences within Challenge 1 treatments.

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, and 21 after secondary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, and 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG.

[2]Challenge 1-1 = 0.5 ml PBS intratracheally (i.t); Challenge 1-2 = 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-4 = 0.1 mg HuSA in 0.5 ml PBS i.t.

[3]Challenge 2-1 = 0.1 mg HuSA+0.5 mg LPS+ 0.1 mg RGG i.t.; Challenge 2-2 = 0.5 mg LPS+0.1 mg RGG; Challenge 2-3 = 0.1 mg HuSA+0.1 mg RGG i.t.

[4]Main effects: Group = Group effect; Time = Time effect; Group × Time = Group × Time interaction.

** $P < 0.01$, *** $P < 0.0001$

(Non)-Specific Antibody Titers to RGG

The kinetics of Total Ab, and isotype-specific IgM, and IgG titers to RGG in plasma from birds concurrently immunized i.t. with combinations of LPS and HuSA at 3 wk of age, and combinations of LPS, HuSA and RGG at 7 wk of age are shown in Figure 3. Least square means of mean total (IgT) and isotype (IgM, IgG)-specific Ab titers to RGG for 4 weeks after Challenge 1 and 3 weeks after Challenge 2 with different combinations of LPS, HuSA and RGG are shown in Table 6 (3 wk) and Table 7 (7 wk).

Non-Specific Total and Isotype Specific Antibody Responses to RGG at 3 wk of Age

Total and isotype-specific antibody responses to RGG at 3 wk of age increased with time (data not shown). Titers were not affected by a Challenge 1 treatment effect or by a Challenge 1 treatment × Time interaction (Table 6). No significant differences were found for total or IgM Ab. At d 14, birds that received LPS (Challenge 1-2 and 1-4) showed lower levels of IgG Ab binding RGG, whereas at d 21, LPS (Challenge 1-2 and 1-4) significantly enhanced IgG Ab binding RGG (data not shown)

Total Specific Antibody Responses to RGG at 7 wk of Age

Total Ab titers to RGG peaked at 10 days after challenge at 7 wk of age in all Groups, except for Group 2 which peaked at 14 days after challenge (Figure 3A). Total Ab titers to RGG at 7 wk of age were significantly affected by a Group × Time interaction (Table 7; $P <$ 0.05), and by Group ($P < 0.05$; Table 7). The highest titers were found in Group 4 (which received LPS at 3 wk of age), followed by Group 1 (PBS at 3 wk of age and RGG + HuSA + LPS at 7 wk of age), Group 5 (HuSA + LPS at 3 wk of age and RGG + LPS at 7 wk of age), Group 6 (LPS at 3 wk of age and LPS + RGG at 7 wk of age), and Group 7 (HuSA at 3 wk and HuSA + RGG + LPS at 7 wk of age), followed by all other Groups. Groups 8, 9, 10, and 11 (all Groups that did not receive LPS at 7 wk of age) showed lowest titers to RGG at 7 wk of age (Table 7). A significant difference was found between Group 4 (LPS at 3 wk of age) and Group 3 (HuSA + LPS at 3 wk of age). At d 7 and 10, significant highest total Ab to RGG were found in birds that solely received LPS at 3 wk (Group 4; Figure 3A).

Table 6. Total (IgT) and isotype (IgM, IgG) plasma antibody titers to Rabbit Gamma Globulin (RGG) for 4 weeks after Challenge 1 at 3 weeks of age in the presence of PBS, human serum albumin (HuSA), and lipopolysaccharide (LPS)[1].

Groups	Challenge 1[2]	IgT	IgM	IgG
1,2,10	1. PBS	2.78	5.20	3.51
3,5,8	2. HuSA+LPS	2.75	5.14	3.60
4,6,11	3. LPS	2.70	5.25	3.89
7,9	4. HuSA	2.72	5.18	3.65
SE		0.11	0.15	0.16
Challenge-1[3]		NS	NS	NS
Time		***	***	***
Challenge-1×Time		NS	NS	***

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, and 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG.

[2]Challenge 1-1 = 0.5 ml PBS intratracheally (i.t); Challenge 1-2 = 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-4 = 0.1 mg HuSA in 0.5 ml PBS i.t.

[3]Main effects: Challenge 1 = Challenge 1 treatment effect, Time = Time effect, Challenge 1 × Time = Challenge 1 treatment × Time interaction.

*** $P < 0.0001$.

Isotype-Specific to RGG at 7 wk of Age

Immunoglobulin M isotype-specific Ab titers to RGG and IgG-isotype specific Ab titers to RGG peaked at 10 days after challenge at 7 wk of age in all Groups (Figures 3B and 3C). Immunoglobulin M isotype-specific primary Ab titers to RGG and IgG-isotype specific primary Ab titers to RGG were affected by a Group × Time interaction, but no significant Group effect was found (Table 7). The highest IgG titers to RGG were found in Groups 1 and 4, namely, birds that received LPS + HuSA + RGG at 7 wk, or LPS at 3 wk and LPS + HuSA + RGG at 7 wk (Figure 3C).
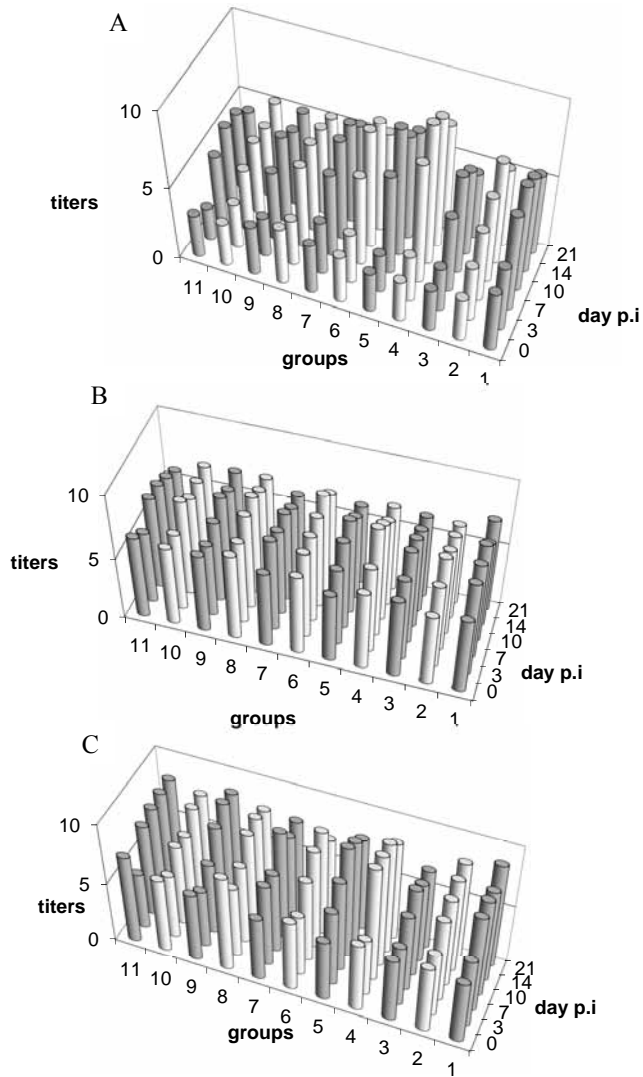
Figure 3. The time course of the primary total (A), IgM (B) and IgG (C) antibody titers (y axis) to RGG of birds immunized intratracheally (i.t.) with HuSA + LPS + RGG (Groups 1, 3, 4, 7), or LPS + RGG (Groups 2, 5, 6), or HuSA + RGG (Groups 8, 9, 10, 11) x axis, respectively, at 7 weeks of age. Data represent mean antibody titers at d 0, 3, 7, 10, 14, and 21 (z axis) after immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted rabbit anti-chicken IgG$_{H+L}$ coupled to peroxidase (RACh/IgG$_{H+L}$/PO; Nordic, Tilburg, The Netherlands) for total antibodies, 1:20,000 diluted goat anti-chicken IgM coupled to PO (GACh/IgM/PO; Bethyl, Montgomery, TX) for IgM, or 1:20,000 diluted goat anti-chicken IgG$_{Fc}$ coupled to PO (GACh/IgG$_{Fc}$/PO; Bethyl) for IgG. Pi = post immunization.

Table 7. Total (IgT) and isotype (IgM, IgG) plasma antibody titers to Rabbit Gamma Globulin (RGG) during 3 weeks after primary immunization with RGG at 7 weeks of age in the presence of human serum albumin (HuSA), lipopolysaccharide (LPS), and RGG[1].

Group	Challenge 1[2]	Challenge 2[3]	IgT	IgM	IgG
1	PBS	1. HuSA+LPS+RGG	[xy]5.70[ab]	[x]7.29	7.25
3	HuSA+LPS	1. HuSA+LPS+RGG	[y]4.65[b]	[xy]6.82	6.39
4	LPS	1. HuSA+LPS+RGG	[x]6.64[a]	[xy]7.18	7.94
7	HuSA	1. HuSA+LPS+RGG	[xy]5.18[ab]	[y]6.54	7.47
2	PBS	2. LPS+RGG	[y]5.01	[xy]6.68	7.00
5	HuSA+LPS	2. LPS+RGG	[xy]5.81	[xy]6.60	7.61
6	LPS	2. LPS+RGG	[xy]5.59	[xy]6.90	7.01
8	HuSA+LPS	3. HuSA+RGG	[y]4.93	[xy]7.03	7.49
9	HuSA	3. HuSA+RGG	[y]4.42	[y]6.34	6.96
10	PBS	3. HuSA+RGG	[y]4.33	[xy]6.65	6.85
11	LPS	3. HuSA+RGG	[y]4.19	[y]6.54	7.41
SE			0.52	0.25	0.41
Group[4]			*	NS	NS
			4≥1,5,6,7≥2,3		
			,8,9,10,11		
Time			***	***	***
Group×Time			***	***	***

[a,b]Means within a colum with different superscripts denote significant differences within Challenge 1 treatments.

[x,y]Means within a colum with different superscripts denote significant differences between (all) Challenge 2 treatments receiving RGG.

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, and 21 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, and 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG.

[2]Challenge 1-1 = 0.5 ml PBS intratracheally (i.t); Challenge 1-2 = 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-4 = 0.1 mg HuSA in 0.5 ml PBS i.t.

[3]Challenge 2-1 = 0.1 mg HuSA+0.5 mg LPS+ 0.1 mg RGG i.t.; Challenge 2-2 = 0.5 mg LPS+0.1 mg RGG; Challenge 2-3 = 0.1 mg HuSA+0.1 mg RGG i.t.

[4]Main effects: Group = Group effect, Time = Time effect, Group × Time = Group × Time interaction.

*$P < 0.05$, *** $P < 0.0001$.

Effects of Intratracheal Challenges on Body Weight Gain

Body weight gain at 24 h after primary Challenge 1 at 3 wk of age with various combinations of LPS and HuSA was significantly affected by Challenge 1 treatment ($P <$ 0.05; Table 8). A significantly lower BWG after Challenge 1 at 3 wk of age was found in birds that were challenged with LPS (Challenge 1-3) or LPS + HuSA (Challenge 1-2) as compared to the groups that were challenged solely with HuSA (Challenge 1-4) or PBS (Challenge 1-1). Body weight gain at 24 h after intratracheal Challenge 2 at 7 wk of age was significantly affected by Group (Table 9). The lowest BWG at 24 h after challenge at 7 wk of age was found in Group 4 (LPS at 3 wk and HuSA + LPS + RGG at 7 wk of age), Group 6 (LPS at 3 wk and LPS + RGG at 7 wk of age), Group 7 (HuSA at 3 wk and HuSA + LPS + RGG at 7 wk of age), and Group 9 (HuSA at 3 wk and HuSA + RGG at 7 wk of age). The highest BWG was found in Group 1 (PBS at 3 wk and HuSA + LPS + RGG at 7 wk of age). Significant differences were found between Groups 4 and 7 versus Group 1, and between Group 6 versus Groups 2 and 5. No significant differences were found between Groups 8, 9, 10, and 11 (i.e. all birds that did not receive LPS at 7 wk of age).

Table 8. Body weight gain (g) 1 day after Challenge 1 (BWG-I) at 3 weeks of age
in the present of PBS, human serum albumin (HuSA), and lipopolysaccharide (LPS).

Groups	Challenge 1[1]	BWG-I
1,2,10	1. PBS	37.7[a]
3,5,8	2. HuSA+LPS	30.0[bc]
4,6,11	3. LPS	27.8[c]
7,9	4. HuSA	34.0[ab]
SE		2.0
Challenge 1[2]		**
		1≥4≥2≥3

[a-c]Means within a colum with different superscripts denote significant differences between Challenge 1 treatments.

[1]Challenge 1-1 = 0.5 ml PBS intratracheally (i.t); Challenge 1-2 = 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-4 = 0.1 mg HuSA in 0.5 ml PBS i.t.

[2]Main effects: Challenge 1 = Challenge 1 treatment effect.

** $P < 0.01$

Table 9. Body weight gain (BWG; g) 1 day after Challenge 2 at 7 weeks of age (BWG-II), and body weight gain during 10 weeks (BWG-T).

Group	Challenge 1[1]	Challenge 2[2]	BWG-II	BWG-T
1	PBS	1. HuSA+LPS+RGG	[x]68.3[a]	[xy]3091
3	HuSA+LPS	1. HuSA+LPS+RGG	[xyz]39.4[ab]	[xy]3119
4	LPS	1. HuSA+LPS+RGG	[yz]29.4[b]	[xy]3096
7	HuSA	1. HuSA+LPS+RGG	[yz]21.8[b]	[xy]3270
2	PBS	2. LPS+RGG	[xy]55.5[a]	[y]3028
5	HuSA+LPS	2. LPS+RGG	[xy]52.7[a]	[xy]3244
6	LPS	2. LPS+RGG	[z]12.4[b]	[xy]3191
8	HuSA+LPS	3. HuSA+RGG	[xy]45.5	[xy]3099
9	HuSA	3. HuSA+RGG	[yz]31.4	[xy]3217
10	PBS	3. HuSA+RGG	[xyz]40.3	[x]3300
11	LPS	3. HuSA+RGG	[xyz]38.3	[xy]3210
SE			11.0	94
Group[3]			*	NS
			1≥2,5,8≥3,10,11≥ 4,6,7,9	

[a,b]Means within a colum with different superscripts denote significant differences within Challenge 1 treatments.

[x-z]Means within a colum with different superscripts denote significant differences between (all) Challenge 2 treatments.

[1]Challenge 1-1= 0.5 ml PBS intratracheally (i.t); Challenge 1-2 = 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-3 = 0.5 mg LPS in 0.5 ml PBS i.t.; Challenge 1-4 = 0.1 mg HuSA in 0.5 ml PBS i.t.

[2]Challenge 2-1 = 0.1 mg HuSA+0.5 mg LPS+ 0.1 mg RGG i.t.; Challenge 2-2 = 0.5 mg LPS+0.1 mg RGG; Challenge 2-3 = 0.1 mg HuSA+0.1 mg RGG i.t.

[3]Main effects: Group = Group effect.

* $P < 0.05$

DISCUSSION

We evaluated modulatory features of challenge with LPS on specific primary Ab responses to HuSA or RGG, and secondary Ab responses to HuSA, decreased BWG after i.t. challenge of growing broilers with combinations of LPS and HuSA at 3 wk of age, and combinations of challenging with LPS + HuSA + RGG at 7 wk of age. Lipopolysaccharide is a major constituent of airborne dust in chicken houses, and like other components of dust, may modulate immune responses (for instance to vaccines and infection) and growth of broilers via the respiratory tract. Previously, we found that (combinations of) LPS, LTA, β-glucan and

chitin as important and representative PAMP from gram-negative bacteria (LPS), gram-positive bacteria (LTA), yeast (β-glucan), and arthropods (chitin), respectively, also affected specific immune responses of broilers (Lai et al., 2009) and layers (Ploegaert at al., 2007) after i.t. challenge. We chose HuSA and RGG as model antigens since they are not part of poultry vaccines (additives). Antibody responses were measured because they provide information on kinetics of the specific humoral immune system. Isotype-specific IgM and IgG titers to these antigens were measured since they may shed light on the involvement of T cells (IgG Ab) as well as memory responses, especially after secondary challenge (to HuSA). Furthermore, it was proposed earlier that broilers as opposed to layers are characterized by short-term IgM responses, whereas layers mount long-term IgG responses upon exposure to a T-cell-dependent antigen (Koenen et al. 2002). The challenge doses of HuSA and RGG were chosen such that specific Ab responses could be either enhanced or accelerated (Parmentier et al., 2008), or decreased and delayed. The single challenge dose for LPS at 3 and 7 wk of age was 500 times higher per day than the amount that is normally inhaled by a healthy chicken per day (estimated at approximately 1 μg) when kept under routine housing (floor) conditions. However, it should be kept in mind that at 7 wk of age levels of airborne LPS in the chicken house might have affected immune responsiveness of the birds differently as compared to 3 wk of age, apart from the experimental treatments. Earlier studies with LPS and other PAMP administered to broilers and layers either i.t. or via other routes revealed that repeated challenge with PAMP such as LPS resulted in refractive responses with respect to BW (gain) and immune responsiveness (Korver et al., 1998, Parmentier et al., 2006, 2008) suggesting a form of innate memory. In addition, repeated challenge of poultry with LPS resulted in Ab responses to LPS with characteristics of T-cell dependent immune responses (Parmentier et al., 2008). Pathogen-associated molecular pattern such as LPS bind to Toll like receptors (TLR) present on antigen-presenting cells that release various cytokines there upon, activating various kinds of T cells (Lee and Iwasaki, 2007; Prescott and Dustan, 2005) and providing a direct link between innate and specific immunity. However, TLR were also found on (mammalian) CD4^{+} T regulator cells (Tregs) (Caramalho et al., 2003; Xu et al., 2004), CD8^{+} Tregs (Prescott and Dustan, 2005), and B cells (Quintana et al., 2008; Bernasconi et al., 2003). All these lymphocyte classes responded to PAMP such as LPS. It was proposed that a proper balance of immune responses rests on appropriate microbial exposure of Tregs during early life, which may accelerate maturation of regulatory pathways (Caramalho et al., 2003) in mammals, whereas hypersensitivity, allergy or immune suppression may rest on a lack of (CD8^{+}) Treg maturation by PAMP exposure (Prescott and Dustan, 2005). Recognition of

specific protein antigens leads to antigen-specific memory, but (nonspecific) recognition of danger signals such as LPS by the innate immune system (antigen-presenting cells) or components of specific immunity (T cells) may determine the size, nature, and longevity of a specific (secondary) immune response. Recognition of danger might have long-lasting effects on CD8 memory T cells, specifically enhancing early cytokine release and altering the nature of subsequent immune responses (Noble, 2009). Finally, PAMP activation of TLR on B cells might be involved in the maintenance of specific and non-specific humoral memory (Bernasconi et al., 2003). However, whether these phenomena are present in poultry is unknown.

In the present study, we therefore evaluated 1) the effect of early exposure (at 3 wk of age) to LPS in the presence or absence of a concurrent immunization with a specific antigen (HuSA) to measure the effect of LPS on primary T-cell-dependent specific Ab responses to HuSA (Challenge 1 effect), 2) the effect of repeated exposure to LPS at 7 wk of age in the presence or absence of HuSA to measure LPS effects on secondary Ab responses to HuSA (Group effect), 3) the effect of early (3 wk) prior to, or concurrent challenge of LPS and RGG on primary Ab responses to RGG at 7 wk of age (Group effect), and 4) the effect of the early 3 wk LPS exposure on specific Ab to HuSA or RGG responses 4 weeks later (Group effect). We studied whether LPS affected primary specific T-cell-dependent Ab responses at 3 and 7 wk of age, or whether early exposure to LPS (3 wk of age) also modulated T-cell-dependent Ab responses, either primary or secondary in a non-antigen specific fashion later in life (7 wk of age).

With respect to the first objective, primary Ab responses to HuSA were enhanced in birds after concurrent i.t. challenge with LPS at 3 wk of age. Enhancement of primary Ab responses to a T-cell dependent antigen such as HuSA in the presence of LPS was found previously in broilers (Lai et al., 2009) and layers (Parmentier et al., 2008). Immunoglobulin G, but not IgM primary Ab responses to HuSA were especially enhanced (Table 2) in HuSA-challenged birds, which corresponds with the putative involvement of CD4^{+} T cells being activated by PAMP. Similarly, LPS enhanced primary Total and IgG Ab responses to HuSA (Groups 1 and 4 versus 10 and 11, Table 3) suggesting the involved of CD4^{+} T cells activated by LPS. In addition primary total specific Ab responses to RGG after challenge at 7 wk of age were enhanced by concurrent LPS challenge (Groups 1-7; Table 7). The latter suggests that putative "natural" environmental LPS challenge did not affect the modulatory features of the experimental LPS challenge at 7 wk of age.

With respect to the second objective, the specific total as well as the isotype-specific (IgM, IgG) Ab responses to HuSA at 7 wk of age were affected by the specific antigen, as expected, (secondary) responses were higher in groups that had already received HuSA at 3 wk of age (antigen-specific memory), but surprisingly the Ab responses to HuSA were highest in groups that received HuSA for the first time concurrently with LPS at 7 wk of age, as well as in birds that received LPS without HuSA at 3 wk of age. The significant differences between Group 1 versus Group 2, Group 3 versus Group 5, and Group 4 versus Group 6, respectively, indicated that LPS enhanced primary Ab responses. In addition, the significant differences between Group 3 versus Group 8, Group 4 versus Group 11, and Group 7 versus Group 9 suggested that LPS has a negative effect, or at least no effect, on secondary Ab responses to HuSA (Table 3). It is tempting to speculate that indeed, as suggested for mammals, early and repeated PAMP (LPS) exposure result in two different forms of regulation of antigen specific immune responses (Noble, 2009). The current data suggested that in broilers, exposure to PAMP such as LPS at an early age, via the respiratory route, may affect (enhance) primary and secondary immune responses, and may therefore have consequences for vaccination strategies and health management. For instance, application of PAMP within vaccines, or challenge of birds with PAMP before vaccination may enhance efficacy of vaccination. In the present study, Ab responses to LPS after challenge at both 3 an 7 wk of age were low. The highest responses were found in birds that received LPS twice (Group 6, Table 5). Earlier we also found lower Ab responses to LPS in broilers (Lai et al., 2009) than in layers (Parmentier et al., 2009).

With respect to the third and fourth objectives, total Ab responses to RGG at 7 wk of age were highest in birds that received concurrently LPS and RGG at 7 wk of age (Groups 1 to 7; Table 7), however, the highest response was found in Group 4 birds which received LPS alone 4 wk earlier. This suggests that activation of the immune system with a PAMP, such as LPS at a young age enhances immune responsiveness to specific antigens (HuSA, RGG) at a later age in an antigen-nonspecific (possibly innate) fashion, which was also suggested for the third objective as discussed above.

Finally, all birds treated i.t. with LPS at 3 wk of age showed reduced BWG, as expected. Human serum albumin decreased the loss of BWG at 3 wk of age, suggesting interaction between innate and specific immune responses. Earlier we found that the temporary decrease in BWG after secondary challenge to LPS was much less severe (Lai et al., 2009, Parmentier et al., 2008), suggesting adaptation of the birds to challenge with LPS and final recovery of the birds from i.t. challenges. Therefore, in the present study, we expected lower BWG in

Groups that received LPS for the first time at 7 wk of age (e.g., Groups 1, 2, and 7). However, lower BWG was determined in the groups that received LPS alone (Groups 4, 6; Table 9), or HuSA alone (Groups 7, 9; Table 9) at 3 wk of age, which is difficult to explain. Concurrent challenge at 3 wk of age with an innate antigen (LPS) and a specific antigen (HuSA) (Groups 3, 5, and 8) resulted in less severe loss of BW at 7 wk of age after LPS challenge.

We conclude that LPS, which is the major component of dust present in poultry houses, can modulate specific primary Ab responses to specific antigens administered concurrently (HuSA, RGG) to broilers at 3 and 7 wk of age. However, LPS may also affect specific primary Ab responses to these antigens even when these antigens are administered 4 wk later. This suggests an enhanced sensory status of the immune system. On the other hand, LPS did not enhance, and only slightly affected, secondary Ab responses to specific antigen suggesting that, as proposed for mammals, PAMP are indeed required for maturation of regulation (Noble, 2009). Whether the present findings rest on these mechanisms is subject of future studies. Our data suggest that environmental hygienic conditions may affect not only immune responsiveness, and therefore responses to vaccination or infectious diseases, but also other physiological parameters, such as BWG (albeit temporarily). Further studies are in progress to address these issues.

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CHAPTER 5

EFFECTS OF TWO SIZE CLASSES OF INTRATRACHEALLY ADMINISTERED AIRBORNE DUST PARTICLES ON PRIMARY AND SECONDARY SPECIFIC ANTIBODY RESPONSES AND BODY WEIGHT GAIN OF BROILERS: A PILOT STUDY ON THE EFFECTS OF NATURALLY OCCURRING DUST

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ABSTRACT. We studied the effects of a concurrent challenge on slow growing broilers with 1) airborne particles of 2 sizes: fine dust (FD, smaller than 2.5 µm) and coarse dust (CD, between 2.5 - 10 µm) that were directly collected from a broiler house, and 2) lipopolysaccharide (LPS) on intratracheal immunizations with the specific antigen human serum albumin (HuSA), and measured primary and secondary systemic (total) antibody (Ab) responses and (isotype-specific) IgM, IgG and IgA responses at 3 and 7 weeks of age. All treatments affected immune responses at several ages, heart morphology and body weight gain (BWG), albeit the latter only temporarily. Dust particles significantly decreased primary entibody (IgT and IgG) responses to HuSA at 3 weeks of age, but enhanced IgM responses to HuSA at 7 wk of age. Dust particles decreased secondary Ab responses to HuSA, albeit not significantly. All the birds that were challenged with dust particles showed decreased BWG after primary but not after secondary challenge. Relative heart weight was significantly decreased in birds challenged with CD, FD, LPS and HuSA at 3 weeks of age, but not in birds challenged at 7 weeks of age. Morphology (weight, width, and length) of hearts were also affected by dust challenge at 3 weeks of age. The present results indicate that airborne dust particles obtained from a broiler house when intratracheally administered at an early age affect specific humoral immune responsiveness and BWG of broilers to simultaneously administered antigens differently than when administered at a later age. The hygienic status of broiler houses at a young age may be of importance for growth and immune responsiveness, and consequently, for vaccine efficacy and disease resistance in broilers. The consequences of our findings are discussed.

Keywords: airborne dust, broiler chickens, lipopolysaccharide, modulation, intratracheal

INTRODUCTION

The high concentrations of airborne particles (fine dust) in animal houses has raised questions on the effects of air constituents on the quality of the environment, risks for developing acute or chronic (infectious) diseases, and the welfare of animals and agricultural employees (Donham et al., 1995, Pope et al., 2002, Andersen et al., 2004, Herr et al., 1999, Al Homidan and Robertson, 2003). Most dust, and its components, inside and outside animal houses, originate from manure, bedding, skin, feather and feed (Aarnink et al., 1999, Aarnink et al., 2006a, Takai et al., 1998, Welch). The concentration and composition of dust varies due to animal activity, species, age, light schedule, ventilation, stocking density, and house design (Collins and Algers, 1986). The size of dust particles is important because it influences the aerodynamic behavior and transport, and it may therefore require different control technologies (Zhang, 2004a). Dust particle size also determines the impact of dust on human and animal health (Mercer, 1978). Respirable particles (smaller than 4.0 µm in diameter, i.e. similar to tobacco smoke) are mainly responsible for health problems, because these small particles can travel deep into the lungs (Collins and Algers., 1986). Studies on airborne dust concentrations are mainly focused on small particle sizes, such as particulate matter smaller than 10 µm (PM10) and particulate matter smaller than 2.5 µm (PM2.5). In general, particles from this range of sizes form the majority both in mass (> 50%) and in counts (> 99%) in animal houses, including poultry houses (Lai at al., 2011b). Small particles deposit deeper in the respiratory tract; however, final deposition also depends on the shape and density of the particles and deepness of animal's breathing. Important constituents of (fine) dust found in poultry (and other animal) houses are formed by microbes, skin (Collins and Algers, 1986) and pathogen-associated molecular patterns (**PAMP**) that are either airborne or derived from microbes in feed or feces. These PAMP can bind to specific innate Toll-like receptors (**TLR**) expressed by antigen presenting cells (**APC**), that in mammals, and likely also in poultry, skew specific immune responses towards T-Helper (**TH**) 2-mediated Ab, or TH1-mediated cellular inflammatory responses (De Jong et al., 2002, Kapsenberg, 2003), or to TH17 responses (Betelli et al., 2007), via the release of cytokines. High levels of PAMP are present in chicken houses, either airborne or as part of dust.

Previously we found that PAMP; that is, (combinations of) lipopolyssacharide (**LPS**), lipoteichoic acid (**LTA**), β-glucan and chitin as important and representative PAMP from gram-negative bacteria , gram-positive bacteria , yeast, and arthropods, respectively, affected specific immune responses of broilers (Lai et al., 2009) and layers (Ploegaert at al., 2007)

after an intratracheal challenge. Also, reduced BWG early after exposure (Lai et al., 2009) was found in LPS- and other PAMP-challenged broilers. Both in layers and broilers, the effect of secondary or repeated LPS challenge on BWG appeared less severe, suggesting a refractive response of poultry to LPS (Korver et al., 1998; Parmentier et al., 2006) as a form of memory or adaptation of the birds to LPS by as yet to be defined mechanisms.

The effects of particle size distribution of dust on the immune system of broilers are still unknown. In the present study, we obtained two different sizes of airborne particles, fine dust (**FD**; <2.5 µm), and coarse dust (CD; 2.5-10 µm), from a broiler house and assessed the LPS content in these 2 size classes. Subsequently, we measured the effects of LPS and both dust size classes on primary and secondary humoral immune responses to the model antigen, human serum albumin (**HuSA**) at 2 different ages and BW (gain) in slow-growing broilers.

First, we studied the effect of early exposure (at 3 wk of age) to FD and CD particles or a comparable dose of LPS. Second, we studied the effect of exposure at a later age (7 wk) to these particles. Third, we studied the effect of repeated exposure to dust particles or LPS at 3 and 7 wk of age on secondary Ab responses to HuSA to establish the effects on memory formation. Fourth, we studied whether dust particles or a comparable dose of LPS after an intratracheal challenge affect BW (gain) of broilers. Changes in heart morphology of broilers have been associated with ascites and infectious diseases (Nijdam et al., 2006). Earlier we described an increase in heart weights and changed heart morphology in broilers treated intratracheally with dust components and PAMP (Lai et al., 2009). Therefore, we addressed possible consequences of challenging with dust particles in 2 size classes (FD and CD) and with comparable dose of LPS on morphological parameters of spleen and heart.

MATERIALS AND METHODS

Birds and Husbandry

The experiment was conducted with 96 commercially obtained slow-growing Hubbard ISA JA 957 male broilers (paternal M99 x maternal JA57 lines). The birds were housed in 8 pens of 2.5 m × 3 m × 2.5 m on a solid floor covered with wood shavings. Each pen contained one Group of 12 birds (Groups 1-8) during the entire experimental period. The light regimen was 14 h light and 10 h dark, and temperatures varied between 18 and 24°C during the complete experimental period. The birds were fed ad libitum with standard broiler diet (204 g/kg of Cp, 2,859 kcal/kg of ME). Water was provided ad libitum via drinking nipples. Chicks were vaccinated with (all live) vaccines for Newcastle disease, Infectious Bursa disease, and Infectious Bronchitis at day of hatch.

The experiment was approved by the Animal Welfare Committee of Wageningen University according to Dutch law.

Reagents

Human serum albumin (lot H3383), and *Escherichia coli* derived lipopolysaccharide (lot L2880-017K4097) were obtained from Sigma Chemical Co. (St. Louis, MO). Both FD and CD dust particles were collected on polycarbonate membrane filters with a diameter of 37 mm in the filter holder. Larger particles were separated by installing a cyclone pre-separator (CPS) (URG corp., Chapel Hill, NC, US) as described by Zhao et al. (2009).

The airflow through the cyclone pre-separator was set at 1 m^3/h. Dust particles were collected in a broiler house (floor with bedding system, side inlet, ventilators in end wall) with approximately 3000 broilers, at the Spelderholt broiler facility, Lelystad, the Netherlands. The PM2.5 and PM2.5-10 dust was removed from the filters, and stored at -20 °C until use. A limulus amoebocyte lysate (LAL) assay was conducted to detect and quantify lipopolysaccharide (LPS) in the PM2.5 and PM2.5-10 dust fractions. LPS concentrations were 90 ng / mg dust in both FD and CD fractions. Birds were challenged once or twice with 4 mg dust containing in total 360 ng LPS.

Experimental Design

The experimental design is shown in Table 1. Treatments at 3 weeks of age (Challenge 1) and 7 weeks of age (Challenge 2) were randomly assigned to the different groups of animals (Groups 1-8). At 3 weeks of age, (i.e. Day 0 of the experiment) all birds (12 birds per Group) from Groups 5, 6, 7, and 8 were challenged intratracheally (i.t.) with 0.5 ml PBS. Group 1 was challenged i.t. with 4.0 mg (fine) dust PM2.5 (FD), and 0.1 mg HuSA in 0.5 ml PBS. Group 2 was challenged i.t. with 4 mg course dust PM 2.5-10 (CD), and 0.1 mg HuSA in 0.5 ml PBS. Group 3 was challenged with 360 ng Sigma LPS (dose comparable to the amount of LPS in the dust fractions) and 0.1 mg HuSA in 0.5 ml PBS. Group 4 was challenged with 0.1 mg HuSA in 0.5 ml PBS. Birds were challenged by placing a 1.2 × 60 mm blunted anal canule (InstruVet, Cuijk, The Netherlands) on a 1 ml syringe gently in the trachea of the bird.

At 7 wk of age, birds from Group 1 and Group 7 were i.t. challenged with 4 mg dust PM2.5 (FD), and 0.1 mg HuSA in 0.5 ml PBS. Group 2 and Group 8 were challenged with 4 mg dust PM2.5-10 (CD), and 0.1 mg HuSA in 0.5 ml PBS. Group 3 was challenged with 360 ng LPS (Sigma) and 0.1 mg HuSA in 0.5 ml PBS. Group 4 was challenged with 0.1 mg HuSA in 0.5 ml PBS. Group 5 was challenged with 0,1 mg HuSA in 0.5 ml PBS. Group 6 was challenged with 0.5 ml PBS only. At days 0, 3, 7, 10, 14, 21, 28 after primary i.t. challenge, and at days 0, 3, 7, 10, 14, and 21 after secondary i.t. challenge 0.5 ml of heparinized blood was collected from the wing vein from all birds. Plasma was stored at -20° C until use. Body weight was measured at the day of Challenge 1 or Challenge 2 prior to

and 24 h after challenge at 3 and 7 wk of age. At 12 wk of age, all birds were euthanized and BW (G) was measured.

Table 1. Experimental set up and treatments at 3 and 7 weeks of age.

Group	Challenge 1[1]	Challenge 2[2]
1	Fine dust+HuSA	Fine dust+HuSA
2	Coarse dust+HuSA	Coarse dust+HuSA
3	LPS+HuSA	LPS+HuSA
4	HuSA	HuSA
5	PBS	HuSA
6	PBS	PBS
7	PBS	Fine dust+HuSA
8	PBS	Coarse dust+HuSA

[1]Fine dust (FD): dust in the size range < 2.5µm; 4 mg/bird. Coarse dust (CD): dust in the size range of 2.5µm - 10µm; 4 mg/bird. HuSA = human serum albumin and was given in a dose of 0.1 mg; and LPS = lipopolysaccharide and was given in a dose of 360 ng. See Material and Methods section for details.

Humoral Immune Response to HuSA and LPS

Total Ab titers to HuSA and LPS in plasma from all birds were determined by ELISA at days 0, 3, 7, 10, 14, 21, and 28 after Challenge 1, and at days 0, 3, 7, 10, 14, 21 after Challenge 2. Briefly, 96-well plates were coated with 100 µl either containing 4 µg/ml HuSA, or 4 µg/ml LPS, respectively. After subsequent washing with H_2O containing 0.05% Tween, the plates were incubated for 60 min at room temperature with serial four-step double dilutions of plasma in PBS containing 1% horse serum and 0.05% Tween. Binding of total antibodies to HuSA, or LPS antigen was detected after 1 h of incubation at room temperature with 1:20,000 in PBS (containing 1% horse serum and 0.05 Tween) diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase (RACh/IgG_{H+L}/PO, Nordic, Tilburg, The Netherlands). IgM, IgG, and IgA antibodies binding to HuSA were determined at all days as well. After incubation with serial dilution of plasma and subsequent washing, bound isotype-specific antibodies to HuSA were detected using 1:20,000 diluted goat anti-chicken IgM coupled to PO (GACh/IgM/PO) directed to the µ heavy chain of IgM (Bethyl, Montgomery, TX), or 1:20,000 diluted goat anti-chicken IgG_{Fc} coupled to PO (Bethyl), or 1:20,000 diluted (GACh/IgA/PO) directed to the α heavy chain of IgA (Bethyl), respectively. After washing, tetramethylbenzidine and 0.05% H_2O_2 were added and incubated for 10 min at room temperature. The reaction was stopped with 50 µl H_2SO_4. Extinctions were measured with Multiscan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. A pooled known positive sample for HuSA and LPS was used for HuSA or LPS, respectively. Positive standards were included in each plate. Titers were expressed as $\log_2$ values of the dilutions

that gave an extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of a standard positive (pooled) plasma present on every microtiter plate.

Statistical analysis

Antibody titers (total, IgM, IgG, and IgA) to HuSA and total Ab titers to LPS at 3 wk of age, were analyzed by 2-way ANOVA for the effects of Challenge 1 (two dust size classes with HuSA, or LPS added to HuSA, only HuSA, or only PBS), time, and their interactions using the repeated measurement procedure with a bird nested within Challenge 1.

Antibody titers (total, IgM, IgG, and IgA) to HuSA, and total Ab titers to LPS at 7 wk of age were analyzed by 2-way ANOVA for treatment (8 groups), time and their interactions using the repeated measurement procedure with a bird nested within Group.

At every time point after Challenge 1 at 3 wk of age or Challenge 2 at 7 wk of age, a one way ANOVA was performed for the effect of Challenge 1 (3 wk) or Group (7 wk) on Ab titers to HuSA or LPS. A one-way ANOVA was performed to determine differences in BWG after challenges using Challenge 1 or Group as the explaining factor at 3 and 7 wk of age, respectively. Similarly, a one way ANOVA was performed to measure differences in heart morphology and spleen at slaughter, respectively. All analyses were done with the GLM procedures of SAS (SAS institute Inc., 1990). Multiple comparisons between means of Challenge 1 treatments at 3 wk of age and Groups at 7 wk of age in the repeated measurement procedure, and the one-way ANOVA at every time point were done with Bonferroni's test.

RESULTS

The kinetics of primary and secondary total Ab, IgM, IgG, and IgA titers to HuSA in plasma from birds immunized intratracheally with various fine (FD) or course (CD) dust particles or LPS and HuSA are shown in Figures 1, 2, and 3, respectively. Least square means of mean total (IgT) and isotype (IgM, IgG, IgA)-specific Ab titers to HuSA during 4 weeks after primary and 3 weeks after secondary immunization with different airborne dust or LPS and HuSA are shown in Table 2 and Table 3.

Primary Total Antibody Responses to HuSA at 3 weeks of age

Total primary Ab titers to HuSA were significantly affected by a Treatment × Time interaction (Table 2, $P < 0.05$). Titers were highest at 10 days after primary intratracheal immunization for the PBS+HuSA-challenged birds (Group 4; Figure 1A; P<0.05), whereas titers were highest at 7 days after intratracheal immunization in the FD+HuSA-treated birds (Group 1; Figure 1A), the LPS+HuSA-treated birds (Group 3; Figure 1A), and the CD+HuSA-treated birds (Group 2; Figure 1A). Titers to HuSA were the lowest in the solely PBS-treated (control) birds (Group 5). A significantly lower total Ab titers to HuSA were found in birds treated with FD+HuSA and CD+HuSA, respectively, compared to the birds challenged with PBS+HuSA (Group 4), whereas the LPS+HuSA-challenged birds were in-between (Table 2). All HuSA-challenged groups showed significantly higher total Ab titers to HuSA as opposed to the birds solely challenged with PBS (Group 5; Figure 1A; Table 2). At day 21 significantly higher total Ab titers to HuSA were found in the PBS+HuSA-challenged birds (Group 4) as compared to the FD, CD or LPS+HuSA-treated birds. (Figure 1A). At the other moments, in general, also highest total Ab titers to HuSA were found in the PBS+HuSA-challenged birds.

Table 2. Total (IgT) and isotype (IgM, IgG, IgA) plasma antibody titers to Human serum albumin (HuSA) during 4 weeks after primary intratracheal immunization with HuSA at 3 weeks of age in the presence of coarse dust (CD), fine dust (FD), lipopolysaccharide (LPS), human serum albumin (HuSA), and PBS.

Groups	Challenge 1[1]	IgT	IgM	IgG	IgA
1	1. FD +HuSA	6.32[b]	2.80[a]	5.86[b]	1.83
2	2. CD +HuSA	5.61[b]	3.08[a]	5.50[b]	2.28
3	3. LPS+HuSA	6.66[ab]	2.96[a]	6.47[ab]	1.85
4	4. PBS+HuSA	7.63[a]	3.07[a]	7.03[a]	1.94
5,6,7,8	5. PBS	2.63[c]	2.28[b]	2.38[c]	2.06
SE		0.41	0.21	0.38	0.23
Challenge-1[2]		***	***	***	NS
		4≥3≥1,2>5	1,2,3,4>5	4≥3≥1,2>5	
Time		***	***	***	***
Challenge-1×Time		***	***	***	***

[a-c] denotes significant differences between Challenge 1 treatments

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted $RACh/IgG_{H+L}/PO$ for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, 1:20,000 diluted $GACh/IgG_{Fc}/PO$ for IgG, and 1:20,000 diluted $GACh/IgG_{Fc}/PO$ for IgA.

[2]See table 1 for group treatment details.
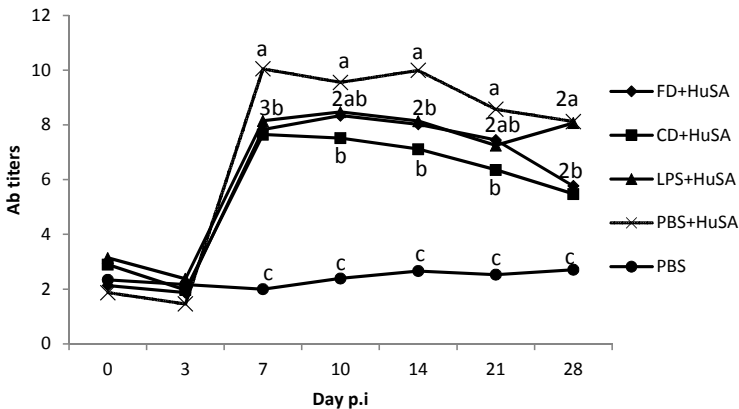
*** $P < 0.0001$

A

B

C

Figure 1. The time course of the primary total (A), IgM (B), and IgG (C) antibody titers to HuSA of birds immunized intratracheally (i.t.) with FD+HuSA (Challenge 1-1); or CD+HuSA (Challenge 1-2); or LPS+HuSA (Challenge 1- 3), or PBS+HuSA (Challenge 1-4); or solely PBS (Challenge 1-5), respectively, at 3 weeks of age. Data represent mean antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for total antibodies, or 1 : 20,000 diluted GACh/IgM/PO for IgM, or 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG. P.i = post immunization. [a-c] denote significant differences between Groups at these ages.

Isotype-Specific Primary Antibody titers to HuSA at 3 weeks of age

The IgM-isotype specific primary Ab responses to HuSA during the observation period were affected by a Treatment × Time interaction (P < 0.05; Table 2) and affected by Treatment (P < 0.05). Titers of IgM binding HuSA were highest at days 7 in birds from Group 4 (PBS+HuSA), Group 2 (CD+HuSA), Group 3 (LPS+HuSA), and Group 1 (FD+HuSA), and the lowest at day 7 for the PBS-treated control birds (Group 5) (Figure 1B). Primary IgM Ab titers were significantly enhanced at day 7 in the HuSA-challenged birds (Group 4), the CD+HuSA-challenged birds (Group 2), the LPS+HuSA-challenged birds (Group 3), and the birds challenged with FD+HuSA (Group 1; P<0.05; Table 2) versus those of the (control) birds solely PBS-challenged (control) birds (Group 5; Figure 1 B). At day 10, IgM Ab titers to HuSA were significant higher in the PBS+HuSA-challenged birds (Group 4), the CD+HuSA-challenged birds (Group 2), and the LPS+HuSA-challenged birds (Group 3) than in the PBS-challenged control birds (Group 5; Figure 1 B). At day 7 and day 28 post challenge, significantly lower IgM titers to HuSA were found in the FD+HuSA-challenged (Group 1) as compared to the PBS+HuSA-challenged birds (Group 4; Figure 1 B). The IgG-isotype specific primary Ab responses to HuSA during the observation period were affected by a Treatment × Time interaction (P < 0.05; Table 2) and affected by Treatment (P < 0.05). Birds challenged with PBS+HuSA (Group 4) showed significantly higher primary IgG antibodies directed to HuSA than birds challenged with FD+HuSA (Group 1) and CD+HuSA (Group 2; Table 2). Primary IgG Ab titers to HuSA were significantly enhanced at days 7, day 10, day 14, day 21, and day 28 in birds from all groups challenged with HuSA versus those of the (control) birds solely challenged with PBS (Group 5; Figure 1C). At days 7, 10, 14, 21, and 28 CD+HuSA-treated birds had significantly lower titers to HuSA than those of the PBS+HuSA-treated birds, which was also true for days 7, 14, and 28 with respect to FD+HuSA-challenged birds (Figure 1C).

Titers of IgA binding HuSA at 3 weeks of age were not affected by a Time × Treatment interaction or Treatment (Table 2). Also no contrasts between treatments at any moment after challenge were found (data not shown).

Primary Total Antibody Responses to HuSA at 7 weeks of age

Total primary Ab titers to HuSA at 7 wk were significantly affected by a Treatment × Time interaction (Table 3). Titers were highest at 10 days after primary intratracheal challenge at 7 week of age for the CD+HuSA-treated birds (Group 8), whereas titers were lowest in the FD+HuSA-treated birds (Group 7; Table 3). At days 7, 10, 14, and 21 significantly higher total Ab titers to HuSA were found in the CD+HuSA-challenged birds (Group 8) as compared to FD+HuSA-treated birds (Group 7), PBS+HuSA-treated birds (Group 5), and solely PBS-treated control birds (Group 6; Figure 2A).

Table 3. Total (IgT) and isotype (IgM, IgG, and IgA) plasma antibody titers to Human serum albumin (HuSA) during 3 weeks after secondary immunization with HuSA at 7 weeks of age in the presence of coarse dust (CD), fine dust (FD), lipopolysaccharide (LPS), human serum albumin (HuSA), and PBS.

Group	Challenge 1[1]	Challenge 2[2]	IgT	IgM	IgG	IgA
1	FD+HuSA	FD+HuSA	7.81[a]	[y]3.08[b]	7.49[a]	1.57[b]
2	CD+HuSA	CD+HuSA	7.67[a]	[x]3.76[b]	7.30[a]	2.11[ab]
3	LPS+HuSA	LPS+HuSA	9.67[a]	[xy]3.29[b]	8.77[a]	2.26[ab]
4	PBS+HuSA	PBS+HuSA	9.69[a]	[xy]3.44[b]	8.89[a]	2.32[ab]
5	PBS	HuSA	3.94[b]	[y]3.54[b]	3.85[b]	2.54[a]
6	PBS	PBS	4.35[b]	[xy]3.85[ab]	3.38[b]	2.59[a]
7	PBS	FD+HuSA	3.66[b]	[xy]3.80[ab]	3.69[b]	2.06[ab]
8	PBS	CD+HuSA	5.51[b]	[x]4.40[a]	4.83[b]	2.18[ab]
SE			0.75	0.22	0.66	0.31
Group3			***	**	***	NS
			1,2,3,4>5,6,7,8	8>6,7>,2>,3,4,5 >1	1,2,3,4>5,6,7,8	
Time			***	***	***	***
Group×Time			***	***	***	***

[a,b] denotes significant differences within Challenge 1 treatments.

[x,y] denotes significant differences between Challenge 2 treatments.

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, and 21 after secondary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT, 1 : 20,000 diluted GACh/IgM/PO for IgM, 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG. and 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgA.

[2]See table 1 for group treatment details.
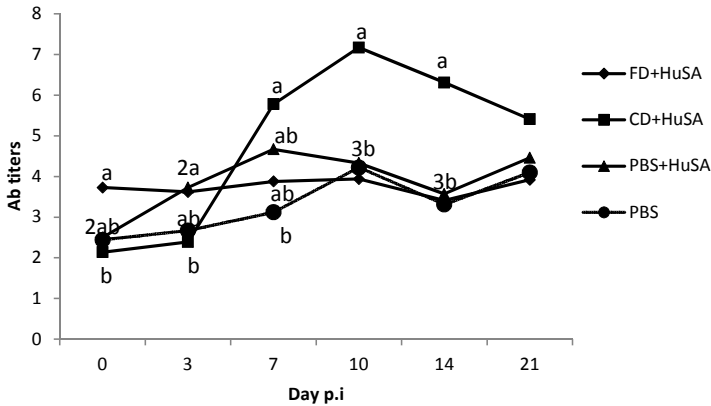
**P < 0.01, *** P < 0.0001

A
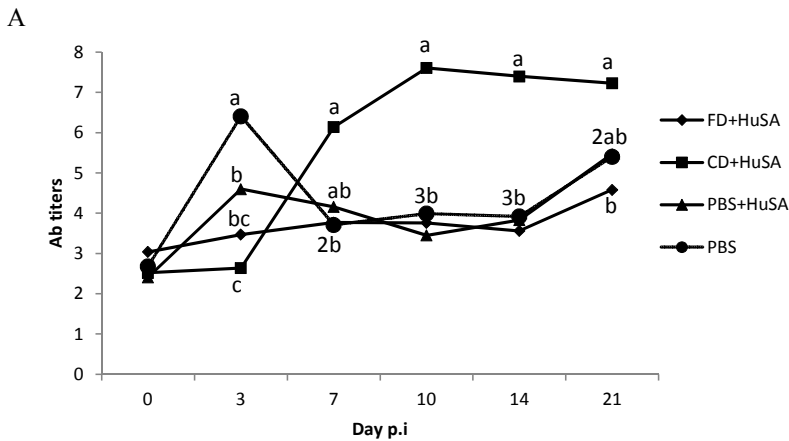
B
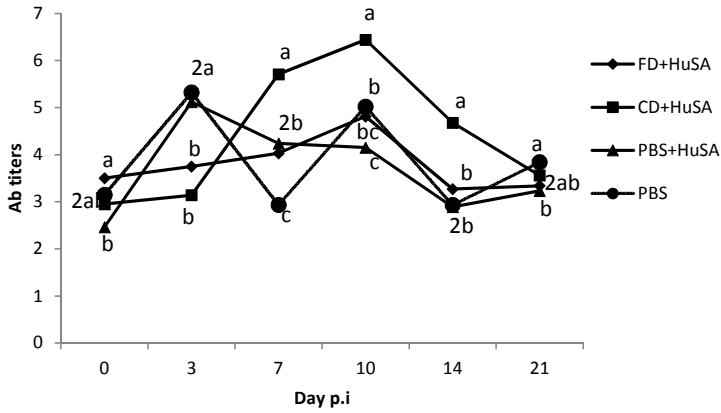
C

Figure 2. The time course of the primary late total (A), IgM (B), and IgG (C), antibody titers to HuSA of birds immunized intratracheally (i.t.) with FD+ HuSA (Group 7), or CD+HuSA (Group 8), or PBS+HuSA (Group 5), or solely PBS (Group 6) respectively, at 7 weeks of age. Data represent mean antibody titers at d 0, 3, 7, 10, 14, and 21 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per Treatment group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for total antibodies, or 1 : 20,000 diluted GACh/IgM/PO for IgM, or 1 : 20,000 diluted GACh/IgG$_{Fc}$/PO for IgG. P.i = post immunization [a-c] denote significant different between Groups at these ages.

Isotype-Specific Primary Antibody titers to HuSA at 7 weeks of age

The IgM-isotype specific primary Ab responses to HuSA during the observation period at 7 wk were affected by a Treatment × Time interaction ($P < 0.05$; Table 3) and affected by treatment ($P < 0.05$). The CD+HuSA-challenged birds had significantly higher IgM titers to HuSA than those of the birds challenged with PBS+HuSA (Table 3). Titers of IgM binding HuSA were highest at days 10 in birds of Group 8 (CD+HuSA), Group 7 (FD+HuSA), Group 5 (LPS+HuSA) and the lowest at day 10 for the non-(PBS)-treated birds (Group 6; Figure 2B). Primary IgM Ab titers were significantly enhanced at day 7, day 10, day 14 in the CD+HuSA-challenged birds (Group 8) versus the FD+HuSA-challenged birds (Group 7), PBS+HuSA-challenged birds, and solely PBS-challenged control birds (Group 6; Figure 2B).

The IgG-isotype specific primary Ab titers to HuSA at 7 wk were significantly affected by a Treatment × Time interaction ($P < 0.05$; Table 3) but there was no significant treatment effect. Titers were highest at 10 days after the primary intratracheal challenge at 7 week of age for the CD+HuSA-treated birds (Group 8), whereas titers were lowest in the solely PBS-treated birds (Group 6; Figure 2C). At days 10 and 14, significantly higher IgG Ab titers to HuSA were found in the CD+HuSA-challenged birds (Group 8) as compared to all other groups (Figure 2C).

Titers of IgA binding HuSA at 7 weeks of age were not affected by a Time × Treatment interaction or Treatment (Table 3). Also, no significant differences between treatments at any moments were found (data not shown).

Secondary Total Antibody Responses to HuSA at 7 weeks of age

Total secondary Ab titers to HuSA were significantly affected by a Treatment × Time interaction (Table 3, $P < 0.05$), but no Treatment effect was found ($P > 0.05$; Table 4). Titers peaked at 7 days Group 2 (twice CD+HuSA) and at 10 days for Group 1 (twice FD+HuSA), Group 3 (twice LPS+HuSA), and Group 4 (twice PBS+HuSA; Figure 3A). At day 3 after secondary challenge, significantly lower total Ab titers to HuSA were found in birds treated twice with FD+HuSA as compared to birds treated twice with PBS+HuSA or LPS+HuSA (Figure 3A).
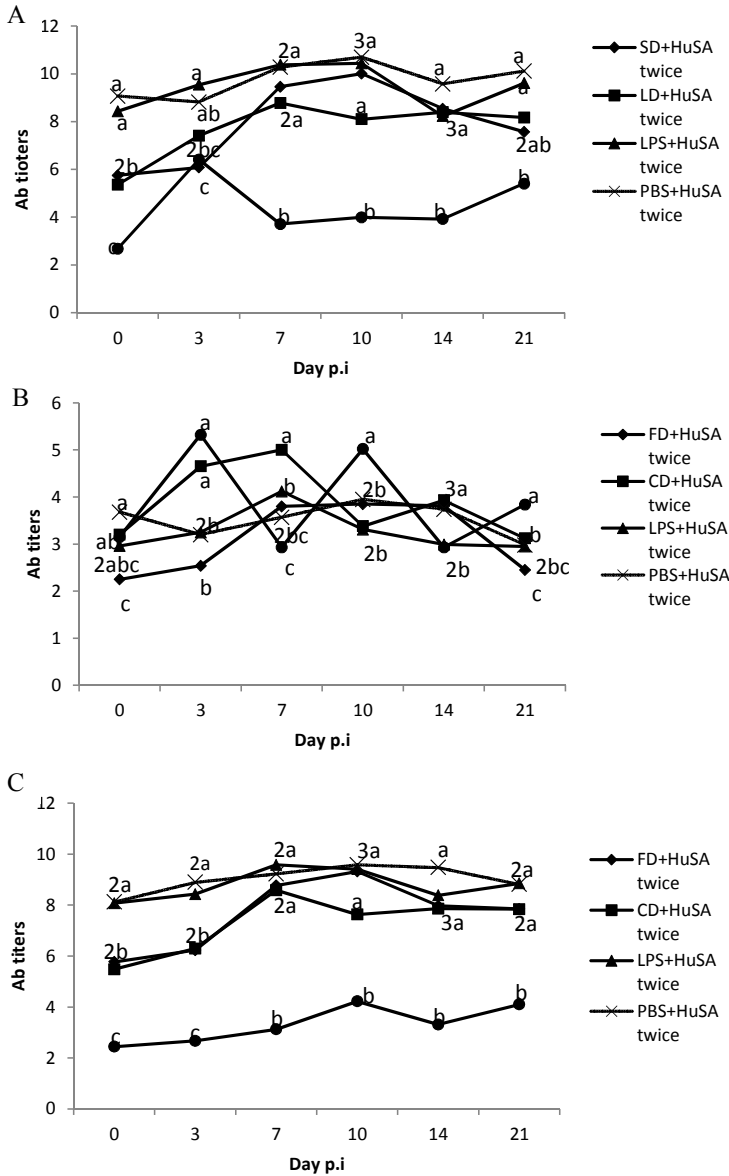
Figure 3. The time course of the secondary total (A), IgM (B), and IgG (C), antibody titers to HuSA of birds immunized intratracheally (i.t.) twice with FD+HuSA (Group 1), or CD+HuSA (Group 2), or LPS+HuSA (Group 3), or PBS+HuSA (Group 4), or solely PBS (Group 6) , respectively, at 7 wk of age. Data represent mean antibody titers at d 0, 3, 7, 10, 14, and 21 after secondary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per Treatment group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for total antibodies, or 1: 20,000 diluted GACh/IgM/PO for IgM, or 1:20,000 diluted GACh/IgG$_{Fc}$/PO for IgG. P.i = post immunization. [a-c] denote significant different between Groups at these ages.

Isotype-specific secondary antibody titers to HuSA at 7 wk of age

The IgM-isotype specific secondary Ab responses to HuSA were affected by a Treatment ×
Time interaction ($P < 0.05$; Table 3), and affected by Treatment ($P < 0.001$). Secondary IgM
Ab titers were significantly higher in the twice CD+HuSA-challenged birds (Group 2) than in
the twice FD+HuSA-challenged birds (Group 1; Table 3). At days 3, 7, and 21 secondary IgM
Ab titers to HuSA were significantly higher in the twice CD+HuSA-treated birds as compared
to the twice FD+HuSA-treated birds. At these days, the titers in the twice CD+HuSA-treated
birds were also significantly higher than in the twice PBS+HuSA-treated birds (Figure 3B).
The IgG-isotype specific secondary Ab responses to HuSA during the observation period
were affected by a Treatment × Time interaction ($P < 0.05$; Table 3), but there was no
significant treatment effect ($P > 0.05$; Table 3). At day 3 after secondary challenge,
significantly lower total Ab titers to HuSA were found in birds treated twice with FD+HuSA
or twice with CD+HuSA as compared with that of birds treated twice with PBS+HuSA
(Figure 3C).

Secondary IgA-isotype specific Ab titers to HuSA were not significantly affected by a
Treatment × Time interaction nor a Treatment effect (Table 3). Also, no differences between
treatments at any moment were found (data not shown).

Antibody Titers to LPS

Primary total antibody titers to LPS at 3 and 7 weeks of age and secondary antibody titers
to LPS at 7 weeks of age increased during the observation periods but were not significantly
affected by Treatment during the complete observation periods, nor at any moment during the
observation periods (Tables 4 and 5).

Table 4. Total (IgT) plasma antibody titers to Lipopolysacharide (LPS) during 4 weeks after primary
intratracheal immunization with LPS at 3 weeks of age in the presence of coarse dust (CD), fine dust (FD),
LPS, human serum albumin HuSA, and PBS[1].

Groups[2]	Challenge 1	IgT
1	1. FD +HuSA	1.08
2	2. CD +HuSA	0.88
3	3. LPS+HuSA	0.75
4	4. PBS+HuSA	0.94
5,6,7,8	5. PBS	0.81
SE		0.13
Challenge-1		NS
Time		***
Challenge-1×Time		***

[1]Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, 21 and 28 after primary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT.

[2]See table 1 for group treatment details.

*** $P < 0.0001$

Table 5. Total (IgT) and isotype antibody titers to lipopolysaccharide (LPS) during 3 weeks after secondary immunization in the presence of coarse dust (CD), fine dust (FD), LPS, human serum albumin (HuSA), and PBS[1].

Group[2]	Challenge 1	Challenge 2	IgT
1	FD+HuSA	FD+HuSA	[xy]2.22[ab]
2	CD+HuSA	CD+HuSA	[y]2.07[b]
3	LPS+HuSA	LPS+HuSA	[xy]2.58[ab]
4	PBS+HuSA	PBS+HuSA	[x]2.85[a]
5	PBS	HuSA	[x]2.94[a]
6	PBS	PBS	[xy]2.81[ab]
7	PBS	FD+HuSA	[y]2.03[b]
8	PBS	CD+HuSA	[xy]2.58[ab]
SE			0.27
Group[3]			NS
Time			***
Group*Time			***

[a,b] denotes significant differences within Challenge 1 treatments.

[x,y] denotes significant differences between (all) Challenge 2 treatments.

[1] Data represent repeated measurements of antibody titers at d 0, 3, 7, 10, 14, and 21 after secondary immunization, as estimated by ELISA of serial dilutions of sera from 12 birds per group and using 1 : 20,000 diluted RACh/IgG$_{H+L}$/PO for IgT.

[2]See table 1 for group treatment details.

*** $P < 0.001$

Effects of Repeated Challenge with Dust Particles and LPS on Body Weight (Gain)

The BWG at 24 h after the primary Challenge at 3 wk of age with various combinations of dust sizes or LPS and HuSA was not significantly affected by Treatment ($P > 0.05$; Table 6), however a significantly lower 24-hour BWG after challenge at 3 wk of age was found in birds challenged with FD+HuSA (Group 1) as compared to the PBS-control and the PBS+HuSA-treated groups. The BWG at 24 h after the intratracheal challenge at 7 wk of age was not significantly affected by Treatment (Table 7). Total BW and BWG at 12 weeks of age were

significantly lower in Groups 2 (CD+HuSA, twice) and Group 7 (FD+HuSA at 7 weeks) as compared to the other groups (Table 7).

Table 6. Body weight gain (g) 1 day after Challenge 1 (BWG-I) at 3 weeks of age in the present of coarse dust (CD), fine dust (FD), lipopolysaccharide (LPS), human serum albumin (HuSA), and PBS.

Groups [1]	Challenge 1	BWG-I
1	1. FD+HuSA	39.8[b]
2	2. CD+HuSA	44.2[ab]
3	3. LPS+HuSA	46.4[ab]
4	4. PBS+HuSA	48.3[a]
5,6,7,8	5. PBS	48.3[a]
SE		2.8
Challenge 1		NS

[a,b] denotes significant differences between Challenge 1 treatments.

[1]See table 1 for group treatment details.

Table 7. Body weight gain (g) 1 day after Challenge 2 at 7 weeks of age (BWG-II), and body weight gain during 12 weeks (BWG-T)[1].

Group[2]	Challenge 1	Challenge 2	BWG-II	BW12w	deltaBW-T
1	FD+HuSA	FD+HuSA	58.5	3291	2681[a]
2	CD+HuSA	CD+HuSA	55.0	2882	2257[b]
3	LPS+HuSA	LPS+HuSA	65.2	3295	2649[a]
4	PBS+HuSA	PBS+HuSA	61.8	3253	2627[a]
5	PBS	PBS+HuSA	47.0	3372	2737[a]
6	PBS	PBS	59.6	3214	2583[a]
7	PBS	FD+HuSA	51.4	2930	2333[b]
8	PBS	CD+HuSA	55.6	3167	2550[a]
SE			8.1	85	78
Group[3]			NS	NS	***
					2,7<1,3,4,5,6,8

[a,b] denotes significant differences within Challenge 2 treatments.

[1]FD = fine dust; HuSA = Human serum albumin; CD = coarse dust; and LPS = lipopolysaccharide

[1]See table 1 for group treatment details.

*** $P < 0.001$

Effects of Repeated Challenge with Dust Particles and LPS on Heart and Spleen Morphology

Measurement of spleen weight revealed that the heaviest spleen weights and relative spleen weights (RSW) were found in Group 8 (CD+HuSA at 7 wk of age), whereas the lowest RSW was found in Group 1 (FD+HuSA twice; Table 8).

Heart weight was significantly decreased in birds that received CD+HuSA at 3 weeks of age, whereas similar treatment at 7 weeks of age resulted in the heaviest heart weights. Birds that were challenged twice (Groups 1, 2, 3, and 4) had significantly lower relative heart weights (RHW) than birds that were challenged only once at 7 weeks of age (Table 8). Heart width was significantly lowest in birds treated with LPS+HuSA twice, but all treatments enhanced heart length as compared to the control birds (PBS twice).

Table 8. Heart, spleen characteristics and relative heart weight (RHW), relative spleen weight (RSW) at slaughter at 12 weeks of age after concurrent intratracheal immunization with HuSA and dust components (DC) at 3 and 7 weeks of age.

Group[1]	Treatment	Spleen (mg)	RSW[2]	Heart Weight (g)	Heart Width (cm)	Heart Length (cm)	RHW[3]
1	FD+HuSA 2x	4.15[ab]	0.0013[b]	18.9[a]	3.69[bc]	5.36[a]	0.00577[b]
2	CD+HuSA 2x	3.93[b]	0.0014[ab]	16.6[b]	3.44[cd]	4.81[bc]	0.00575[b]
3	LPS+HuSA 2x	4.58[ab]	0.0014[ab]	18.2[ab]	3.39[d]	4.54[bc]	0.00552[b]
4	PBS+HuSA 2x	4.54[ab]	0.0014[ab]	17.9[ab]	3.84[ab]	4.47[bc]	0.00550[b]
5	PBS (I) HuSA (II)	4.60[ab]	0.0014[ab]	19.7[a]	3.70[ab]	4.77[bc]	0.00584[ab]
6	PBS 2x	4.60[ab]	0.0014[ab]	19.2[a]	3.68[bc]	4.32[c]	0.00599[ab]
7	PBS(I) FD+HuSA	4.02[ab]	0.0014[ab]	18.0[ab]	3.74[ab]	4.83[b]	0.00617[a]
8	PBS (I) CD+HuSA	4.79[a]	0.0015[a]	20.4[a]	3.99[a]	4.62[bc]	0.00643[a]
SE		0.27	0.00008	0.62	0.09	0.14	0.00022
Main effect							
Treatment		NS	NS	*	**	**	NS
				2<4,7,3< 1,6,5,8	3<2<6,1 <5,7,4<8	6<4,3,8,5,2 <7<1	3,4,1,2<5,6 <7,8

[a-d] denotes significant differences within Challenge 2 treatments.

[1]See table 1 for group treatment details.

[2]RSW = Spleen weight/ body weight at 12 weeks of age

[3]RHW = Heart weight / body weight at 12 weeks of age

* $P < 0.05$; ** $P < 0.01$.

DISCUSSION

In poultry production systems air quality is seriously impaired because of high dust concentrations (Wathes et al., 1997, Takai et al., 1998). The highest concentrations of bacteria (6.4 log10 cfu/m^{-3}) and fungi (4.5 log10 cfu/m^{-3}) were found in broiler houses (Takai et al., 1998, Seedorf and Hartung, 2000). This causes health problems for employees working in this environment but likely also for the animals living in these houses (Al Homidan and Robertson, 2003). Next to composition and concentration, the size of airborne particles is an important characteristic of dust. Size of the particles influences the aerodynamic behavior, transport, and control technologies to reduce the amount of particles in the air (Zhang, 2004a). Particle size determines the impact of dust on human and animal health as well (Mercer, 1978). Particles are often classified as particles smaller than 10 µm (PM10), smaller than 2.5 µm (PM2.5), and smaller than 1.0 µm (PM1), respectively. Particles ranging from 1 to 10 µm are mainly responsible for health problems because they can travel into the respiratory system (Collins and Algers., 1986), albeit smaller particles travel deeper than larger ones, and therefore have greater impact on human and likely animal health. Airborne particles in poultry houses derive from various sources such as, feces, litter, feather debris, and microorganisms. Dust contains high levels of molecular structures derived from micro-organisms with known immunomodulating features. Pathogen associated molecular patterns (PAMP) within dust particles such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), β-Glucans, chitin and many others are present in poultry houses. When intratracheally administered, dust constituents and PAMP-modulated primary and secondary immune responses and negatively affected BWG in broilers (Lai et al., 2009) and layers (Parmentier et al., 2008; Ploegaert et al., 2007).

Deposition of components in the lower airways of poultry depends on size. Especially at young age (< 4 weeks), the increase of particle size resulted in lower deposition in the lungs, but increasing deposition in the air sacs (Corbanie et al., 2006). In the present study we measured the effect of airborne derived particles from two different sizes: FD and CD obtained from a broiler house with known LPS content on the primary and secondary antibody response to the model antigen HuSA and BW (gain) of slow growing broilers. Particles from this range of sizes form the majority both in mass and in counts in animal houses, including poultry houses (more than 90%, Lai et al., 2011b). As a control, the effect of a comparable concentration of pure LPS (360 ng) was measured as well. In the current study, only one concentration of all components (dust particles: 4 mg; LPS: 360 ng and

HuSA: 0.1 mg) was used, thus it is likely that different doses may have resulted in different responses. In an earlier similar experiment levels of LPS derived from FD in a broiler house were 4.6, 6.1, and 8.8 ng/m^3 air at 1, 3, and 7 wk, and levels of LPS derived from CD were 34.9, 63.4, and 169.1 ng/m^3 air at these moments. The HuSA was chosen as model antigen to prevent possible interference with obligatory vaccinations of the birds, given that HuSA is not part of vaccine additives. The current dose of HuSA was chosen because it was unknown whether dust would increase or decrease Ab responses. The Ab responses were measured because they provide information on kinetics of the immune system. Birds were exposed at 2 different ages, to estimate age-dependent sensitivity of the broiler, and to measure the effect of the airborne components on secondary immune responses. Slow-growers were studied to measure immunomodulation of secondary immune responses.

Four questions were addressed. First, we studied the effect of early exposure (at 3 wk of age) to FD CD particles or a comparable doses of LPS on the humoral response to a specific model antigen (HuSA) (Challenge 1 effect). We measured both total and isotype-specific Ab responses to HuSA, given that earlier we found different effects of PAMP on either IgM or IgG. The IgA was measured because we applied a mucosal (respiratory tract) challenge. Second, we studied the effect of exposure at a later age (7 wk) to FD and CD, or LPS on primary antibody responses to HuSA. Earlier we found that challenges of broilers at a later age had different effects than challenges at a young age, suggesting adaptation of the birds (Lai et al., 2009, 2011a). Third, we studied the effect of repeated exposure to dust particles or LPS at 7 wk of age on secondary Ab responses to HuSA (Group effect) to establish the effects on memory formation. Fourth, we studied whether dust particles or a comparable doses of LPS after i.t. challenge affected BW (gain) of broilers. In addition, morphological parameters of the spleen and heart were estimated.

With respect to the first objective, dust particles but not the current comparable dose of LPS decreased (Table 2) primary (total) Ab responses to concurrently intratracheally immunized HuSA at 3 weeks of age, especially at day 7 and day 10 after challenge, indicating that immune modulation by dust particles may occur after primary challenge. Table 2 shows that the dust particles, both FD and CD, induced more pronounced and prolonged negative effects during the complete observation period than a comparable dose (360 ng) of LPS. In earlier studies (Parmentier et al., 2008) we found enhancement of antibody responses in the presence of much higher doses (0.5 - 1 mg) of LPS. The current data cannot discriminate between the effects of LPS within the dust versus other undefined components that are likely to be present in dust. In addition, we tested commercial purified LPS, which may be different

from the naturally obtained LPS in the dust samples, that likely is derived from various gram negative bacteria, and which may be soluble or part of bacterial membrane remnants. Further studies on types and sources of LPS, and likewise the combination of physical dust and bacteria may be required to find more pronounced and prolonged effects of these components as well. On the whole, in the present study with broilers, total primary Ab at 3 weeks of age responses were decreased by FD+HuSA, and the CD+HuSA treatments (Table 2). Treatments not only differed with respect to the moments of effect, but also with respect to the affected Ab isotype. Primary IgM responses to HuSA were affected by FD+HuSA, whereas primary IgG responses to HuSA were affected by CD+HuSA. Despite, primary IgA responses to HuSA were not affected (Table 2).

With respect to the second objective, effects of primary challenge with dust particles and HuSA at a later age (7 wk of age) revealed a pronounced enhancing (Table 3) effect of CD on primary total, IgM, and IgG responses to HuSA. The FD decreased primary total Ab, but enhanced IgM and IgG responses to HuSA. On several moments after the challenge at 7 wk of age, significant enhancing effects of both CD and FD treatments were found as opposed to similar treatments at 3 weeks of age (Table 2). This suggests that the effects of dust or its components on immune responses may depend on the age of the recipient, which in turn may be related with adaptation of the birds to the environment, and as a consequence affect immune responsiveness and sensitivity for LPS and other PAMP. As mentioned above, levels of dust and LPS in broiler houses usually increase in time. Again, no effect of dust or LPS on the IgA levels was found, suggesting that dust or LPS affected immune responses at the systemic, but not the mucosal level.

With respect to the third objective, effects of secondary challenges with dust particle and comparable LPS challenges revealed pronounced effects of FD, CD, and LPS on secondary total Ab, IgM, and IgG responses to HuSA. Total secondary Ab titers and IgG-isotype specific Ab responses to HuSA were significantly affected by a treatment × time interaction, suggesting that dust or its components affected the kinetics of the secondary antibody response. Particularly, secondary IgM Ab titers were significantly higher in the twice CD+HuSA-challenged birds (Table 3) than in the birds that were challenged twice with FD+HuSA (Table 3). The results indicated that dust particles depending on their size may have positive effects on the kinetics of secondary Ab responses to HuSA depending on the isotype. No significant treatment effects of dust particles or LPS on secondary Ab responses was found. Earlier, we found that PAMP treatments affected primary but not secondary responses. Whether this is related with aging of the birds or is based on different or no effects

of PAMP or dust particles on memory cells is as yet unknown. Also in the current study, modulation of secondary Ab responses to HuSA was less pronounced after secondary challenge with dust particles and LPS than after primary challenge.

Finally, with respect to the fourth objective, all birds treated intratracheally with dust and LPS showed a lower BWG early after primary challenge, however only FD+HuSA-challenged birds had significantly lower BWG as compared to the PBS+HuSA- and PBS-control treated group (Table 6). At 24 hours after secondary challenge at 7 weeks of age BWG was not significantly affected by treatments (Table 7). The BW at 3 weeks of age was related with the effects of dust particles treatments at 3 and 7 weeks of age, however, there were much severe effects of FD+HuSA treatments on young birds at 3 weeks (Table 6), as opposed to the other treatments and also opposed to later age at 7 weeks of age (Table 7), suggesting again a refractive response to dust (or its components) as described before (Lai et al., 2009). Total BW and BWG after 12 weeks of age were not significantly affected by the experimental treatments but BWG was significantly lower in Group 2 (CD+HUSA at 3 wk and 7 wk) and group 7 (FD+HuSA at 7 wk) as compared with those of the other Groups. These data suggested specific adaptation of the birds to the challenge with dust particles and final recovery of the birds to intratracheal challenges. At 12 weeks of age, significantly higher spleen weights were found in CD+HuSA-challenged birds treated once at 7 wk as compared with those of the birds that were treated with CD+HuSA twice at 3 wk and 7 wk. In addition, a significantly heavier relative spleen weight (RSW) in group 8 (CD+HuSA at 7 wk) as opposed to group 1 (FD+HuSA twice; Table 8) was found. This is in accordance with the higher Ab responses to HuSA in these birds, suggesting that (CD) dust at later ages enhances immune reactivity.

The morphology of the heart at 12 weeks of age was affected by the intratracheal treatments with dust particles, although we cannot distinguish between primary and secondary challenges. Weight, length and width of the heart of the broilers intratracheally treated with dust particles at 7 weeks of age were different from the other treatments. Higher heart weight and relative heart weight (RHW) were found in Group 8 (CD+HuSA at 7 wk) as compared to Group 1 (FD+HuSA at 3 wk and 7 wk) and Group 2 (CD+HuSA at 3 wk and 7 wk). This result indicated that primary challenge at 7 wk (Table 8) affected heart morphology differently than secondary challenge. Again, effects of secondary challenge was seemingly less pronounced after secondary challenge with dust particles and LPS than after primary challenge.

Taken together, the current study showed that airborne dust particles obtained from a poultry house when given intratracheally affected immune responsiveness and BWG in a negative fashion at 3 wk of age, but may affect immune responsiveness in a positive fashion at 7 wk of age. Thus dust components harvested from a broiler house affected immune responses and BW(G). This suggests that the effects of dust or PAMP or other dust constituents (i.e. hygienic status) are related with the immune maturation or adaptation to the environmental conditions of the bird. Negative or positive effects of dust on immune responsiveness may have consequences for health management (such as moment and type of vaccine administration) of poultry. Further studies on the effect of dust particles and its constituents on immune reactivity of poultry are in progress.

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CHAPTER 6

LOCALISATION AND (SEMI-)QUANTIFICATION OF FLUORESCENT BEADS OF TWO SIZES IN CHICKEN AFTER SIMULTANEOUS INTRATRACHEAL AND CLOACAL ADMINISTRATION

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ABSTRACT. Bacteria and non-degradable particles can enter the chicken via several entry routes. Two of these entry routes, the respiratory- and cloaca entry routes, are known for their capability to activate the immune system. We studied the localization of simultaneous intratracheally and cloacally applied beads of two sizes in the chicken body in time, and when possible, semi-quantified the amount of beads. Next, we studied the role of phagocytic cells as transport vehicle within the chicken body. Ten broiler hens, 3.5 weeks of age, received simultaneously 1.25×10^9 1.0 μm beads and 1.05×10^7 10 μm FITC (green) or TRITC (red) labelled beads. Red beads were given intratracheally, and green beads were given cloacally. Various tissues from two chickens per moment were sampled at 1 h, 6 h, 24 h, 48h and 1 w after challenge. The following tissues were studied for the presence of beads: Bursa of Fabricius, lungs, liver, kidneys, gall bladder, spleen, thymus, small intestine, caecum, intestinal luminal contents, aerated bones, faeces and blood. The presence of beads in a tissue was estimated using fluorescence microscopy. The highest amount of beads was found in the organs after 1 h closest to the application site: i.e. the lungs for red beads, and the Bursa for green beads, respectively. All tissues sampled showed all 4 types of beads within 1 h. Lower levels of beads were found in lungs and Bursa within 6 h and in all other organs after 24 h, except for the kidneys (after 48 h). At 1 h, 1 μm intratracheally applied red beads were also found in the caecal luminal content and caecal tissue, but not in the small intestinal luminal content, suggesting that caeca are capable of excreting small particles entering the body via the respiratory route. Active uptake of 1 μm particles likely by macrophages was observed in the lung, but was not observed in other tissues. Data suggested that uptake via epithelial cells or penetration of the epithelial cells might be another entry way into the chicken. Once inside the tissue, beads likely entered the bloodstream and spread rapidly to all organs. The presence of non-degradable and non-immunogenic beads for 7 d of different sizes in all sampled organs throughout the whole chicken body suggested potentially negative chronic health and welfare risks of the chicken for respiratory tract or cloacal challenges.

Keywords: *Chicken,   Cloaca,   Fluorescein-labelled   polystyrene   bead,   Intratracheal, Localisation.*

INTRODUCTION

Various entry routes of environmental particles in chickens are known. Particles including bacteria may be taken up via the gut, contracted via the eyes, obtained via the cloaca or inhaled via the respiratory tract. The cloaca and the respiratory tract (lungs and air sacs) are likely important entry routes, whereas uptake via the eyes is probably relatively limited. Uptake via the gut is strongly regulated by the enterocytes and in addition compensated and partly neutralised by the gut microbiota. In addition, the intestinal tract is protected by an extensive immune system. Various studies reported immune responses to model specific antigens and innate antigens or pathogen-associated molecular patterns (**PAMP**) or modulation of immune responses after cloacal and bursal challenges with bacteria and PAMP (Ekino et al., 1979; 1985; Matsuda et al., 1976). 'Cloacal drinking' (uptake of particles by contractions of the cloaca can lead to antigenic challenge of the Bursa of Fabricius, resulting in an effective systemic antibody response (Sorvari et al., 1975). Into the cloaca lumen injected sheep red blood (**SRBC**) initiated secondary-like antibody response to subsequently intravenously injected SRBC, indicating that a systemic antibody response can be induced via the cloaca (Ekino et al., 1979). These systemic reactions suggested uptake of antigenic material applied via the cloaca. However, little is known of the processes underlying uptake via the cloaca, the final localisation of the antigens or particles in time and the way these components activate the immune system. An earlier study with fluorescein-labelled (FITC) polystyrene beads applied to the cloaca of broilers, revealed that 27% of all beads localised in the lumen of the Bursa (Van der Sluis et al., 2009) and 28.3% of all beads was traced in the content of ileum and rectum. Localization, digestion or expulsion of the rest of the cloacally applied beads remained unknown.

Challenge of the respiratory tract of poultry with antigens, bacteria and PAMP was subject of various studies (Parmentier et al., 2008a, Wideman et al., 2004, Lai 2009, 2010). Primary and secondary antibody responses to intratracheally (**i.t.**) challenged antigens and modulatory effects of simultaneously i.t. administered PAMPs were reported for layers (Parmentier et al., 2008; Ploegaert et al., 2007) and broilers (Lai et al., 2009; 2010). These findings suggested that both specific antigens as well as PAMP were systemically taken up and immunologically processed. However, as is true for the cloaca, little is known of the processes underlying the systemic immune responses to antigens obtained via the respiratory tract. High levels of dust particles ranging from smaller than 1 μm to larger than 10 μm were found in poultry houses (Lai et al.,

submitted). In experimentally challenged 2- and 4 wk old broilers, FITC-labelled particles larger than 10 µm were almost absent in the lower and upper respiratory tract, particles between 5 µm and 10 µm were mainly found in the nose and eyes, and in small amounts in the lower respiratory tracts. Particles smaller than 5 µm were more homogeneously deposited in the complete respiratory tract. This suggested that smaller beads may enter the chicken body more effectively, and thus form a higher risk health than larger beads. However, not all beads were located in the respiratory tract (roughly 80%) (Corbanie et al., 2006).

We studied the localization of two different sizes of non-immunogenic particles in chickens applied simultaneously via the respiratory tract and the cloaca as a model for entry of dust particles in chicken houses. Particles were traced back in different tissues in time and when possible (semi-)quantified. We expected 1) that different sized foreign particles may locate in different tissues or in different concentrations, 2) a higher concentration of beads is located in immunologically important tissues or in tissues with many phagocytic cells, e.g. spleen and liver, 3) intratracheally applied beads locate in different tissues and concentrations than cloacally applied beads, 4) distribution of foreign particles may rest on different transport mechanisms: via the blood, via cells (phagocytes), air sac penetration (trachea) or mechanical transport (cloaca). Knowledge of localisation of fluorescent particles may be a good model for the studying the entry route, and final localization of dust and other particles in chickens, that may add to optimizing vaccine and health managements as well as a model for vaccination. As is true for cloacal uptake, it remains unknown where particles finally ended up.

MATERIALS AND METHODS

Birds and housing

Ten one-day-old Ross female broilers were commercially obtained (Morren Hatchery, Lunteren, The Netherlands). All birds were kept in one group in a roofed saw dust-covered pen (1.75 x 1.15 x 0.80 m^3). Temperatures varied between 25°C and 32.4°C (d 0-6), 22°C and 30°C (d 7-13), 20°C and 24°C (d 14-20) and 20°C and 23.1°C (d 21-29). *Ad libitum* water and feed (standard broiler diet; 204.2 g/kg of CP, 2,859 kcal/kg of ME) were accessible for the birds. Light regimen was 16/8 h light/dark. The birds were vaccinated at d 13 of age with Newcastle Disease vaccine.

The experiment was approved by the Animal Welfare Committee of Wageningen University according to Dutch law.

Reagents

Fluorescein-labelled polystyrene beads (FluoSpheres® polystyrene microspheres: 1.0 µm yellow-green (FITC) fluorescent, F13081, Lot 780720; 1.0 µm red (TRITC) fluorescent, F13083, Lot 834676; 10 µm yellow-green fluorescent, F8836, Lot 805261 (3x); 10 µm red fluorescent, F8834, Lot 756069 (2x), Lot 839851 (1x) and Lot 880479 (1x)) were all from Invitrogen (Breda, The Netherlands).

Experimental design

At 3.5 wk of age, all birds received i.t. 0.5 ml PBS with 1.25×10^9 1.0 µm (TRITC) red beads and 1.05×10^7 10 µm red beads. A few minutes later, birds were held upside down and the abdomen was stimulated to defecate. After defecating, 0.5 ml PBS with 1.25×10^9 1.0 µm green (FITC) beads and 1.05×10^7 10 µm green beads were administered to the cloaca. Birds were held upside down until all fluid was taken up by the cloaca via cloacal reflexes. Both i.t. and cloacal samples were prepared one day in advance and stored at 4°C. Before use, the samples containing the beads were sonicated with a Sonomatic 150 S to agitate the beads and prevent them from adhering to the tube and clotting.

Two birds were killed by cervical dislocation randomly at 5 moments post challenge (**p.c.**): 1 h, 6 h, 24 h, 48h and 1 wk p.c. The abdomen was carefully opened and the sternum was lifted for approximately 2 cm to collect the air sacs. The air sacs were put on glass slides and stored in the dark at room temperature. After removing tissue, the following tissues were collected, weighed, snap frozen in liquid nitrogen, and stored at -80°C until use: Bursa of Fabricius, gall bladder, kidneys, liver, lungs, spleen, thymus, approximately the first 4 cm of the small intestine (without luminal content), the middle part of a caecum (without luminal content), 1.5 cm of the humerus bone, and 1.5 cm of the radius bone. The luminal content of the small intestine and the caeca were removed by placing two tweezers at one end and pulling one to the other end simultaneously squeezing and collecting the luminal content in a separate tube. The weight of the luminal content of the small intestine and the caecum, as well as a faecal sample (either collected during slaughtering or during collection of the organs) were measured and stored at -20°C until use. Blood was collected from all birds 30 min. prior to slaughter. Five blood smears per chicken were made. After drying, smears were stored in the dark at room temperature. Plasma was stored at -20°C until use.

Preparation of tissues and detection of fluorescent particles

Frozen bones were flushed with PBS and a syringe to collect bone marrow. The eluted fluid was homogenised and a droplet of 10 µl was put on a slide. Intestinal parts and content (small intestine, caeca and faeces) were taken and weighted. Samples were dissolved in 200 µl PBS. A slide for each sample was made by allowing 10 µl of the fluid to dry.

From all tissues, 7 µm thick frozen sections were made with a microtome Leica CM 3050S cryostate (Leica Microsystems, Germany) at -20°C. Slices were put on a microscope slide and were air dried. After drying, the slides were coloured with Hemacolor® (Merck, Germany) and stored at -20°C until use. In addition, a small piece of all other collected frozen tissues was taken at -20°C. Special attention was given to the gall bladder to avoid release and loss of frozen bile. These pieces were weighted and defrozen. To remove all luminal content of the small intestine and the caeca, the luminal site of the samples were washed in PBS and gently squeezed with tweezers before use. All samples were separately squeezed by cutting it first and then squeezing it through a PA Nylon gauze of 100 µm (Stokvis & Smits N.V., IJmuiden, The Netherlands) with the help of the plunger of a syringe. PBS was used to wet the nylon and the sample. PBS was also used to spray through the squeezed sample. After this, the fluid containing the sample was collected and centrifuged with an Eppendorf centrifuge for 2 min at 3.0 rcf. The supernatant was poured in a new tube and centrifuged for 2 min at 5.0 rcf. Next, 200 µl of PBS was added to the pellet was dissolved again. The fluid of this tube was then added to the other tube and the pellet was dissolved. A microscope slide was made for each sample by allowing 10 µl of the fluid of each sample to dry.

All slides for microscopical evaluation (air sacs, blood smears, bone marrow, tissues and intestinal contents) were examined using a fluorescence microscope (Zeiss, West-Germany and Nikon Instruments Europe B.V., The Netherlands) for the presence of fluorescent particles at 505 nm excitation and 515 nm emission (for green FITC-fluorescent beads) and at 580 nm excitation and 605 nm emission (for red (TRITC-)fluorescent beads), respectively. Pictures were taken with a DP50 digital camera (Olympus, Germany) connected to the fluorescence microscope.

(Semi-)quantification of fluorescent particles

All air sac samples (ranging from 4 to 14 samples per bird with a median of 10) and all five blood smears were examined by fluorescence microscopy. Only one sample per bird was examined for the luminal content of the small intestine, the caeca and the faecal sample. When no beads were present in a blood, bone marrow or tissue sample, no further counting was done. If more than 30 beads (of one colour and size) were present, four more samples were made to estimate the average amount of beads per sample. If arbitrary very high numbers of beads were present, the original sample was diluted until a reasonable amount of beads (arbitrary) allowed quantification. The number of each of the four types of beads (colour and size) was counted and calculated to a corrected estimated total amount of that type of bead per total organ (amount of beads x weight of total organ / weight of sample used). The number of the different beads in the air sacs, the intestinal contents, blood and bone marrow samples could not be calculated to a standard amount and were therefore semi-quantified. Quantification for these samples was

done on a qualitative scale: 0 (no beads), 1 (low amount of beads; <100), 2 (>100), 3 (>1,000) and 4 (very high amount of beads; not countable and/or rateable). The number of found beads in the humerus and radius bones was quantified on a different qualitative scale: 0 (no beads), 1 (<10), 2 (<50), 3 (<100) and 4 (>100). The number of found beads in 10 µl blood was also quantified on a different qualitative scale: 0 (no beads), 1 (<1), 2 (<10), 3 (<100) and 4 (>100).

Phagocytosis test

A phagocytosis test with peripheral blood leukocytes (PBL) was performed as follows.
Blood 0.5 ml) was diluted 1:1 with PBS and subsequently 1:1 diluted with Histopaque® 1119 (Lot 100M6171; Sigma-Aldrich Co., St. Louis, MO). White blood cells were collected from the interphase after centrifuging for 2.5 min at 12,500 rpm in an Eppendorf centrifuge. PBL were washed twice by adding 1 ml PBS and centrifuging for 1 min at 12,500 rpm. Finally the pellet was dissolved in 1 ml PBS. Two tubes of 190 µl of the PBL mixture were used. Ten µl containing approximately 1×10^8 1.0 µm red fluorescent beads, was added to one tube with PBL. Ten µl containing 3.6×10^4 beads of 10 µm red fluorescent beads was added to the other tube with PBL. The tubes were incubated at 37°C. A µl droplet from both tubes was taken before (0 min), and 5, 10 and 15 min, respectively after adding the beads. The droplet was put on a microscope slide and air dried. The slides were coloured using Hemacolor®. Phagocytosis was examined by fluorescence microscopy at 580 nm excitation and 605 nm emission.

RESULTS

Aerosol size distribution

Detection and (semi-)quantification of fluorescent particles

Results of the presence and the (semi-) quantification of beads in various tissues are shown in Graphs 1-16. Pictures of the tissue examination are shown in Figures 1, 2, and 3. Very high numbers (up to an estimated 11.33% of the total applied 10 µm red beads and an estimated 14.57% of the total applied 1 µm red beads) were located in the respiratory tract. Also the number of green beads in the Bursa was relatively high: an estimated 2.64% of the total applied 1 µm green beads and an estimated 0.34% of the total applied 10 µm green fluorescence beads were located in the Bursa. Other tissues never showed more than an estimated 1% of the total amount of administered beads. The total amount of located 1 µm red beads in the respiratory tract varied between 0.00% and an estimated 14.57% (after 1 h) between the two birds sampled at that moment. The total amount of located 1 µm green beads in the Bursa varied between 0.00% and 2.64% (after 1 h) between the two birds sampled at that moment. The total amount of located 10 µm red beads in the respiratory tract varied between 0.00% and 11.33% (after 1 h) between the two birds sampled at that
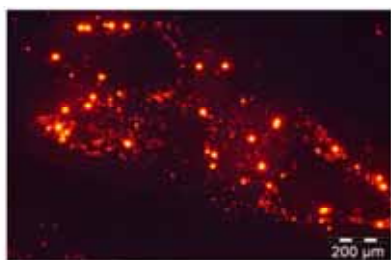
Figure 1. 7 µm thick slider of lung tissue containing 1 µm red fluorescence beads (small red dots) and 10 µm red fluorescence beads (large red dots)
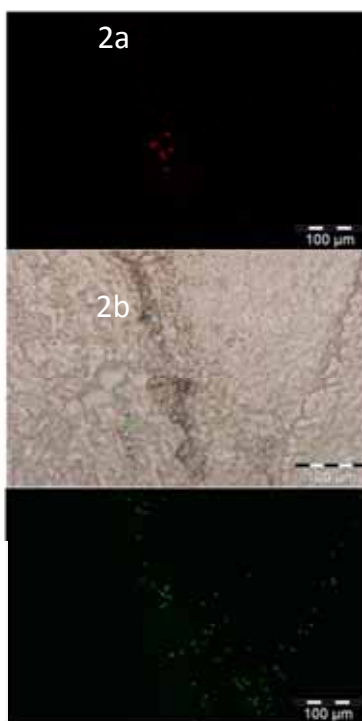

Figure 2. 7 µm thick slide of Bursal folding containing beads. a; Bursal folding with red fluorescence, visualising 1 µm red fluorescence beads. b; Bursal folding (same as a) without fluorescence. c; Bursal folding (same as a) with green fluorescence, visualising 1 µm green fluorescent beads.

moment, and the total amount of located 10 µm green beads in the Bursa varied between 0.00% and 0.34% (after 1 h) between the two birds sampled at that moment.

As expected, red beads located mainly in the lungs at all sample moments (on average an estimated 78.70% 10 µm beads and an estimated 86.84% 1 µm beads of the estimated total located red beads in the chicken body). Red bead clearance in the lungs likely occurred already before 6 hours. At 24 h relatively small amounts (an estimated 0.21% and 0.00% for 10 µm beads and an estimated 0.26% and 0.00% for 1 µm beads of the total of applied red beads) were still present in the lungs. Relatively high amounts of red particles of both sizes were found in the kidneys at all sample moments after challenge. Although lower, still relatively high levels of red beads from both sizes were located in the liver and in the thymus. Small numbers of beads were found in the gall bladder and the Bursa. Already within one h red beads were present in the Bursa, and thus probably travelled throughout the whole body. The spleen did not contain many numbers of red beads.

Low levels of 10 µm red beads were observed in the lumen and wall of the small intestine and in the wall of the caecum. Higher levels were, however, observed in the lumen of the caecum, with a peak at 24 and 48 h p.c. Ten µm red beads were also found at 6 h p.c. in the faeces and then numbers gradually decreased. One µm red beads were found in the wall of the small intestine and the caecum at all moments, but numbers decreased after 24 h p.c.. The luminal content of the small intestine showed no 1 µm red beads at 1 h, but low amounts were found at the following sample moments. The luminal content of the caecum showed 1 µm beads at all moments with a peak at 24 h p.c.. The faeces contained no 1 µm red beads at 1 h after i.t. challenge, but very high amounts of 1 µm red beads at 6 and 24 h after i.t. challenge. Also at 48 h and 1 wk after i.t. challenge there were still many 1 µm red beads present in the faeces.

High numbers of red beads (both 1 µm and 10 µm) were also observed in the air sacs, which gradually decreased in time. This was also observed for the humerus and radius, albeit to a lesser extent and with a higher clearance rate.

Localisation of green beads, as expected, occurred mainly in the Bursa at all sample moments (on average an estimated 58.45% 10 µm green beads and an estimated 71.67% 1 µm green beads of the total located green beads in the chicken body). Relatively high numbers of green beads of both sizes were detected in the lungs, already at 1 h after cloacal challenge. Also the liver and consequently the gall bladder contained high numbers of 1 µm particles, especially at 1 h after

challenge. In the kidney 1 µm green beads were present. At 1 and 6 h after cloacal challenge, the thymus showed a very constant level of 1 µm green beads for both chickens sampled (17,000-18,000 1 µm beads). However, almost no amounts of 10 µm green beads were found in the liver, gall bladder, kidney and thymus. No 10 µm green beads were found in the wall of the small intestine and the caeca, or in the luminal content of the small intestine. Ten µm green beads showed a constant level within the luminal content of the caeca, but numbers were lower after 1 wk. A decrease was also observed for these 10 µm green beads in the faeces starting at 1 h after cloacal challenge and decreasing to almost no green 10 µm beads after 1 week. One µm green beads showed a constant level in the small intestinal and caeca wall, although a small peak was observed at 1 and 6 h after cloacal challenge. The lumen of the small intestine also showed a constant, but low level of 10 µm green beads. The caecal lumen showed very high amount of 10 µm green beads: first increasing to very high levels at 24 h, followed by a small decrease at 48 h and 1 wk. One µm green beads showed high presence in faecal samples, especially at 1 and 6 h p.c.. Green beads deposition in the air sacs was observed at all sample moments. Overall, green beads showed a very constant level of deposition, although at 6 h a small peak was found for 1 µm green beads. Within the bone marrow 1 µm green beads were slightly more present than 1 µm red beads. In the blood smears, 1 µm red beads were found in slightly higher amounts than green beads. On the whole, most tissues showed a more or less equal division between the 10 µm and 1 µm beads of both colours. Most tissues also showed lowering numbers of beads in time suggesting bead clearance already at 24 h, except for the kidney where bead clearance was indicated at 48 h.
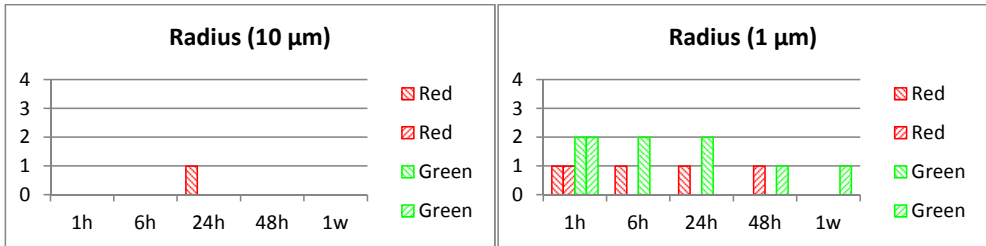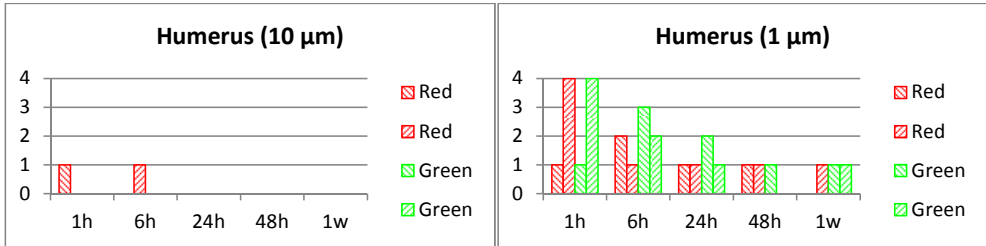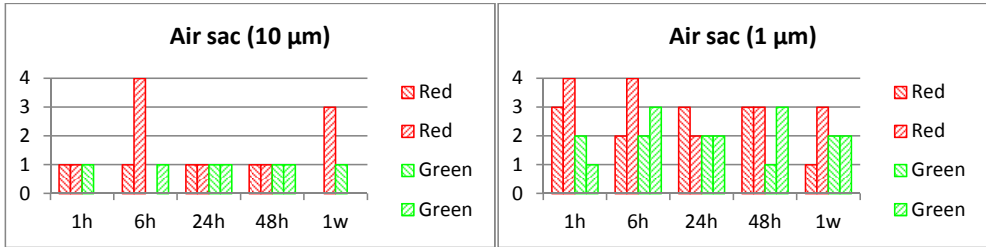
a

b

a   b
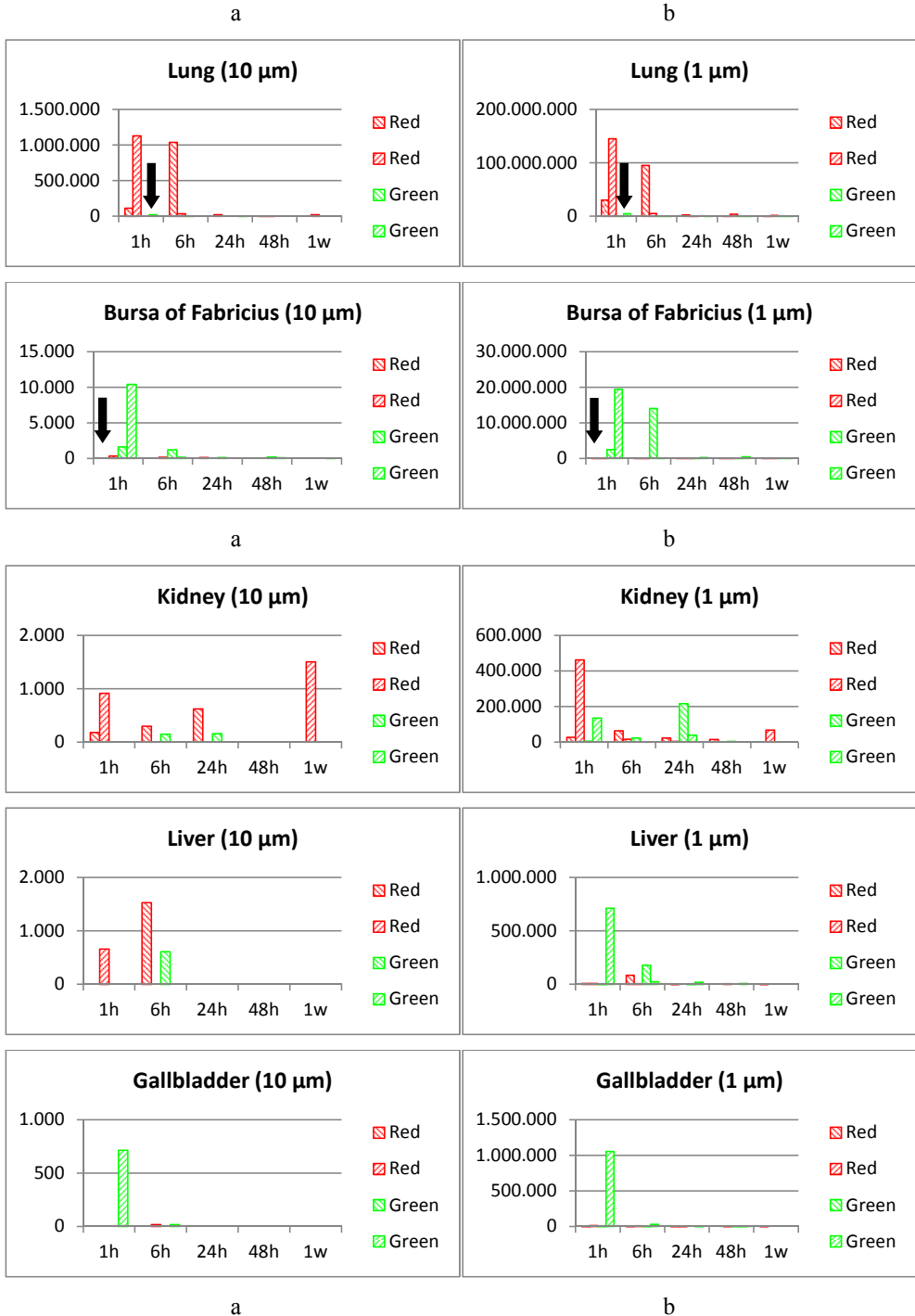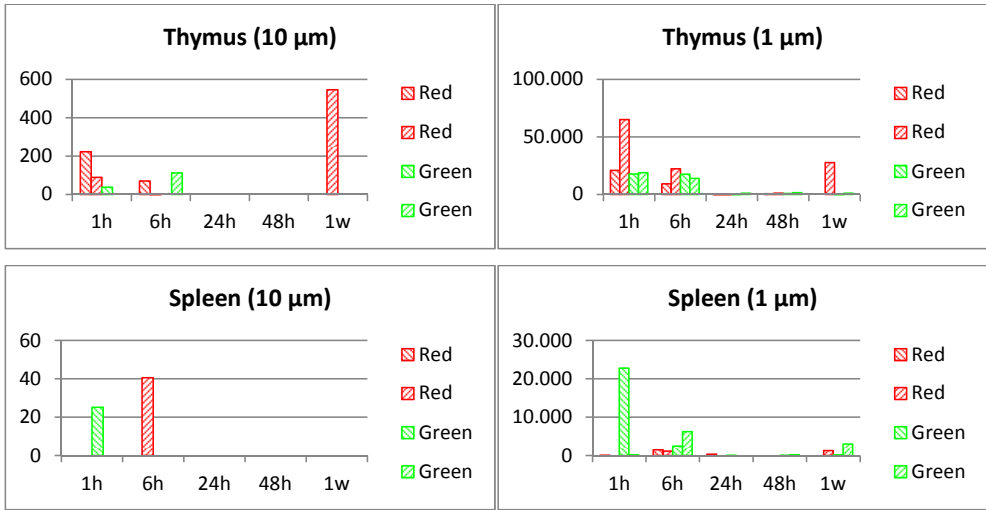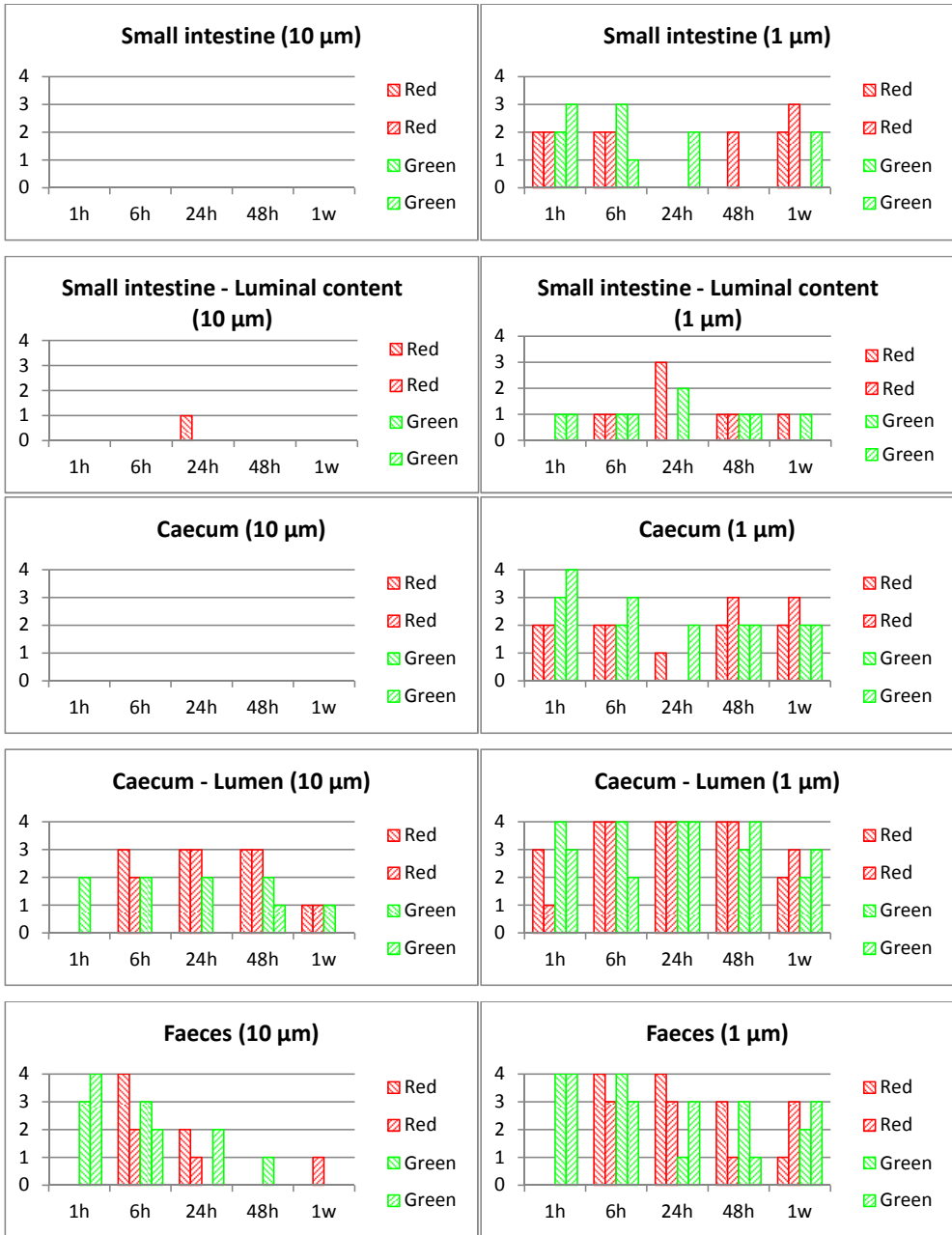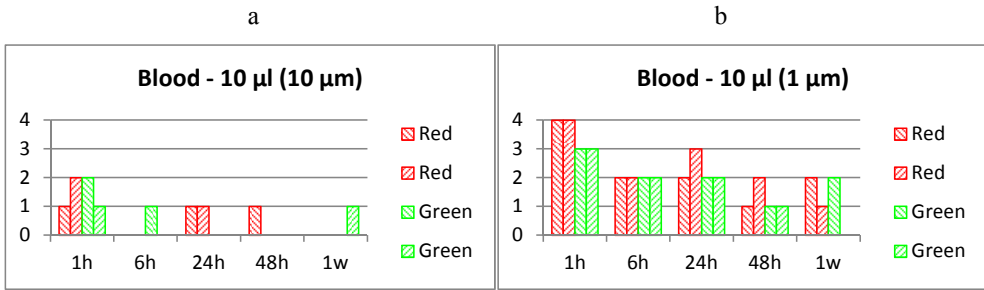
Graphs 1-7. Estimation of total beads (a: 10 µm, b: 1 µm) present in total organ of two chicken per treatment based on quantitative scale (1: Lung, 2: Bursa of Fabricius, 3: Kidney, 4: Liver, 5: Gallbladder; 6: Thymus, 7: Spleen). Same pattern within same time indicates same animal. Arrows indicate presence of the other coloured and sized beads, which are not visualised in the graphs due to scaling.

Graphs 8-16. Estimation of total beads (a: 10 µm, b: 1 µm) present in total organ of two chicken per treatment based on an ordinal qualitative scale (8: Air sac, 9: Humerus, 10: Radius, 11: Small intestine, 12: Small intestine - Luminal content, 13: Caecum, 14: Caecum - Luminal content, 15: Faeces, 16: Blood (10µl)). Same pattern within same time indicates same animal. Scale: 0 (no beads), 1 (<100 beads), 2 (>100 beads), 3 (>1,000) and 4 (not countable and/or rateable). Scale humerus and radius: 0 (no beads), 1 (<10 beads), 2 (<50 beads), 3 (<100 beads) and 4 (>100 beads). Scale blood: 0 (no beads), 1 (<1 beads), 2 (<10 beads), 3 (<100 beads) and 4 (>100 beads).

Phagocytosis test

The capacity of chicken PBL to phagocytose fluorescent beads of different size and therefore suggest their role as a possible carrier in the blood, was examined. Monocytes and probably also heterophils were able to take up 1 µm beads already at 5 min after incubation with most phagocytosis at 15 min after incubation (Figure 3). However, 10 µm beads were not observed to be taken up by any type of leukocyte at any moment after incubation (data not shown).
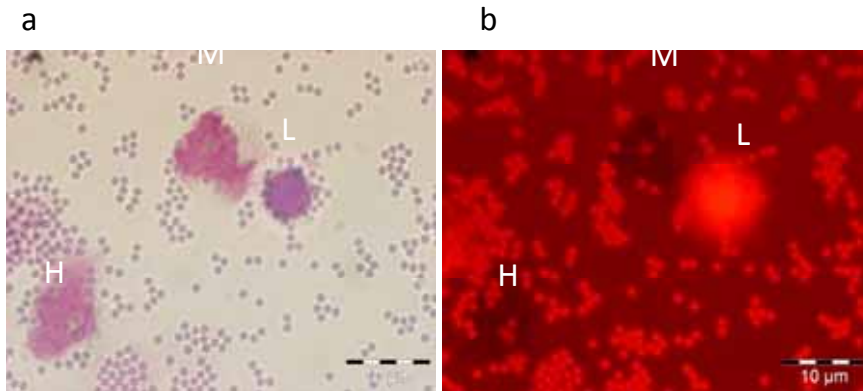

Figure 4. Uptake of 1 µm beads by leukocytes at 15 min after incubation. a; From left to right: heterophil (H), monocyte (M) with phagocytised beads and lymphocyte (L). b; Same picture as a with fluorescence.
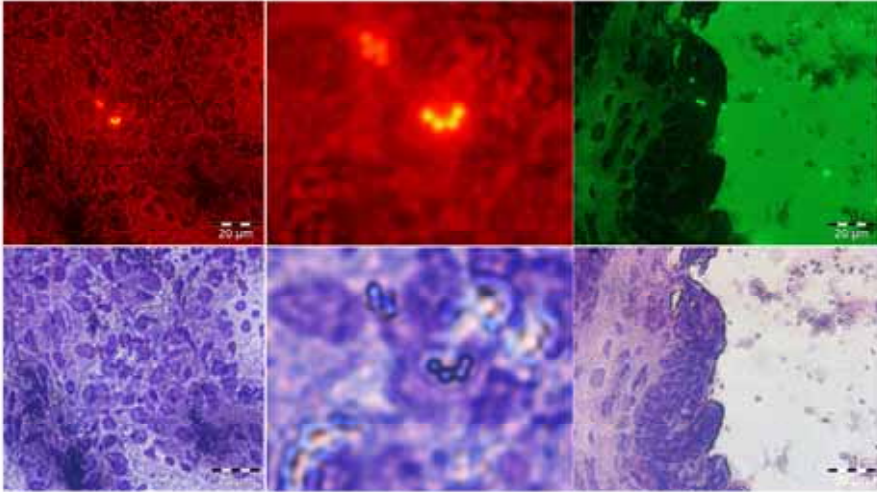
Figure 3. Uptake of fluorescent particles after 1 hour. a; Macrophage and four taken up 1 µm red particles in lung tissue. b; Same as a, without fluorescence. c; Detail of a. d; Detail of b. e; Possible uptake of 1 µm green fluorescence particles by cells in the bursa of Fabricius. f; Same as e, without fluorescence.

DISCUSSION

Next to the digestive tract, the respiratory tract and the cloacal route are likely important routes of antigenic entry in the chicken. Surprisingly little is known of the mechanisms by which specific and innate antigens or microbes penetrate the chicken body, the final destiny and localisation, and the clearance or immune processing of antigens by the chicken. Since hygiene in a chicken house most likely (in)directly affects immune responsiveness and as a consequence responses to vaccines, production (egg lay, body weight gain) levels, behaviour, and disease resistance, more knowledge on the capacity of microbes to penetrate the chicken is required. A limited number of studies was directed on the fate of particles after experimental injection in the respiratory tract or the cloaca of the chicken. These studies were limited to sampling shortly after challenge. In the present study we incorporated longer periods after challenge, since a continuous presence of invading microbes may affect subsequent innate or specific immune responses. We used non-immunogenic (polystyrene) beads to avoid immune mediated clearance or degradation, and limited our study to evaluate the final destination and distribution of non-degradable beads from two different sizes after i.t. or cloacal challenge. In this respect, our results do not reveal the fate of particles with immunogenic features or sensitive to digestion during phagocytosis or complement activity.

Earlier studies indicated that beads either are already removed within one hour or fail to penetrate the chicken body. Already 44.7% of cloacally applied 1 µm beads was lost after 15 min (Van der Sluis et al., 2009), whereas recovery rates averaged between 73% and 82% at 20 min after a respiratory challenge (Corbanie et al., 2006). In the present experiment an experimental group sampled earlier than one h might have resulted in a higher recovery rate of applied beads. However, assuming that (specific) immune responses require the entrance into the body and the presence of antigen more than an hour, we focussed our study on later moments than the earlier studies. In general, using two chickens per sample moment resulted in a high variation for the tissues based on a quantitative scale as well as the presence of beads. However, on the whole, beads of both colours and both sizes were always found in an individual challenged bird, albeit distribution of the two sizes and two colours of beads could differ between the two animals (Graphs 1-16). Variation may be explained by the chicken uniqueness to process or clear the applied beads, but loss of i.t. applied beads due to coughing or dropping from the cloaca cannot be excluded. This likely resulted in different final amounts of beads applied to the desired target location of administration. Most studied tissues showed a more or less equal distribution (and relative levels) between the 10 µm and the 1 µm

beads, indicating that challenge was indeed successful. It was found before (Corbanie et al., 2006), that the penetration capacity of 10 μm beads differed from that of 1 μm beads. This was true both for i.t. administered as well as for the cloacally administered beads. It has to be kept in mind, however, that in the present study the 1 μm beads of both colours were administered in a 100 fold higher concentration than the 10 μm beads of both colours. However, more than 100 fold differences in relative numbers and distribution of the beads in almost all tissues were observed. This was probably also true for the air sacs, bones and small intestine, but a good comparison for these tissues on a qualitative scale remained difficult. In some samples of the lung, content of the caecum, and the faeces a 100 fold difference was found, which may reflect the almost direct location of administration.

No large numbers of all types of beads were found back in the chicken body, suggesting that that within 1 h, most beads were cleared. Especially green beads may have been removed quickly after cloacal administration. Most particles administered via the cloaca, but also administered to the respiratory tract, were likely removed within one h. On the other hand, all types of beads resided in the chicken body for at least a week. This suggested that beads or particles present in the chicken's environment (e.g. bacteria, dust, PAMPs or others), which are not immunologically or mechanically cleared in the chicken body can remain there for a prolonged period, and thus could have a chronic effect on health and welfare of the chicken. It should be kept in mind that the used beads were non-immunogenic (polystyrene), whereas immunogenic beads and/or organic matter might have degraded and cleared much faster, or may have located differently. Future studies should therefore include either degradable components, or inert beads labelled with specific or innate antigens or both.

As expected, red beads of both sizes located mainly in the lungs at all sample moments. Red bead clearance in the lungs likely occurred within 6 h p.c. This is consistent with reported macrophage activity in the lungs (Ochs et al., 1988). We hypothesise that via the lungs, the red beads went to the air sacs and consequently to the marrow of the humerus and radius, and probably also to other aerated bones. Whether the beads entered the body via uptake by phagocytes in the lungs or the air sacs and subsequently were transported phagocytes remained unknown. Within 1 h p.c. red beads were found in various tissues: Bursa, liver, thymus, kidney, spleen and gall bladder suggesting transport via the blood stream. The 1 μm red beads were also observed in the intestinal and caecum walls. With respect to the caecum, the 1 μm red beads were found in the caecum content after 1 h, but not in the lumen of the small intestine or in the faeces. This suggested that after clearance in the lungs, these beads did not reach the small intestine via an oral/intestinal route. It is therefore tempting to

speculate that the caeca are capable of excreting small particles. The described route was also more or less the route that the 10 µm red beads followed in smaller quantities. The larger red beads were observed in the gall bladder, and spleen at 6 h, and in the luminal content of the small intestine at 24 h, but were never observed in the wall of the small intestine and the caeca. They were, however, found in relatively high numbers in the caecum content and faeces at 6 and 24 h p.c. and 48 h and 1 wk p.c. in the caecum content. Relatively high numbers of red beads of both sizes were found in the kidneys at all sample moments. This suggested that the kidneys are a natural clearance route of particles that were taken up in the respiratory tract. Since we are not aware of a direct contact between air sacs and kidneys, these particles were likely transferred to the kidneys via the blood stream, either by cells or not. The effective pore size of the glomerular wall is approximately 8 nm, which may not facilitate the passage of the current beads. However, the kidney may (temporarily) function as a repository, which explains the relative high levels of beads at all sample moments. Though lower, also relatively high levels of red beads from both sizes were found back in the liver and the thymus. The liver is a very important organ for clearance of non-self material, although especially originating from the digestive tract. The opposite is true for the thymus, which is generally considered as a 'closed' immune privileged site for non-self structures. Surprisingly high levels of beads were found in the thymus, especially in the outer layer of the thymus or in blood vessels within the thymus, and thus probably not in direct contact with the naïve T cells. Small numbers of red beads were found in the gall bladder and in the Bursa. The gallbladder functions as a drain for the liver, indeed a high correlation in the amount of red beads within liver and bile was found. Already within one h the red beads were present throughout the whole body, which likely rests on transport via the blood stream. Surprisingly, little numbers of red beads were found in the spleen at any moment. Whether this was related with the non-immunogenic nature of the beads remained to be elucidated.

As expected large amount of green beads were located in the Bursa, which is an important immune organ, being the closest organ with respect to cloacal challenge and thus a likely deposition organ regardless of its function. Location of green beads occurred mainly in the Bursa at all sample moments after cloacal administration. Like in the respiratory tract for red beads, clearance of green bead in the Bursa occurred at 6 h. With respect to other tissues, relatively high numbers of green beads of both sizes were detected in the lungs, especially at 1 h after cloacal administration. The numbers of green beads in the lungs were equal or even higher than the number of red beads in the Bursa. The Bursa-to-lung route may be more direct than the lung-to-Bursa route, for instance since beads administered i.t. may have been diluted

among the air sacs. Also, clearance of beads via the Bursa may be easier or more efficient than clearance via the lungs. The liver and consequently the gall bladder contained many 1 µm green beads, especially after 1 h, indicating that the liver functions as a drain for more blood vessels than those originating from the digestive tract. The liver, showed most deposited green beads, which may be related to its size and vasculature. As was true for the red beads i.t. administered, the smallest numbers of green beads after cloacal administration were found in the spleen.

The hypothesised route that 1 µm green beads followed after cloacal administration starts in the cloaca. Via cloacal drinking, the green beads were either deposited in the Bursa of Fabricius, the intestines (or the oviduct). The location of beads in the Bursa (Figure 4) suggested phagocytosis, which may result in active uptake of particles into the body and subsequently active exclusion of particles in the intestine via faeces. Cloacally administered green beads also located in the air sacs, the humerus and the radius bone marrow, as was also observed for the i.t. administered red beads. It remains unclear how the green beads located in these tissues: via mechanical penetration or excretion via lung tissue and air sac tissue or via the vasculature in the bone marrow or a combination of both. By intestinal peristaltic (reflux) movements, material present at the end of the intestine can be transported back to the caeca and even further into the small intestines. After 1 h green beads were found in very high numbers in the caecum lumen and also, though in less numbers in the lumen of the small intestine. However, like red beads administered i.t., green beads administered cloacally were also present in the walls of the small intestine and of the caecum. This suggests that the green beads were first taken up via the Bursa and then excreted by the caeca, like proposed for the red beads. Alternatively, the caeca were capable of taking up particles from the caecal lumen. A similar route was probably followed by 10 µm green beads, though in smaller quantities, as well. The large green beads were also observed in the kidney (at 6 and 24 h), liver (at 6 h) and spleen only (at 6 h). Ten µm green beads were not observed in the bone marrow, but were observed in the lungs and the air sacs (at all sample moments). This indicated that the 1 µm green beads found in the bones, were probably transported to the bones via the lungs and/or air sacs. The large green beads were not observed in the wall and luminal content of the small intestine, nor were they observed in the wall of the caeca. They were, however, observed in the caecal lumen, indicating that reflux of 10 µm particles from the cloaca to caeca can occur, but that large particles cannot be transported to the small intestines as opposed to smaller particles (1 µm).

Finally, we tested whether transport of beads of the current sizes could rest on phagocytosis. A phagocytosis test with peripheral blood leukocytes (**PBL**s) showed that 1 µm beads were taken up by heterophils and monocytes. Free avian respiratory macrophages (**FARM**s, Toth and Siegel, 1986) are able to cross the lung epithelium (Oláh and Vervelde, 2008). FARMs are also present in the air sacs, where they clear trapped particles. Clearance via FARMs is probably the only mechanism in the air sac, as transportation of particles back to the bronchi has not been demonstrated. Clearance via FARMs is significantly slower in the air sacs than in the lungs (Ficken et al., 1986, Mensah and Brain, 1982). Other parts of the respiratory tract show additional clearance mechanisms besides FARMs, such as trapping particles in the mucus, oral mucociliary transport and swallowing of trapped material. In this perspective FARMs might also transport trapped particles via the mucus to the oesophagus, instead of transportation throughout the body (Kothlow and Kaspers, 2008). Whether these underlie the presence of both 10 µm red and 1 µm red beads originating from the cloaca in the small intestine after 6 h remains to be studied. The absence of red beads after 1 h in the gut indicated that the beads did not yet reach the small intestine via a supposedly oral route. Also macrophages in the Bursa are capable of crossing membranes in the Bursa (Oláh and Vervelde, 2008). However, little is known of their possibilities to cross membranes outside the Bursa, their exact functioning and their role in particle clearance. Ten µm beads were not phagocytosed by PBLs. This corresponded with the observation of single, lose 10 µm beads in the organs and in the blood.

Our data suggest that uptake of beads via the respiratory tract occurred actively via inhalation and subsequently phagocytosis by leukocytes and/or epithelial cells (small beads) and mechanical penetration (large beads and possibly small beads). Beads are then transferred throughout the body via the blood stream via cells (small beads) or free (small and large beads) and/or contraction of cloaca (cloacal drinking). Macrophages do seem capable of taking up small particles, but do not transport the beads throughout the chicken body. The i.t. administered beads are likely cleared via the liver and bile, via the kidney, via the oral route (clearance of the respiratory tract) and finally via the intestinal tract and the faeces. A similar route may be true for cloacally administered beads, which showed a similar distribution as i.t. administered beads. The latter was uncertain for the air sacs. The air sacs were directly collected from the chicken and placed on a slide. We were not able to identify the inside and the outside of air sacs with certainty. Thus we cannot exclude that red beads were found within the air sacs and green (and red) beads were found outside the air sacs.

In summary, beads of different sizes, but non-immune of nature were found throughout the chicken body in various tissues for a prolonged period. The distribution was not in contrast with earlier findings that i.t. administered antigens (Lai et al., 2009, Parmentier et al., 2008a) or cloacally administered antigens (Ekino et al., 1979) may provoke innate and specific immune responses and suggests that the chicken is highly sensitive for environmental (airborne or floor-derived) antigens apart from those being in the diet. More knowledge on the distribution and final clearance of antigenic components should add to optimisation of chicken health procedures.

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CHAPTER 7

GENERAL DISCUSSION

Intensive housing systems are applied in poultry production in order to be able to keep large numbers of birds under climate-controlled conditions for higher productivity. Since the industrialization of poultry production, poultry houses have been a source of numerous airborne contaminants, including dust (from different sources and with different biological components), gases (ammonia, odorous compounds, methane), and microorganisms. High dust concentrations inside poultry houses and high emissions of dust from poultry houses are the main problems in poultry production. It is estimated that in Europe, dust from poultry houses contributes approximately 50% of the total particulate matter (PM) emission from agriculture (EMEP-CORINAIR., 2007), and in the Netherlands agriculture contributes approximately 25% of the PM in ambient air (Chardon and Van de Hoek, 2002). the dust concentrations in animal houses, especially in poultry and pig houses, are much higher than concentrations in the outdoor environment (Zhang, 2004a). Dust of different sizes and of different components can, together with ammonia, significantly affect human and animal health (Cambra-López et al., 2009, Collins and Algers., 1986). It is desirable for poultry livestock production to evolve to systems that fulfill the requirements for animal welfare and health and also environmental protection. The role of research is to provide knowledge that can help to reach this aim. Within this study we focused on defining the problem of the existing air quality in broiler houses and its effect on animal health and on the immunological reactions of the birds. From earlier studies it is known that bad air quality can have a big influence on the immune system of the housed birds and on their health and productivity (Homidan et al., 2003, Parmentier et al., 2008b). How dust components may play a role in these effects, and to what extent, is still unclear. Furthermore, the effects are of particles of different sizes on deposition in the broiler's body are not yet fully understood.

The aim of this project was to obtain a better understanding of dust particle size distribution in counts and in mass in poultry houses and compare them with those in other animal houses and with outside air, and to get insight into the travelling of particles of different sizes (fluorescent beads) in the broiler body. This study yielded information on the effects of airborne particles and of dust composition on broilers' health that lead to changes in body weight and heart parameters. It also contributed to knowledge about the relationship between aerial pollutants in animal houses and the immunological reactions of the birds. In this chapter, the main findings from the studies reported in this thesis are discussed in a broader context and their implications for animal husbandry and for future research are given.

Dust characterization

In this thesis, effects of airborne dust on health of broiler chickens were assessed, and a first attempt was made to evaluate the localization of dust particles and consequently their possible mode of action. As yet, little is known about the effect of air pollution, including of dust, on the health of food animals in general and in poultry in particular. Dust in animal houses consists of or is commonly contaminated with microorganisms, such as bacteria, viruses and fungi, and different gases (Hartung, 1992, Seedorf, 2004, Harry, 1978, Muller and Wieser, 1987). Dust can also act as a vehicle for carrying microorganisms in the air (Zhao, 2011) One important major risk from dust pollution is its role in the transmission of infectious diseases (Zhao et al., 2011b, Harry, 1978), another is the mechanical damage it causes to the airways, thereby enhancing the risk of infection. Dust samples from poultry houses have been found to contain more bacteria (in counts and in grams weight) than litter (Dennis and Gee, 1973). A noticeable relationship has been reported between dust and the airborne microorganisms emitted from animal houses (Muller and Wieser, 1987). The particles can also adsorb gases such as NH3, and odorous compounds (Razote et al., 2004, Redwine et al., 2002).

The harmful effects of airborne particles depend on several factors, including the components of dust and particle size (Zhang, 2004a). The dust particles inside animal houses have been found to be significantly correlated with respiratory diseases in humans (Andersen et al., 2004) and in animals (Al Homidan and Robertson, 2003). Dust particles not only affect animal respiratory health, but also influence animal welfare and productivity by inducing diseases and by increasing mortality (Donham, 1989). Pope (2000) found that particles smaller than 2.5 µm adversely affected health in humans. However, little is known of the mechanisms by which particles affect human and animal health. In addition, the health effects depend on the concentration of particulate matter, and length of exposure. In general, long-term exposure has larger and more persistent cumulative effects than short-term exposure (Pope, 2007).

In humans, fine dust (PM2.5) and coarse dust (PM2.5-10) have been related to lung cancer and cardiovascular disease, and to mortality, after short- and long-term exposure (Pope et al., 2002). The results from Pope's study showed the potential danger of particle inhalation. Harrison and Yin (2000) found that the potential health risk associated with particles is related to particle number and mass. The present thesis reports that most particles in the dust counts of the animal houses were in the size range of fine dust. On average, 92% were smaller than 2.5 µm in diameter, and these particles contributed only 2.6% to particle mass (Lai et al.,

2011b. submitted). Other researchers have reported finding very high numbers of particles in the lowest size ranges in broiler houses: Yoder and Vanwicklen (1988) found that most particles were in the size range of between 1 and 2 µm. Large dust particles, however, seem to have a higher chance of containing microorganisms (Zhao et al., 2011a) . In laying hen houses, 99% of the total airborne particles in counts were smaller than 10 µm, while 97% of the total particles were smaller than 5 µm (Maghirang et al., 1991). Mass size distribution is generally totally different from size distribution in counts. The small dust particles generally contribute little to mass, but contribute greatly to the total number of particles in the air. Heber et al. (1988a) found that in pig farms, airborne particles larger than 4 µm contributed more than 80% to mass, but less than 30% to counts.

Particle size distribution must be known in order to understand the potential deposition of particles in human and animal respiratory tracts. In general, smaller particles are able to penetrate deeper in the respiratory tract (Alfoldy et al., 2009). Particles are deposited within the respiratory systems by three mechanisms, which depend on particle size: i.e. 1) a diffusion mechanism for particles smaller than 0.1 µm; 2) diffusion and sedimentation mechanisms for particles between 0.1 and 1 µm; and 3)   sedimentation and impaction mechanisms for particles larger than 1 µm (Zhang, 2004a). Corbanie et al. (2006) defined the deposition of particles as the amount of particles deposited in the respiratory tract. They found that the deposition pattern of particles depends both on particle size and on the depth of breathing, i.e. strong breathing will result in a deeper deposition of particles. The results presented in this thesis can be used in future studies to detect the mechanisms of particle deposition in the respiratory airways and to determine the effects of different particle constituents and sizes in poultry. Whether the particles are deposited in the upper airways or lower airways (in the lungs), they have potential to cause damage locally (in the head airway, or in the lung) or systemically (particles that remain for a long time are more likely to cause disease). In the research described in Chapter 6 we studied the deposition of non-degradable beads of 1.0 µm and 10 µm sizes when applied intratracheally (red beads) or cloacally (green beads). We recovered 78.7% of the 10 µm red beads and 86.8% of the 1 µm red beads from the chicken body, compared with 58.5% of the 10 µm green beads and 71.7% of the 1.0 µm green beads. These results show the importance of the access routes of particles via the respiratory (and also cloacal) routes, and may contribute to further studies to evaluate the relationship between deposition mechanisms in the respiratory tract and the subsequent localization in the body of different particle types. All types of beads remained in the chicken body for at least 1 week. Our results suggest that after intratracheal exposure, beads or particles commonly present in

chicken houses (dust, bacteria, Pathogen Associate Molecular Pattern – PAMP)   can penetrate the chicken's body and remain there for prolonged periods, and thus may chronically affect the bird's health and welfare. It would be worth-while repeating the study but using degradable components that are or are not bound to specific or innate antigens. Such studies could help to characterize the responses of the birds to environmental airborne conditions, and to identify the immunomodulatory features of airborne PAMP, as suggested in Chapters 3 and 4.

Airborne particles in intensive livestock systems are found in high concentrations and distributed over a large range of sizes (Zhang, 2004a). Dust concentrations have been studied in various animals (Takai et al., 1998). In the present project, we measured the concentrations and dust particle sizes in and around 26 animal houses (poultry, pig, cattle, and mink houses), in spring and summer. The results reported in Chapter 2 show that the concentrations and particle size distribution in livestock houses differed among the farms. We reported that there were large variations between animal houses and animal species in particle counts and mass in the different size ranges. Mass concentrations of particles varied between 0.83 and 4.6 mg m^{-3} in poultry houses. They were highest in floor systems for layers, followed by aviary systems for layers, turkey floor system, and broiler floor system. For the other animal farms, mass concentrations varied between 0.13 to 1.62 mg m^{-3} in pig houses, and 0.02 to 0.12 mg m^{-3} for mink and cattle houses (Lai et al., 2011b. submitted). Knowledge of particle size distribution and concentrations is useful in order to understand the potential health effects associated with particle size ranges and exposure to high concentrations of the particles. In this respect, poultry might be affected more chronically by the higher concentrations of dust, and thus might experience more immunomodulation by dust constituents than the other species tested. However, the measured concentrations of dust in animal houses depend not only on animal type but also on factors such as housing system, feed management, season, sampling periods, temperature and humidity, animal activity, and animal density.

The results reported in Chapter 2 show that a floor system for layers contained higher concentrations of dust than an aviary system (Lai et al., 2011b. submitted), whereas the dust concentrations in aviary systems were higher than in a cage system (Takai et al., 1998). This is probably because layers in floor and aviary systems are in direct contact with the litter on the floor, in which they can dust bathe. The litter area per layer is much higher in floor systems than in aviary systems. Bedded housing systems generally have more air quality problems than housing systems without bedding material. Aarnink et al. (2004) found that the use of bedding increased dust concentrations in pig houses. The type of bedding material may

also influence the concentration of dust in the air (Madelin and Wathes, 1989a, Kim et al., 2008). However, Van Harn et al (2011) found no difference in PM10 and PM2.5 concentrations and emissions in broiler houses when wood shavings, chopped wheat straw, ground rapeseed straw or silage maize was used as bedding material. Banhazi et al. (2008) found that ventilation rate, type of bedding, temperature, and building design noticeably affected inhalable and respirable dust concentrations in pig houses.

There are different and opposite effects of ventilation rate on dust concentration. On one hand, there is the diluting effect: a higher ventilation rate dilutes the airborne particles; on the other hand, a higher ventilation rate reduces the deposition of airborne particles and this partly offsets the dilution effect (Aarnink and Ellen, 2007, Gustafsson, 1999). Duchaine et al. (2000) reported that concentrations of dust can be reduced by increasing the ventilation rate in livestock production systems. Gustafsson (1999) reported that in houses for growing-finishing pigs about 60% of total airborne dust settled again at a ventilation rate below 100 m^3h^{-1}. The sedimentation rate was reduced at higher ventilation rates, leading to a lower dilution effect. When the set-point temperature of a climate control unit was set 2^oC above the recommended level, causing the ventilation rate to decrease, dust concentrations increased (Al Homidan et al., 1997). High outside temperatures may affect dust concentration inside animal houses indirectly, by increasing ventilation rates and activating wet cooling systems. In humid environments, dust concentrations will be reduced, because dust particles will absorb water from the air, thereby increasing in size and in weight. This will cause particles to settle at a faster rate. Takai et al. (1998) found that concentrations of inhalable and respirable dust in poultry houses were significantly different between seasons. This can also be explained by the fact that during the summer, when temperatures are high, ventilation rates are higher than in winter (Redwine et al., 2002). More research is needed in order to determine the exact effects of temperature and relative humidity on dust concentrations and emissions from animal houses.

Animal properties – i.e. stocking density, activity, age, and weight – can affect dust levels. For pigs, it was found that the concentration of dust generally increased parallel with increasing stocking density (Gustafsson, 1999). Dust concentrations are generally higher during daytime, when the animals are more active, than during the night (Seedorf et al., 1998). Madelin and Wathes (1989b) found a decrease in dust concentrations in the late fattening period of broilers, whereas a study of Hinz and Linke (1998) reported that the concentration of dust generally increased with animal age and weight. Dust concentrations and emission rates increased during the growing period of broilers (Aarnink and Ellen, 2007,

Redwine et al., 2002). The contribution of PM2.5 to PM10 mass increased during the growing period of broilers (Van Harn et al., 2011). Broiler age may affect dust concentrations because of an increase in dried manure, and an increase in animal weight that causes more dust production from the animal body, and also because of increased dust emission from the bedding caused by increased animal activity. At the end of the growing period of the broilers, however, the birds generally become less active again. The exact relationship between animal activity, age, and weight on dust concentrations, however, is still not fully understood, largely because it is also influenced by the litter characteristics

Finding a way to reduce the dust emitted from animal houses such as those for poultry or pigs is a challenge for researchers. Airborne dust can be reduced at source, for instance by using low-dust feed, or dedusted bedding material, applying bedding material thickly, or spraying oil, or purifying the air from animal houses by ionization and air scrubbers (Aarnink et al., 2005). Acid scrubbers may reduce airborne bacteria by 70%(Aarnink et al., 2011a). In their study, Seedorf and Hartung (1999) found that biological scrubbers removed total dust in the range between 22 to 96%, but were not consistent in reducing microorganisms. Aarnink et al. (2011b) reported that PM10 and PM2.5 dust concentrations in broiler houses were reduced by 44% and 68% respectively, when rapeseed oil was sprayed at a rate of 6 ml $m^{-2}d^{-1}$. The reduction increased to 82% for PM10 and remained similar for PM2.5 . At an application rate of 24 ml $m^{-2}d^{-1}$, air ionization reduced PM10 by an average of 36% and PM2.5 by an average of 10%. Reduction rates were related to particle size (higher reductions for larger particles). However, ionization had no noticeable effect on concentrations of microorganisms (Cambra-Lopez et al., 2009). Data on particle size distribution in livestock houses is important for the development of particle reduction techniques. It is important to identify which particle ranges remain, and which are removed from the air. Therefore, further research is needed to develop good techniques that not only reduce dust concentrations but also reduce microorganisms, fungi, and gaseous pollutants. Further improvement of air quality in animal houses is one of the biggest challenges in livestock research for the future.

Relation between dust components and the immune system

In general, the immune system of chickens can, like that of mammals, be typed as having three levels of defense. The first lines of defense are physical barriers like skin, and mucous membranes of the digestive, respiratory, and reproductive tracts. Once invaders break through these, they encounter the second line of defense: the innate immune system. The innate immune system is rapid acting. It is not antigen-specific, reacts similarly to a variety of

organisms and does not exhibit memory responses. It consists of cells: granulocytes, monocytes-macrophages, dendritic cells, and natural killer cells, and humoral components such as acute phase proteins, natural antibodies, and complement components. The third level of defense is the adaptive immune system; it reacts specifically to particular antigens. It can provide long-lasting protection based on memory (Beutler, 2004). Immune defenses can affect other host parameters, such as growth and reproduction (Bonneaud et al., 2003), but, most importantly, they determine mortality and survival.

To our knowledge, the effects of combined intratracheal challenges with dust components and/or dust particles on the immune system of broilers have never been studied before. Knowledge of the interaction between airborne dust and the immune system of broilers is valuable because of the high levels of dust found in broiler houses as described above and in Chapter 2 of this thesis, and also because the dust contains many immune modulating components: so-called pathogen associated molecular patterns or PAMP.

Dust particles have been found to be associated with PAMP that have strong immune modulating features on the immune system of the chicken. Maldonado et al. (2005) found that PAMP such as LPS or LTA significantly influenced the immune responses of layers. Intratracheal LPS challenges of layers caused sickness symptoms and modulated (enhanced) specific antibody responses in a dose-dependent fashion (Parmentier et al., 2004).

The results presented in Chapter 3 showed that components commonly present in dust can modulate specific antibody responses to the intratracheally administered model antigen human serum albumin (HuSA). LPS, LTA, β-glucan (BGL), chitin, NH3, and that heated dust enhanced primary Ab responses to HuSA. LPS and BGL in particular also noticeably enhanced primary and secondary total antibody and IgM and IgG antibody responses to HuSA. All birds challenged with dust, PAMP or NH3 showed a decreased BWG after both primary and secondary challenge. The hearts of dust-challenged birds were larger and heavier than those of the birds challenged solely with HuSA (Chapter 3). This indicated that airborne PAMP, as part component of dust, has immunomodulatory properties. The change in heart morphology is attributable to the inflammation in the lung (data not shown) after intratracheal treatments with PAMP or dust particles. Dust affects human lungs in the third stage, resulting in lung inflammation, and so it seems likely that animals will also suffer from lesions. It has to be kept in mind, however, that the experiments in this thesis focused on antibody responses, and that modulation was done with a limited set of PAMP and a limited range of concentrations.

Immune modulation may change the kinetics of an immune response: it may be enhanced, or decreased, or accelerated, or remain normal after primary or secondary challenge. Measuring antibodies in plasma allows kinetic studies of the humoral immune responsiveness. Whether the dust components also affected cellular immune responses remains to be studied. From all birds studied spleens were frozen for future cytokine measurements, thus effects of dust on the cellular part of the immune system may be subject of future studies.

Vaccines often consist of one or more proteins derived from the pathogen, or from inactivated or weakened pathogens, plus an adjuvant. Adjuvants perform a depot function (based on oil) but also may include PAMP (such as mycobacteria). Airborne PAMP (LPS, LTA or BGL) present in animal houses may thus interact with vaccination, and may change vaccine efficiency by changing the T cell and B cell responses in a negative or positive fashion. In the research presented in this thesis we found that the primary responses were usually affected differently by the experimental treatments than the secondary responses. This suggests that primary vaccination may be affected differently by dust components than secondary vaccination or actual infection. This argues for future studies on the effects of dust and its constituents on the immune system of poultry.

Chapter 4 described how a model antigen challenge with mixtures of LPS and HuSA or RGG was given to chickens in early life at 3 weeks of age, and at 7 weeks of age. HuSA was administered together with LPS at 3 weeks of age to study the effects of dust components during early life conditions on immune responses to the T cell dependent antigen HuSA. A similar challenge was done again at 7 weeks of age. Three-week-old birds were much more prone to immunomodulation with LPS than 7-week-old birds, and this was true for both primary and secondary immune responses at 7 weeks of age, which suggests that the birds adapted to LPS with age. Similar effects were found for LPS on BWG, with a much greater effect at 3 weeks of age. The results of challenging the birds with LPS at two ages, with or without the presence of specific antigens, showed that LPS, which is a very important component of dust, can modulate the immune system of the chicken at 3 weeks of age and later at 7 weeks of age even in the absence of specific antigens.

The first experiments in this thesis (Chapter 3) suggested that dust components such as LPS modulate the immune system more at 3 weeks of age than at 7 weeks of age. LPS administered intratracheally has been found to induce pulmonary hypertension in poultry (Lorenzoni and Wideman, 2008). Parmentier et al. (2008c) reported that T cell dependent humoral immune responses of layers were positively affected but BWG negatively affected LPS administered intratracheally. Secondary challenge of layers with LPS did not enhance

specific antibody responses nor did it affect BWG, which suggests adaptation of the birds (Parmentier et al., 2008c). Repeated LPS challenge may cause birds to become refractory to LPS (Korver et al., 1998). Prescott and Dunstan (2005) showed that LPS bind to TLR present on the antigen-presenting cells of mammals that release various cytokines, but also bind to TLR on T-cells, and by doing this provide a link between innate immunity and specific immunity. LPS binding to CD4+ T cells was required to establish Treg cells that regulated secondary specific immune (antibody) responses within homeostatic boundaries. Our studies showed that the broilers responded differently to the second dose of antigen than to the first. After secondary challenge, specific antibodies occurred quickly, reached higher levels, and lasted for a longer period, all suggesting antigen-specific memory responses. In Chapter 4 we reported that intratracheal challenge solely with LPS at 3 weeks of age also affected antigen specific primary and secondary antibody responses at 7 weeks of age, which suggests that similar forms of regulation of immune responses by Tregs (T-regulatory) activated by innate antigens such as LPS may occur in poultry as well. Whether the effects of LPS on antibody responses to HuSA and RGG is based on the presence of TLR on T-cells and B-cells leading to accelerated primary antibody responses to simultaneously administered specific antigens or to non-specific activation of lymphocytes remains to be studied.

Differences between immunomodulation by fine dust and by coarse dust

House dust mites are potential sources of molecules which might induce the release of multiple pro-inflammatory cytokines and chemokines from epidermal keratinocytes and dermal fibroblasts (Arlian and Morgan, 2011). Avoiding skin contact with house dust mites would reduce the likelihood of mite-induced inflammation in the skin (Arlian and Morgan, 2011). Protection against the allergy induced by the environment particular to farms is interesting, as this can lead to reduction of allergic diseases in humans during the first month of life and even during pregnancy (Vuitton and Dalphin, 2006). Laboratory studies continue to demonstrate significant shifts in the immune system from Th2 toward Th1 responses due to environmental factors. Clones of T-cells with oligoclonality in their antigen receptor (TCR) have been found in human peripheral blood after chronic stimulation with house dust mite and dermatophagocides (Ohehir et al., 1993). Bronchoalveolar leukocytes elicited by coal mine dust has been found to modulate immunity by releasing interleukin-1 and enhancing lymphocytes (Kusaka et al., 1990). The mechanisms of how dust affects human health are not fully explained, but a consistent and probable causal relation can be determined between fine and coarse dust and respiratory system diseases (Dockery et al., 1993, Hoek et al., 2000).

PM10 particles affected respiratory diseases more strongly than they affected fatal cardiovascular diseases (Castillejos et al., 2000). Exposure to PM10 for up to 5 days has been reported to increase the incidence of chronic pulmonary diseases by 2.5% and the incidence of cases of pneumonia by 1.95% (Zanobetti et al., 2000). Furthermore, the changes in cardiac autonomic function reflected by changes in mean heart rate (HR) and heart rate variability (HRV) may be part of the pathophysiologic mechanisms or pathways linking cardiovascular mortality with particulate air pollution (Pope et al., 1999).

Little is known of the direct or indirect effectiveness of the dust components on the immune system of broilers. Chapter 5 demonstrated the effects of fine dust (FD, particles < 2.5 μm) and coarse dust (CD, particles from 2.5 to < 10 μm) on the immune system of broilers. When given intratracheally, airborne dust particles collected from poultry houses affected immune responsiveness and BWG negatively at 3 weeks of age, but affected immune responsiveness positively at 7 weeks of age. Both FD and CD induced more pronounced and prolonged negative effects during the entire observation period than a comparable dose of LPS. At 7 weeks of age the effects of primary challenge with dust particles and simultaneously administered HuSA revealed that CD enhanced primary total, IgM and IgG antibody responses to HuSA. FD decreased primary total antibody responses, but enhanced IgM and IgG antibody responses to HuSA. Secondary challenges with dust particles and comparable LPS doses showed pronounced effects of FD and CD. Interestingly, all birds challenged with FD, or CD or LPS had a lower body weight gain after primary challenge. In addition, only FD+HuSA challenged birds had significantly lower body weight gain compared to the PBS+HuSA and PBS control treated group. This suggests that FD has more negative effects on broiler health than CD or LPS. The results presented in Chapter 5 indicate that CD enhanced antibody responses to HuSA, whereas FD decreased primary responses to HuSA. After 7 weeks of age, both CD and FD had significantly enhancing effects. These results also strongly suggest that immune responsiveness adapts during aging and repeated innate antigenic challenge. Whether this rests on innate activation of Treg, as suggested above, requires further study.

Challenge dose for dust components and its particle sizes.

The results presented in this thesis provide evidence that the effects of dust on the broiler immune system are related to the size of the dust particles, and also depend on the age of the bird. It is unknown, however, whether the age plays a role via the maturation of the immune system or via the size and surface of the respiratory tract or its sensitivity to penetration.

The doses used for primary and secondary intratracheal challenges to the chickens in the studies described in Chapters 3 and 4 (0.5 mg LPS, LTA, β-glucans, chitin) were approximately 500 times higher than a healthy chicken would normally inhale per day. Such high doses were used to overcome possible effects of the increased levels of airborne LPS (and other PAMP) in the chicken house. Chronic stimulation might have affected the immune responsiveness of the birds differently when they were 7 weeks old than when they were 3 weeks old. In Chapter 5 the dose of LPS used was only 360 ng, as compared to 4 mg of fine dust or coarse dust, and 0.1 mg HuSA. These differences in the magnitude of the dose may have been why the responses reported in Chapters 3 and 4 differed from those reported in Chapter 5. In addition, the results presented in Chapter 5 may also reflect the mechanical properties of dust particles, which may have overruled immunogenetic modulation by the lower dose of LPS as compared to the Chapters 3 and 4. The purified LPS used in the study described in Chapter 5 might also have differed from the naturally obtained (possibly heterogenetic) LPS present in dust samples. In Chapter 5 the levels of LPS challenge were based on the measured LPS levels in the dust size groups. Based on measurements of LPS harvested in poultry houses, levels of LPS derived from FD in broiler houses were 4.6; 6.1; 8.8 ng m^{-3} at 7 weeks and levels of LPS derived from CD were 34.9; 63.4; and 169.1 ng m^{-3} of air. The LPS dose used in Chapter 5 was thus much lower than the doses used in Chapters 3 and 4. In the study described in Chapter 3, heated dust had no effects on chicken antibody responses to HuSA, whereas in Chapter 5 we reported that dust particles could influence chicken immune responses. In the study described in Chapter 3, birds were challenged with only 1 mg heated dust, but in the study described in Chapter 5 we challenged birds with a much higher dose (4 mg dust). In addition, the dust particles used in that study were collected from a broiler house, and as well as containing LPS they probably contained other unknown dust components (LTA, BGL, etc.). However, regardless of the contradictory results, dust particles and LPS were found to modulate the chickens' immune responses to an extent that depended on the particle size, LPS concentration, route of application, and age of the birds.

The implications of the findings for the broiler husbandry

Animal welfare is becoming a topic requiring increasing research attention. Animal husbandry conditions with high animal densities have enhanced risks of infectious diseases, and housing characterized by higher airborne concentrations of immune modulating or inflammatory inducing agents, which probably affect the health and growth of the birds and also their welfare. This argues for more insight into husbandry management. This thesis

provides evidence that birds are potentially able to adapt to unfavorable conditions and may even use immune modulators to maintain homeostasis within the immune system. Challenge at an early age with immune modulatory constituents resulted in lower sensitivity to these components at a later age, and also enhanced immune responsiveness. This suggests that a challenge at a young age might have positive effects later in life, providing that the dose is optimal. The results presented in Chapters 3 and 5 showed that challenging birds with dust components and dust particles can change their heart morphology. Dust components and particles may prohibit absorption of oxygen (O_2) and release of carbon dioxide (CO_2), which may be an animal health and welfare issue that future studies should investigate.

MAIN CONCLUSIONS FROM THIS THESIS

- Dust concentrations in different particle size ranges were high in poultry houses and these were higher than in pig houses, followed by cattle houses and mink houses (particles in counts and in mass). This suggests that poultry are at greater risk of pulmonary disease and immune modulation.

- In term of counts, particles in animal houses in the size ranges smaller than 2.5 µm (92%) were the greatest proportion, whereas particle mass was highest in the size ranges larger than 2.5 µm (97%). Since small particles are known to penetrate much deeper in the respiratory tract than larger particles, this implies further risks of pulmonary problems.

- Large variations in particle counts and mass in different size ranges are found in animal houses. These variations are largely attributable to animal species/housing combination, and outside temperature and humidity.

- Dust and its probable components such as lipopolysaccharides (LPS), lipoteichoic acid (LTA), chitin, and β-glucans (BGL) enhanced primary total antibody responses to a model antigen such as HuSA. LPS and BGL initiated the most pronounced and prolonged effects under experimental conditions. Secondary immune responses revealed more pronounced effects of LPS and BGL on antibody responses, but less than after primary challenge. This suggests that as they grew older, the birds adapted and were thus able to cope with secondary challenges of dust or its components. This also suggests that airborne PAMP in dust might potentially interfere with the immune competence of the birds and, as a consequence, with responses to vaccination and resistance to infection or susceptibility to inflammation.

- Body weight gain of broilers was significantly reduced after primary challenge with dust and dust components compared with that of birds not challenged with PAMP. Again, the effects were less or absent after secondary challenge.

- The morphology of the hearts of broilers treated with PAMPs was changed (in terms of weight, length, and width of heart) compared to that of the group not treated with PAMP. This may have been caused by heart activity increasing in response to the environmental immune-enhancing stimuli (PAMPs) and respiratory capacity decreasing due to lung inflammation or deposition of dust particles in the lung.

- LPS (endotoxin), an important component of airborne dust present in poultry houses, modulated specific primary antibody responses to model antigens of broilers at different ages. The birds also showed higher or modulated immune responses to model antigens than birds not challenged at 3 weeks of age with LPS. This indicates that it is important to assess the presence of airborne LPS because of its modulatory features that either enhance or suppress immune responses.

- 'Natural' airborne dust particles collected from a broiler house negatively affected immune responsiveness and BWG at 3 weeks of age, but positively affected immune responsiveness at 7 weeks of age. Consequently, the effects of dust or PAMPs were related with the maturation of the broilers' immunity or their adaptation to their environmental conditions.

- A challenge with beads of 1 µm and 10 µm that represented dust particles resulted in the maximum amounts of the beads being found in various tissues throughout the body (e.g. bursa of Fabricius, lungs, liver, kidneys, gall bladder, spleen, thymus, small intestine, caecum, intestine luminal contents, aerated bones, feces, and blood) only 1 hour later. The beads remained present in all tissues for at least one week after intratracheal (and also cloacal) administration. They might have been taken up by phagocytic cells and/or have been transferred via the bloodstream.

RECOMMENDATIONS FOR FUTURE RESEARCH TOPICS

- Most attention in the present thesis was given to LPS, which is one of the major components in dust. Future studies should also address and incorporate the levels of LTA, chitin, BGL, and NH_3, inside and outside animal houses, and their immunomodulatory capacities.

- Further studies should address types, species and sources of LPS; likewise, combinations of physical dust and bacteria may be required to find more pronounced and prolonged effects of these components as well.

- Instead of intratracheal challenge using a needle, a spraying challenge of the birds may be a good and more natural alternative to challenge the birds.

- In the present thesis only two sizes of dust and beads were studied for their effects on immune responses, BWG, and localization in broilers. These effects of particles should be studied in more size ranges.

- In the present study, inert non-degradable beads were studied for their localization in the chicken's body after challenge. This facilitated long-term studies; however, it should be investigated whether such challenges resemble natural challenging with degradable particles.

- The present findings also suggest that the relative effects of coarse (PM10) and fine (PM2.5) particles on the immune system should be examined in more animal species and under a wider variety of animal housing conditions, population characteristics, and air pollutants.

In general it can be concluded from this thesis that airborne dust and its likely components have strong immunomodulatory effects on the immune system of broilers. Dust particles or dust components may localize throughout the chicken's body early on, and may remain present for a prolonged period. Young chickens seem more prone to immune modulation than older chickens, which suggests that adaptation has occurred. In addition, challenges with a PAMP such as LPS at 3 weeks of age affected birds to such an extent that the effects were still present 4 weeks later. This suggests that dust components such as LPS enhance the alertness of the chicken immune system, as exemplified by the primary immune responses being higher, but not the secondary memory responses, not even when antigens were given 4 weeks later. This is not in contradiction with the hypothesis that danger signals are initially involved in the activation of the immune system but that later on are required for regulation of immunity. On the other hand, dust or its components negatively affected growth, albeit temporarily. These findings argue for further studies on the role of airborne dust on the maturation of immunocompetence but also on the effects of such dust on health management procedures and the welfare of poultry and other food animals.

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SUMMARY

One of the main problems within intensive poultry production in temperate areas is the bad air quality inside the animal house. The dust concentrations are higher in poultry houses than in any other livestock housing and are especially high in floor systems with bedding. The dust in livestock houses can be characterized with respect to its concentration, composition, particle shape, particle sizes, particle density, and the source of dust. The airborne dust particles contain many microorganisms: bacteria, fungi, and viruses or parts thereof. These microorganisms and their components may affect human and animal health. Chief among the harmful components of airborne dust are endotoxins or lipopolysaccharides (LPS), lipoteichoic acid (LTA), β-glucans (BGL), chitin, and ammonia (NH_3). The particle size distribution (PSD) of dust determines the potential impact on human and animal health. The size of a dust particle affects its behavior in the air, as well as where it will be deposited in the human or animal respiratory tract when it is inhaled. In general, the smaller particles travel deeper into the respiratory system and are more likely to cause lung diseases. Besides dust, ammonia is one of the major components determining air quality inside poultry houses.

The relationship between airborne dust (in terms of concentration, size distribution or components) and broiler health (including immune responses) is largely unknown. Neither are the effects of air quality on broilers' lung health and other diseases known. Heart morphology and physiology/functioning are frequently related with infection and ascites. However, little is known about the interactions between inhaled particles and the immune system and their consequences for body weight gain, heart parameters, and disease resistance.

The aim of the research described in this thesis was to study the impact of air quality on the immune-related health of broilers. This was done in relation to the concentrations and composition of dust, and dust particle size distributions in poultry houses in the Netherlands. In addition, to enhance our understanding of the effects of dust components and particle size on the immune system of poultry we also studied the influence of the age of the birds and adaptability of the birds using repeated challenge models.

DUST CONCENTRATION AND ITS PARTICLE SIZE DISTRIBUTION (CHAPTER 2)

The concentration and size distribution of airborne particles were measured inside and outside typical animal houses for: broilers, broiler breeders (floor housing with litter); layers (floor housing system and aviary housing system); turkeys (floor housing with litter); pigs: fattening pigs (traditional houses, low emission houses with dry feed, and low emission houses with wet feed), piglets, sows (individual and group housing); cattle (cubicle house), and mink (cages). The differences in dust concentration of each house showed that PM10

(particles < 10 µm) mass concentrations were highest in poultry houses: 0.83-4.60 mg m^{-3}, followed by pig houses: 0.13-1.62 mg m^{-3}, cattle houses: 0.02-0.12 mg m^{-3}, and mink houses: 0.04-0.12 mg m^{-3}. The results revealed that inside animal houses on average 92% of particles in counts were in the PM2.5 class (particles < 2.5 µm), but this accounted for only 2.6% of the total mass. In addition, the small number of particles larger than 2.5 µm (7.6%), accounted for 97% of the total mass. In outside ambient air, 99% of the number of particles were in the PM1 (particles < 1.0 µm) class, which contributed about 11% to the total mass. Although particle counts in different size range varied greatly, for all particle classes except the particles in the 0.25 - 1.0 µm range, most variation could be accounted for by species/housing combination and outside temperature and relative humidity.

THE EFFECTS OF DUST COMPONENTS ON THE IMMUNE SYSTEM (CHAPTER 3)

The aim of the second experiment was to establish the influence of different dust components on the innate and specific immunity of broilers, and the relation between dust components and body weight gain or heart morphology. Slow-growing broilers from 3 weeks of age were housed in the same facilities within 9 pens. On days 0, 3, 7, 10, 14, 21 after primary challenge with various dust components and days 0, 3, 7, 10, 14, 21 after secondary challenge with the same components, blood samples were collected. Plasma was used to study total antibody, and isotype-specific IgM and IgG antibody responses to the model antigen human serum albumin (HuSA) and to LPS. Body weight was measured on day 0 before primary and secondary challenges and again on day 2 after each challenge. The results showed that all dust components, such as LPS, LTA, BGL, chitin, heated-dust, and NH$_3$ enhanced the total antibody responses to HuSA after primary and secondary challenge. However, the effects induced by LPS and BGL were more pronounced and prolonged. Enhanced antibody responses to LPS were found in the group of birds challenged with LPS. Challenging the birds with NH$_3$, LTA, BGL, and dust also enhanced antibody responses to LPS. This suggests that the birds were stimulated by these components of airborne LPS that were already present in the chicken house. This study showed that all birds challenged concurrently with dust components and HuSA gained less body weight after primary challenge than the solely HuSA-challenged birds. Interestingly, only a cocktail-treated group was still affected after secondary challenge, which suggests that the birds had adapted to individual dust components. The results showed that the weight, length, and width of the hearts of the broilers that had been treated with BGL, LPS or NH$_3$ and HuSA were

significantly larger than those of the hearts of solely HuSA-treated birds, which suggests that environmental air quality might affect heart morphology and activity.

THE EFFECTS OF REPEATED LPS CHALLENGE ON THE IMMUNE SYSTEM (CHAPTER 4)

The aim of the third experiment was to determine the adaptability of broilers to LPS early in their life (at 3 weeks of age) and later in life (at 7 weeks of age). The experiment was conducted with slow-growing broilers (Hubbard ISA) housed in the same facility within 11 pens. On days 0, 3, 7, 10, 14, 21 after primary challenge and days 0, 3, 7, 10, 14, 21 after secondary challenge, blood samples were collected. Plasma was used to study total antibody and isotype antibody responses of broilers to HuSA and rabbit gamma globulin (RGG) after primary and secondary challenges at different ages. Body weight was measured at 3 and 7 weeks of age on the day of challenge and 24 hours after challenge. It was hypothesized that broilers would be able to cope with environmental LPS, and that the coping mechanism might be based on the initiation of regulation mechanisms at two different ages. We hoped to be able to identify these mechanisms. In general, LPS treatments affected immune responses at several time points, and affected body weight gain. LPS enhanced antibody responses to HuSA and RGG after secondary challenges, but also when the specific antigens (HuSA, RGG) were given four weeks later for the first time. These results suggest that exposure to LPS at an early age via the respiratory tract may enhance immune responses to specific antigens at later ages in an antigen nonspecific fashion. This emphasizes the importance of immunomodulating dust components in the air in the modulation of immune responses of poultry, and may have consequences for vaccine and housing management.

THE EFFECTS OF DUST PARTICLE SIZE (CHAPTER 5)

The aim of the fourth experiment was to establish the effects of fine dust (FD) (PM2.5: particles < 2.5 µm) and coarse dust (CD) (PM2.5-10: particle size from 2.5 – 10 µm) collected from broiler houses and corresponding concentrations of pure LPS, as determined in the dust samples, on the immune system of broilers. On days 0, 3, 7, 10, 14, 21 after primary challenge at 3 weeks of age and days 0, 3, 7, 10, 14, 21 after secondary challenge at 7 weeks of age, blood samples were collected. Plasma was used to study the total antibody, and isotype-specific antibody responses of broilers to HuSA and LPS. Body weight was measured on the day of challenge and 24 hours after challenge at 3 and 7 weeks of age. It was expected that FD and CD would reduce the immune competence of broilers, and FD would affect the birds much more than CD. In general, both FD and CD negatively affected immune responses and

body weight gain at young age (3 weeks of age), whereas enhanced immune responses were found at later age (7 weeks of age). In addition, the heart size (weight, length, width) was increased of the broilers treated with dust (FD and CD) at 7 weeks of age. The results also showed that secondary exposure had fewer effects than primary challenge, suggesting that the birds had adapted to environmental conditions.

LOCALIZATION AND QUALIFICATION OF FLUORESCENT BEADS IN CHICKEN (CHAPTER 6)

The aim of the fifth experiment was to localize non-immunogenic particles of two different sizes (1 µm and 10 µm) in chickens challenged via the respiratory tract and the cloaca. Ten one-day-old Ross broilers were housed in one group in a roofed pen with sawdust bedding. At 3.5 weeks of age all birds received intratracheally 1.25×10^9 red beads with a diameter of 1.0 µm and 1.05×10^7 red beads with a diameter of 10.0 µm. At the same time, 1.25×10^9 green beads with a diameter of 1.0 µm and 1.05×10^7 green beads with a diameter of 10.0 µm were administered to the cloaca. At 1 hour, 6 hours, 24 hours, 48 hours, and 1 week thereafter, two randomly selected birds were dissected in order to collect various tissue samples, which were examined using fluorescence microscopy.

Beads of different size ranges were found throughout the chicken body in various tissues: air sacs, bursa, liver, thymus, kidney, spleen, gall bladder, bone marrow, intestines and blood. The data suggested that beads had been transported throughout the chicken's body via the bloodstream, probably either within phagocytic cells (1 µm beads) or on their own (10 µm beads). Beads administered to both locations were still found in the chicken's body one week after administration. We conclude that airborne material can easily invade the chicken's body (systemically), which may account for the systemic antibody responses to specific antigens after intratracheal challenge we found, and may also cause the immune modulatory effects of intratracheally administered dust or dust components.

GENERAL CONCLUSION

Dust in livestock houses is different from dust present in outside air in terms of its concentration and particle size distribution. In the research presented in this thesis we found that the dust concentration in livestock houses was highest in poultry houses, followed by pig houses, while there were much lower concentrations in cattle houses and mink houses. The highest proportion of particle counts was in sizes smaller than 2.5 µm. Airborne particles of different sizes and with different components could trigger the immune system of broilers, as exemplified by the enhanced primary responses that were antigen-nonspecific. On the other

hand, the absence of major effects of dust components on secondary immune responses may indicate that dust components can regulate the immune system and that birds adapt to the environment. Dust and/or its components, however, had an important negative impact on body weight gain and heart parameters. These findings suggest there are relationships between (changing) hygienic conditions in animal houses and the activation and the functioning of the chicken's immune system which may have consequences for disease susceptibility and vaccine efficiency. This argues for further studies on the microbial composition of dust and its physical features and the effects on the immune system of poultry or other livestock reared for food.

Samenvatting

Een groot probleem in de intensieve pluimveehouderij in gematigde klimaatzones is de slechte luchtkwaliteit in de stallen. In pluimveestallen zijn de stofconcentraties hoger dan in alle andere stallen, vooral in de stallen met strooisel. Stof in stallen kan beschreven worden in termen van concentratie, samenstelling, vorm van de deeltjes, deeltjesgrootte, dichtheid van de deeltjes en de bron van de deeltjes. De stofdeeltjes kunnen verschillende micro-organismen bevatten: bacteriën, schimmels en virussen en onderdelen daarvan. Deze micro-organismen en de componenten daarvan beïnvloeden de gezondheid van mens en dier. De belangrijkste schadelijke componenten in het stof zijn endotoxinen ofwel lipopolysacchariden (LPS), lipoteichoine zuur (LTA), β-glucanen (BGL), chitine en ammoniak (NH_3). De deeltjesgrootteverdeling van het stof bepaalt de potentiële impact op de gezondheid van mens en dier. De grootte van een stofdeeltje bepaalt het gedrag van dit deeltje in de lucht en waar het na inademing terecht komt in de luchtwegen van mens en dier. In het algemeen kan gesteld worden dat des te kleiner het deeltje is, des te dieper het zal doordringen in de luchtwegen. Kleinere deeltjes zullen daardoor eerder longaandoeningen veroorzaken. Naast stof is ammoniak één van de belangrijkste componenten die de luchtkwaliteit in pluimveestallen bepaalt.

Er is weinig bekend over de relatie tussen stof in de lucht (in termen van concentratie, deeltjesgrootteverdeling of samenstelling) en de gezondheid van vleeskuikens (inclusief het effect op het immuunsysteem). Ook is er weinig bekend over het effect van luchtkwaliteit in het algemeen op de longgezondheid en andere ziekten bij vleeskuikens. De morfologie en fysiologie / functioneren van het hart worden vaak in verband gebracht met infecties en ascites. Echter, er is weinig bekend over de interactie tussen ingeademd stof en het immuunsysteem en de consequenties voor groei, hart parameters en ziekteresistentie.

Het doel van het onderzoek beschreven in dit proefschrift was om het effect vast te stellen van luchtkwaliteit op de immuun gerelateerde gezondheid van vleeskuikens. Dit tegen de achtergrond van de stofconcentraties en -samenstelling en de deeltjesgrootteverdeling van stof in pluimveestallen in Nederland. Om meer begrip te krijgen van de effecten van stofcomponenten en de deeltjesgrootte op het immuunsysteem van pluimvee hebben we ook het effect onderzocht van de leeftijd en het aanpassingsvermogen van de vleeskuikens door de dieren herhaaldelijk bloot te stellen aan stofdeeltjes of stofcomponenten.

STOFCONCENTRATIE EN DEELTJESGROOTTEVERDELING (HOOFDSTUK 2)

De stofconcentratie en deeltjesgrootteverdeling van stof werd gemeten in verschillende stallen voor: vleeskuikens, vleeskuikenouderdieren (beiden strooiselstallen); leghennen

(scharrelstal en volièrestal); kalkoenen (strooiselstal); varkens: vleesvarkens (traditionele stallen, emissiearme stallen met droogvoer en emissiearme stallen met brijvoer), biggen, zeugen (individueel en in groepshuisvesting); melkvee (ligboxenstallen), en nertsen (kooien). De stofconcentraties in de verschillende stallen lieten de hoogste PM10 (deeltjes < 10 µm) massa concentraties zien in pluimveestallen: 0,83-4,60 mg m^{-3}, gevolgd door varkensstallen: 0,13-1,62 mg m^{-3}, melkveestallen: 0,02-0,12 mg m^{-3} en nertsenstallen: 0,04-0,12 mg m^{-3}. Uit de resultaten blijkt dat in de stallen gemiddeld 92% van het aantal deeltjes in de PM2,5 klasse (deeltjes < 2,5 µm) viel, maar deze deeltjes droegen voor slechts 2,6% bij aan de totale massa stof. Dit betekent dat dit relatief kleine aantal deeltjes groter dan 2,5 µm (7,6%), voor gemiddeld 97% bijdroeg aan de totale massa stof. In de buitenlucht vielen 99% van het aantal deeltjes in de PM1 (deeltjes < 1.0 µm) klasse en deze deeltjes droegen voor 11% bij aan de totale massa stof. Hoewel de aantallen deeltjes in alle klassen, behalve voor de deeltjes in de klasse 0,25 – 1,0 µm, sterk varieerden, kon een belangrijk deel van de variatie worden verklaard uit de combinatie diersoort/huisvestingssysteem en de temperatuur en relatieve luchtvochtigheid van de buitenlucht.

EFFECT VAN STOFCOMPONENTEN OP HET IMMUUNSYSTEEM (HOOFDSTUK 3)

Het doel van het tweede experiment was te bepalen wat de invloed was van verschillende stofcomponenten op het aangeboren en specifieke immuunsysteem van vleeskuikens en de invloed van stofcomponenten op de groei van de dieren en de morfologie van het hart. Langzaam groeiende vleeskuikens van 3 weken oud werden gehuisvest in 9 hokken in dezelfde stal. Op dag 0, 3, 7, 10, 14, 21 na primaire immunisatie met verschillende stofcomponenten en op dag 0, 3, 7, 10, 14, 21 na secundaire immunisatie met dezelfde componenten, werden bloedmonsters afgenomen. Het bloedplasma werd gebruikt voor bepaling van totaal antilichaamtiters en isotype specifieke IgM en IgG antilichaamtiters tegen het model antigeen humaan serum albumine (HuSA) en op LPS. Lichaamsgewicht werd bepaald op dag 0 voor de primaire en secundaire immunisatie en vervolgens op dag 2 na iedere immunisatie. De resultaten lieten zien dat alle stofcomponenten, zoals LPS, LTA, BGL, chitine, verhit stof en NH$_3$, een verhoogde antilichaamrespons op HuSA gaven na primaire en secundaire immunisatie. Echter, de respons als gevolg van LPS en BGL toediening waren sterker en langduriger dan bij de andere componenten. De antilichaamrespons op LPS was verhoogd bij vleeskuikens die waren geïmmuniseerd met LPS, maar ook bij vleeskuikens die waren geïmmuniseerd met NH$_3$, LTA, BGL en stof induceerden een verhoogde antilichaamrespons tegen LPS. Dit suggereert dat de vleeskuikens

al gestimuleerd waren door LPS in het stof dat al aanwezig was in de lucht van de stal waarin ze waren gehuisvest. Deze studie liet zien dat alle vleeskuikens die gelijktijdig geïmmuniseerd werden met stofcomponenten en HuSA een lagere groei hadden na primaire immunisatie dan de vleeskuikens die alleen met HuSA werden geïmmuniseerd. Interessant genoeg, hadden na secundaire immunisatie alleen de vleeskuikens die een cocktail van de verschillende componenten kregen toegediend een verlaagde groei. Dit suggereert dat de vleeskuikens zich hadden aangepast aan de individuele stofcomponenten. De resultaten lieten verder zien dat de vleeskuikens die BGL, LPS of NH_3 in combinatie met HuSA kregen toegediend, een significant groter hart hadden (uitgedrukt in gewicht, lengte en breedte) dan de vleeskuikens die alleen HuSA toegediend hadden gekregen. Dit suggereert dat luchtkwaliteit in stallen de hartmorfologie en –activiteit kan beïnvloeden.

EFFECTEN VAN HERHAALDE LPS TOEDIENING OP HET IMMUUN SYSTEEM (HOOFDSTUK 4)

De doelstelling van het derde experiment was te bepalen in welke mate vleeskuikens zich kunnen aanpassen aan LPS, bij toediening op een jonge leeftijd (na 3 weken) en na toediening op latere leeftijd (na 7 weken). Het experiment werd uitgevoerd met langzaam groeiende vleeskuikens (Hubbard ISA) die waren gehuisvest in 11 hokken in dezelfde stal. Op dag 0, 3, 7, 10, 14, 21 na primaire immunisatie en op dag 0, 3, 7, 10, 14, 21 na secundaire immunisatie, werden bloedmonsters genomen. Het bloedplasma werd gebruikt voor bepaling van de totaal en de isotype antilichaamresponses van de vleeskuikens op HuSA en konijnen gamma globuline (RGG) na primaire en secundaire immunisatie op beide leeftijden. Lichaamsgewicht werd bepaald op 3 en 7 weken leeftijd op de dag van immunisatie en 24 uur later. De hypothese was dat vleeskuikens zich kunnen aanpassen aan LPS in stallucht en dat het aanpassingsmechanisme gebaseerd is op de initiatie van de regulerende mechanismen op beide leeftijden. In deze studie hopen we inzicht te krijgen in deze mechanismen. De resultaten laten zien dat de LPS behandelingen de immuunrespons op verschillende momenten beïnvloedde en tevens de groei van de dieren beïnvloedde. LPS stimuleerde de antilichaamrespons op HuSA en RGG na secundaire toediening, zelfs wanneer de specifieke antigenen (HuSA, RGG) vier weken later voor het eerst werden toegediend. Deze resultaten suggereren dat blootstelling aan LPS op een jonge leeftijd via de luchtpijp een verhoogde immuunrespons geeft op specifieke antigenen op latere leeftijd, op een antigeen aspecifieke wijze. Dit toont het belang aan van stofcomponenten in de lucht op de modulatie van de immuunrespons bij pluimvee. Dit kan gevolgen hebben voor de wijze waarop de dieren moeten worden gevaccineerd en gehuisvest.

EFFECT VAN DEELTJESGROOTTE VAN STOF (HOOFDSTUK 5)

De doelstelling van het vierde experiment was om het effect vast te stellen van fijn stof (PM2,5: deeltjes < 2.5 µm) en grof stof (PM2,5-10: deeltjes in de range van 2,5 – 10 µm) en corresponderende concentraties puur LPS, zoals bepaald in de stofmonsters, op het immuunsysteem van vleeskuikens. Het stof werd verzameld in een vleeskuikenstal. Op dag 0, 3, 7, 10, 14, 21 na primaire immunisatie op 3 weken leeftijd en dag 0, 3, 7, 10, 14, 21 na secundaire immunisatie op een leeftijd van 7 weken, werden bloedmonsters genomen. Bloedplasma werd gebruikt om de totale en isotype-specifieke antilichaamresponsen bij vleeskuiken op HuSA en LPS te bepalen. Het lichaamsgewicht werd bepaald op de dag van immunisatie op 3 en 7 weken leeftijd en 24 uur hierna. De verwachting was dat FD en CD de immuuncompetentie van vleeskuikens zou verlagen en dat FD een grotere invloed zou hebben op de vleeskuikens dan CD. Uit de resultaten bleek dat zowel FD als CD een negatieve invloed had op de immuunrespons en op de groei van de dieren op jonge leeftijd (3 weken leeftijd), terwijl een verhoogde immuunrespons werd gevonden op latere leeftijd (7 weken leeftijd). Verder was het hart vergroot van vleeskuikens behandeld met stof (FD en CD) op een leeftijd van 7 weken. De resultaten lieten ook zien dat de secundaire blootstelling aan stof minder effect had dan de primaire blootstelling. Dit suggereert dat de vleeskuikens zich hadden aangepast aan de omgevingscondities.

VERSPREIDING EN KWALIFICATIE VAN FLUORESCERENDE DEELTJES IN KIPPEN (HOOFDSTUK 6)

De doelstelling van het vijfde experiment was de verspreiding te bepalen van niet-immunogene deeltjes van twee verschillende groottes (1 µm en 10 µm) die via de luchtpijp en de cloaca werden toegediend. Tien eendagskuikens (Ross) werden gehuisvest in één groep in een hok met strooisel. Op een leeftijd van 3,5 week kregen de vleeskuikens $1,25 \times 10^9$ rode inerte deeltjes met een diameter van 1.0 µm en 1.05×10^7 rode inerte deeltjes met een diameter van 10.0 µm toegediend via de luchtpijp. Op hetzelfde moment werden $1,25 \times 10^9$ groene inerte deeltjes met een diameter van 1.0 µm en 1.05×10^7 groene inerte deeltjes met een diameter van 10.0 µm toegediend via de cloaca. Na 1, 6, 24 en 48 uren en na 1 week na toediening werden twee aselect gekozen vleeskuikens ontleed om verschillende weefselmonsters te nemen voor analyse met de fluorescentie microscoop.

De inerte deeltjes van beide groottes werden verspreid over het hele lichaam terug gevonden in de volgende weefsels: luchtzakken, Bursa van Fabricius, lever, thymus, nieren, milt, galblaas, beenmerg, darmen en bloed. De data laten zien dat de inerte deeltjes via het

bloed door het gehele lichaam zijn verspreid, waarschijnlijk in fagocyterende cellen (1 µm deeltjes) of op zichzelf (10 µm deeltjes). Deeltjes die via de luchtpijp en de cloaca werden toegediend, werden ook na één week nog terug gevonden in het lichaam van de kippen. Uit dit onderzoek concluderen wij dat deeltjes in de lucht gemakkelijk het lichaam van kippen kunnen binnendringen. Dit zou de systemische antilichaam reactie op specifieke antigenen na toediening via de luchtpijp kunnen verklaren, zoals gevonden in voorgaande onderzoeken. Dit zou tevens de immuun modulerende werking van stof en stofcomponenten, toegediend via de luchtpijp, kunnen verklaren.

ALGEMENE CONCLUSIE

Stof in stallen verschilt van stof in de buitenlucht ten aanzien van concentratie en deeltjesgrootte verdeling. In het onderzoek gerapporteerd in dit proefschrift vonden we dat de stofconcentraties het hoogst waren in pluimveestallen, gevolgd door varkensstallen, terwijl de concentraties beduidend lager waren in melkveestallen en nertsenstallen. Het grootste deel van het aantal deeltjes was kleiner dan 2,5 µm. Stofdeeltjes in de lucht van verschillende grootte en met verschillende componenten kunnen het immuunsysteem van vleeskuikens stimuleren, zoals geïllustreerd door de verhoogde aspecifieke primaire responses op toegediende antigenen. Aan de andere kant laat de geringe reactie van het immuunsysteem op secundaire blootstelling aan stofcomponenten zien dat deze stofcomponenten het immuunsysteem kunnen reguleren en dat de dieren zich kunnen aanpassen. Stof en/of de stofcomponenten hadden echter een belangrijk negatief effect op de groei van de dieren en op de hart parameters. Deze bevindingen suggereren dat er een relatie bestaat tussen (veranderende) hygiënische condities in stallen en de activering en het functioneren van het immuunsysteem bij kippen. Dit kan gevolgen hebben voor de gevoeligheid voor ziektes en de efficiëntie van vaccinaties. Dit is een belangrijk argument om verder onderzoek te doen naar de microbiologische samenstelling van stof en de fysische karakteristieken van het stof en de effecten daarvan op het immuunsysteem van pluimvee en andere landbouwhuisdieren.

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INTERNAL REPORTS

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My experiments for the thesis were set up in animal houses (De Haar) on the Zodiac farm, and the measurements were done in Spelderholt in Lelystad. My thanks go to the co-workers over there.

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Hà), sisters, brothers, nephews, nieces and my friends who always help me and have taken care of me and my family over last few years.

Con không biết dùng lời nào để nói lên hết lòng biết ơn của con đến với cha mẹ, cha mẹ đã vất vả và hy sinh rất nhiều cho thành công của chúng con ngày hôm nay. Quyển luận văn này con dành tặng cha mẹ với lòng biết ơn sâu sắc nhất. Cha từng nói rằng mơ ước lớn nhất của cuộc đời là thấy con cái học tập nên người và thành đạt. Con kính mong hương hồn cha siêu thoát và yên tâm an nghỉ nơi cõi vĩnh hằng.

Quyển luận văn này em dành tặng anh, anh đã gánh vác công việc gia đình, chăm sóc con và dành thời gian động viên em trong suốt 4 năm qua để em yên tâm học tập. Thời gian 4 năm qua thật khó khăn vất vả cho gia đình mình, anh em và con đã cùng nhau dành được kết quả ngày hôm nay. Cảm ơn anh thật nhiều.

Ngô Trí Huy, con là tài sản quý giá là động lực của mẹ với lời hứa hai mẹ con mình cùng học tập thật tốt. Mẹ cảm ơn con trai của mẹ đã rất trưởng thành, cố gắng học tập trong thời gian mẹ không ở nhà.

Lời cảm ơn đến với các anh chị em trong gia đình: chị Mai, anh Lưu, em Công, em Thành, em Đàm, chị Hương, anh Bình, chị Ngọc, chị Hải, anh Tính, các cháu và những người đã luôn giúp đỡ và động viên.

Lai Thi Lan Huong
30 May 2012
Wageningen, the Netherlands.

ABOUT THE AUTHOR

Lai Thi Lan Huong was born on February 13, 1977 in Trau Quy, Gia Lam, Hanoi, VietNam. She spent 12 years on her junior and senior education in her hometown. In 1995, she went to Hanoi University of Agriculture in Hanoi (HUA) to study Veterinary Medicine. After graduating with a Veterinarian in 2000, she continued to study her Master degree in Hanoi University of Agriculture in 2001. She received her MSc diploma in 2004. In 2008, she was given the opportunity to work as a PhD student in the Animal Sciences Group of Wageningen University and Research Centre (WUR) in the Netherlands. The topic of her research was "Effects of air quality on chicken health". In June 2012 she will go back to Hanoi University of Agriculture (HUA) in Vietnam and work at Animal Anatomy and Histology in Veterinary Medicine faculty of Hanoi University of Agriculture.

TRAINING AND SUPERVISION PLAN

Training and Supervision Plan			Graduate School WIAS
Name PhD student	Lai Thi Lan Huong		
Project title	Effects of air quality on chicken health		
Group	Animal Sciences Group		
Daily supervisor(s)	Dr. Ir. André Aarnink & Dr. Ir. Henk Parmentier		
Supervisor(s)	Prof. Bas Kemp		
Project term	From Apr. 2008	Until Apr. 2012	
Submitted	Jan. 2012	Certificate	

EDUCATION AND TRAINING		
The Basic Package	year	credits *
WIAS Introduction Course <Sept 23-26>	2008	
Course on philosophy of science and/or ethics, Wageningen, April ethics "Biology underpinning animal sciences: Broaden your Horizon" <Mar. Apr>	2009	
Introduction interview with WIAS scientific director and secretary: <May>	2008	
Introduction interview with WIAS education coordinator: <May 16>	2008	
Introduction interview with WIAS PhD students confidant: <May 16>	2008	
Subtotal Basic Package		**3**
Scientific Exposure (conferences, seminars and presentations)		
International conferences		
< full name of conference, place, start and end dates >		
Conference of immunology, "the genetic bases of resistance and immunity of avian diseases <1 oral >, Arkansas, USA, Oct 2-5	2009	
Avian Immunology research group meeting <1 poster>, Budapest, Hungary, Oct 7-10	2010	
Animal Hygiene and Sustainable Livestock Production <1 poster>, Vienna, Austria, Jul 3-7	2011	
Seminars and workshops		
< full name of seminar / workshop, place, date >		
WIAS science day, Wageningen, The Netherlands, Mar 9	2009	
Low frequency electromagnetic field exposure and modulation of cellular functions - WIAS seminar	2010	
It makes sense to know your enemy, WIAS seminar	2010	
Resilience of fish in intensive system, WIAS seminar	2010	
Sole culture in the Netherlands, past, present and future, WIAS seminar Nov 5	2010	
Airborne transmission of pathogens in livestock production: its impact on human and animals Apr 24	2011	
Healthy as sport hourse Wageningen, The Netherlands, May 26	2011	
5th Workshop on Fundamental Physiology and Perinatal Development in Poultry, Wageningen, The Netherlands	2011	
Learning how to eat like a pig, Wageningen, The Netherlands, Sep 22	2011	
Writing a PhD thesis, Wageningen, The Netherlands, Oct17-19	2011	
WIAS science day, Wageningen, The Netherlands, Feb 2	2012	
Presentations		
< title of presentation, place, date, oral / poster >		
WIAS science day, Wageningen, The Netherlands, Mar 13 <1 oral>	2008	
Conference of immunology, Oct 2009 "the genetic bases of resistance and immunity of avian disease <oral presentation>, Arkansas, USA, Oct 2-5	2009	
Avian Immunology group meeting, <poster presentation>, Budapest, Hungary, Oct 7-10	2010	
Animal Hygiene and Sustainable Livestock Production, <poster presentation>, Vienna, Austria, Jul 3-7	2011	
WIAS science day <poster presentation>, Wageningen, The Netherlands, Feb 2	2012	

Subtotal Scientific Exposure		**11.20**
In-Depth Studies		
Advanced Immunology course University Medical Center Utrecht, Utrecht, Jan 3-7	2011	
Advanced visualisation, integration and biological interpretation of -omics data, Wageningen, Nov 7-9	2011	
Epigenesis and epigenetics, perinatal nutritional and physiological consequenses, Wageningen, Nov 21-23	2011	
Advanced statistics courses		
Statistics for the Sciences, Wageningen, May 28 - Jun 4	2008	
WIAS Advanced Statistics Course: Design of Animal Experiments, Wageningen, Dec 8-10	2010	
Statistics for the Life Sciences, Wageningen, May 30, 31 and Jun 1, 4, 5	2007	
MSc level courses		
Subtotal In-Depth Studies		**6.10**
Statutory Courses		
International course on laboratory animal science, Utrecht University, Utrecht, Sep 8-19	2009	
Laboratory Use of Isotopes (mandatory when working with radio isotopes)		
		3.00
Professional Skills Support Courses		
Information Literacy including introduction Endnote, Wageningen, Nov 27-28	2008	
Project and time management,Wageningen, Mar 9-20 April	2010	
Techniques for writing and presenting a scientific paper, Wageningen, Aug 31-Sep 3	2010	
Interpersonal PhD communication, Wageningen, Oct 26-27	2010	
Writting for academic publication, Wageningen, Sep 9- Nov 25	2011	
Subtotal Professional Skills Support Courses		**6.90**
Research Skills Training		
Preparing own PhD research proposal	2006	
Subtotal Research Skills Training		**6**
Didactic Skills Training		
Supervising practicals and excursions		
Supervising theses		
Subtotal Didactic Skills Training		
Management Skills Training (optional)		
Organisation of seminars and courses		
Organisation of WIAS Science day, Wageningen, Feb 2	2012	
		2.0
Education and Training Total		**38.20**

* one ECTS credit equals a study load of approximately 28 hours