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Investigation of the humoral and cellular immune responses of chickens to Salmonella typhimurium live vaccine

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Contents

LIST OF ABBREVIATIONS

Abs	Antibodies
Ag	Antigen
APC	Antigen Pressenting cells
BSA	Bovine serum albumin
C.T.	Ceacal Tonsil
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
ELISA	Enzyme–linked immunosorbant assay
FACS	Fluorescence activated cell scanner
FAE	Follicle associsted epithelium
FCS	Fetal calf serum
GALT	Gut associated lymphoid tissue
1.B.I	First booster immunization
IgG, IgA, IgM	Immunglobulin class G, A and M
IL	Interleukin
LPS	Lypopolysaccharide
M.C	Mucosal cell
MHC	Major histocompatability complex
M.O	Microorganism
MV	Mean value
NK	Natural killer cell
PBS	Phosphate puffer saline
POD	Peroxidase
PP	Payer's patches
SD	Standard deviation
sIgA	Secretory immunoglobulin A
S.t.	Salmnella typhimurium
TCR	T cell receptor
TLRs	Toll like preceptor
TNF	Tumor necrosis factor

1 INTRODUCTION

Salmonella infections are still a serious health hazard worldwide, affecting both humans and animals. Infections with Salmonella cause a variety of acute and chronic diseases in poultry and significant economical problems. Moreover, infected birds comprise one of most important reservoirs of Salmonella that can be transmitted through the food chain to humans. Isolation of Salmonella is reported more often from poultry and poultry products than from any other animal species. This probably reflects the high prevalence of Salmonella infections in poultry.

Salmonella parasites may be divided into three categories on the basis of the diseases caused, their host species range and invasiveness. The first group comprises the highly pathogenic host adapted Salmonella strains S. gallinarum and S. pullorum which cause fowl typhoid and pullorum disease seen in poultry flocks worldwide. The second group includes the invasive serotypes of Salmonella, most importantly S. enteritidis and S. typhimurium. The third group is called the noninvasive Salmonella, which do not usually cause illness in birds or humans.

Group one Salmonella species are not prevalent in Germany and of minor concern to the German poultry industry. In contrast, the invasive Salmonella species are of significant interest since they are still widespread and can provoke diseases in poultry flocks. Thr role of poultry as a principal source of Salmonella infections in human has been acknowledged previously. Edwards et al. (1981) reported that poultry products play an important role in human Salmonellosis as domestic poultry constitute the largest single reservoir of Salmonella through contaminated eggshell and poultry carcasses, which are contaminated at slaughter. However, with the exception of very young chicks, S. typhimurium or S. enteritidis rarely causes clinical diseases, but can colonize the gut of poultry as inapparent carriers and shed the Salmonella in the faeces that lead to horizontal transmission to other birds by the oral route and contamination of meat at the time of slaughter (BARROW et al., 1987 and 1990.)

The public health importance of Salmonella strongly argues for the need for control measures for Salmonella in poultry to prevent these organism from entering the food chain. Efforts on the national and European level include improved hygiene and

husbandry conditions on the farm, the selection of more resistant birds and prevention of infection by vaccination programs (BERNDT and METHNER, 2001). Vaccination against Salmonella is implemented by law in Germany and applied to control S. typhimurium and S. enteritidis infection in many countries throughout Europa.

Previous work has shown, that immunization with live attenuated Salmonella vaccine confers much better protection than vaccination with killed bacteria (KANTELE, et al. (1991). This superior efficacy of live over killed vaccines is believed to be due to the ability of live vaccine to elicit cell mediated immunity in addition to an antibody response. The nature of this cellular response remains controversial, in particular the identity of antigen involved. Evidence has been presented for both protein and carbohydrate determinants including lypopolysaccaride (LPS) antigen, which have been involved in the cell mediated response. LEE et al., (1983) found that the clearance of Salmonella typhimurium in chickens correlated with cell mediated immunity rather than the humoral immune response.

The aim of this study was to obtain new insights into the humoral and cellular immune responses to vaccination with live attenuated S. typhimurium.

2 REVIEW OF LITERATURE

The interaction of Salmonella parasites with their host and the resulting immune responses have been studied most intensively in mice. This is mainly due to the availability of several inbred strains and genetically modified animals which enable the characterization of immune effector mechanisms. Therefore, this review of the literature will first focus on the knowledge obtained from mouse studies and subsequently discuss the literature on host-pathogen interaction in the chicken.

2.1 The interaction of S. typhimurium with the immune system of mice

The infection of mice with S. typhimurium, which causes murine typhoid fever, is one of the best characterized disease models and was established for two reasons. First, the murine pathogen replicates in the host and causes a systemic infection, which can readily be established by inoculation of small numbers of bacteria. As a consequence, the host responses can be studied in detail in a situation, in which no septic shock is induced (HSU.et.al., 1989). In addition, mouse strains are available which show resistance and susceptibility to parasite infection. These strains have been used extensively and have lead to the identification of important cytokines in the defence to S. typhimurium. This includes proinflammatory cytokines such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (LANGERMANS et al., 1994).

Salmonella species are enteroinvasive, facultative intracellular bacteria, that are initially acquired through the ingestion of contaminated food or water. These bacteria cross the epithelial barrier at the level of the ileum or colon by invading M cells, which routinely sample luminal antigen for transport to nearby antigen presenting cells. M cells are located in the follicle-associated epithelium (FAE), which covers organized lymphoid tissue in the gut, such as the payer's patches (PP).

The mechanism by which Salmonella species adhere to the epithelium is not well understood. HULTGERN et al. (1993) discussed, that the colonization or invasion of gastrointestinal mucosal surfaces by enteric pathogens often involves microbial lectin-like adhesins that recognize specific glycoconjugates on intestinal epithelial cells. In the mammalian intestine, carbohydrate structures on the epithelial cell surfaces vary among

species and among different cell types and even among enterocytes on a single villous (FALK et al., 1994 and MAIURI et al., 1993). There is some evidence that the selectivity of microbial adhesins for specific epithelial surface carbohydrate structures may determine the patterns of susceptibility of certain species and individuals to Salmonella infec

tion and the regional distribution of infection within the host gastrointestinal tract. The epithelial cell molecules that serve as receptors are largely unknown (BOREN et al., 1993 and LINDSTEDT et al., 1991).

Genetic studies of S. typhimurium indicate complex processes of adherence and cell invasion. Fimbria, which are the subject of intensive genetic and functional studies, are thought to play a role in the initial adherence of S. typhimurium to M cells of FAE (MULLER et al, 1991), and may also adhere to absorptive enterocytes. Relatively little is known about the composition of the M cell membrane, however, the initial interaction of S. typhimurium with an epithelial cell induces profound changes in the apical cell surface (FRANCIS et al., 1990 and TAKEUCHI et al., 1966). It has been suggested that these changes could facilitate adherence and invasion of the organisms on the respective cell, which could explain in part why different Salmonella species tend to infect certain target epithelial cells, while leaving other cells untouched. (FRANCIS et al., 1990).

Murine Salmonellosis is a multi-stage infection, in which the intestinal infection leads to a transient tissue phase and bacteraemia, followed by several days of intracellular growth of bacteria in the macrophages of liver and spleen. Subsequently, large numbers of bacteria are liberated into the blood, leading to toxic effects (MACKANESS et al., 1966). Protection against Salmonella can be measured as the reduction of the growth rate of the bacteria in the liver (HOBSON et al., 1957).0

The structure of S. typhimurium has been studied intensively to understand the molecular mechanisms which elicit an immune response directed against bacteria. LPS, also known as endotoxin, is the major surface molecule and pathogenic factor of gram negative bacteria, and one of the best studied microbial products that potently activate the innate immune response. LPS consists of three distinct structural regions: the O-antigen, the core and lipid A. The O-antigen and the core consist of polysaccharide

chains, whereas lipid A comprises the fatty acid and phosphate substituents (ULEVITCH et al, 1995). The host receptors for LPS have been characterized only Recently, and this discovery has significantly influenced our current concept of host-pathogen-interaction.

The immune system in vertebrates consists of two related components: the innate and the adaptive immune system, which function together to protect the body against infections (MEDZHITOV et al., 2000). Because of the adaptive immune response being a time-consuming process, requiring weeks to be fully developed, infections must be initially held in check by the innate immune system, which is the first line of defence against microbial pathogens. The innate immune system slows the rate of multiplication of the pathogens to allow time for the more specific adaptive immune system to be activated (JANEWAY et al., 2002 and NAGAI et al., 2002).To achieve this, several components of the innate immune system are necessary, including the complement system, lysosoms, lactoferrin and an array of antimicrobial peptides, which have antimicrobial activity. In addition, effector cells such as neutrophils, macrophages and natural killer (NK) cells play critical roles in pathogen detection and elimination (FIERER et al., 2001). During bacterial infection, Salmonella encounters an extensive network of leukocytes in the lamina propria of the intestine. Macrophages are amoung the first cells encountered by Salmonella after successful invasion of the gut tissue. LPS released from the bacterial surface first binds to LPS binding protein (LBP) to form an LPS-LBP complex, which associates with a membrane protein complex on macrophages. This includes the CD14 molecule and the recently identified LPS-receptor Toll-like-receptor 4 (TLR-4). TLR-4 signals the presence of LPS to the macrophage cytosol and activates these cells (QURESHI et al., 1999). These signals ultimately help to initiate the innate and specific immune response to clear bacterial infections (SUFFREDINI et al., 1999).

Instead of being destroyed by phagocytes upon engulfment, Salmonella have developed several mechanisms to survive in phagosomal compartments (GALLOIS et al., 2001). This phagosome can be cytotoxic to macrophages by inducing apoptosis, as has been shown in vitro (GUICHON et al., 2001). The ability of S. typhimurium to survive and replicate within macrophages in a manner unlike to other intracellular bacteria is an essential prerequisite for virulence of Salmonella (FIELDS et al., 1986). This limits the effectiveness of the antibodies and neutrophil-mediated damage and thus

allows the pathogen to disseminate systemically from the mucosal site (ELHOFY et al., 1999).

Significant progress has been made toward understanding how pathogenic bacteria promote their survival within the host through the regulated expression of bacterial virulence genes (ROSENBERG et al., 2000). The organisms that successfully survive in the macrophages vary in their approach with which they deal with the intracellular environment as they combat nutrient limitation, fusion with lysosoms and phagosome acidification. (DENSIN et al., 1990). Vacuoles containing live S. typhimurium delay and attenuate phagosomal acidification, in contrast to heat killed organisms. The rate of acidification depends on the accumulation of the proton-ATPases, which reflects the volume of the phagosome. Large vacuoles as in the case of those phagosomes containing live S. typhimurium require more protons and more time to acidify, and this explains their lower grade of acidification. In addition to that, a low pH serves as an intracellular signal that plays an integral part in transcriptional regulation of genes, whose products are essential for intramacrophage survival of the bacteria. (RATHMAN et al., 1996)

In addition to the resident tissue macrophages, neutrophils play a critical role in the restriction of microbial replication and spread of bacteria early after pathogen entry. These cells are the most efficient phagocytes of the immune system and the first cells attracted to the site of infection (JONES et al., 1999). In this context, neutrophils exhibit a dual function. First, they phagocytose the pathogen efficiently and exhibit potent microbicidal activities mediated by granular enzymes, anti-microbial peptides and reactive intermediates of both oxygen and nitrogen (ELSBACH et al., 1985 and DEVI et al., 1995). The uptake and degradation of bacteria is facilitated by antibody-mediated phagocytosis and opsonisation of bacteria by complement components. (WARREN et al., 2002). Secondly, neutrophils release an array of cytokines and chemokines and attract other cells of the innate as well as the adaptive immune system. Therefore, neutrophils not only contribute to immediate pathogen restriction, but also focus the specific immune response to the site of infections, which ultimately achieve control of pathogens (ROSENBERG et al., 1999 and GALLIN et al., 1985). Comparisons with infections caused by other intracellular bacteria have confirmed that neutrophils are critical for the control of fast-replicating intracellular bacteria such as Listeria monocytogenes and S. typhimurium (CONLAN et al., 1997 and ROGERS et al., 1993),

while the control of slow -replicating bacteria such as M. tuberculosis and M. bovis is largely neutrophil independent (SEILER et al., 2000). An additional effector mechanism proposed for neutrophils is an early lytic engagement with phagocytosis of the hepatocytes, before pathogens can grow to large numbers inside highly permissive host cells. This, neutrophil combat early hepatosplenic infection and also produce several macrophage-activating cytokines including TNF-α and IFN-γ (CONLAN et al , 1994).

Dendritic cells (DCs) are the most potent APCs for the activation of naive T cells and are a crucial link between peripheral sites and secondary lymphoid organs. Moreover, they provide a key link between the innate and adaptive immune system (BANCHEREAU et al., 2000 and 1998). As discussed before, the penetration of the gut mucosa by pathogens expressing invasion genes is believed to occur mainly through specialized epithelial M cells. However, S. typhimurium bacteria which are deficient in invasion genes encoded by Salmonella pathogencity island 1 (spI1) are still able to reach the spleen after oral administration. This suggests the existence of an alternative route for bacterial invasion which is independent of M cells. RESCIGNO et al. (2001) have reported a new mechanism for bacterial uptake into mucosal tissues that is mediated by DCs. DCs that are located in the sub-epithelial dome of payer's patches as immature cells can penetrate the intestinal epithelial cells to sample bacteria. These DCs subsequently act as vehicles for the dissemination of Salmonella parasites (CHEMINAY et al., 2002). Immature DCs are characterized by a high capacity for antigen uptake but are rather poor at activating T-cells. Inflammatory stimuli and exposure to infectious agents trigger an irreversible differentiation into mature DCs. During maturation the endocytosis activity is lost and antigens are processed and presented by both MHC-class I and MHC-class II molecules at the cell surface. In addition, cytokines are released and co stimulatory molecules (CD, CD86 and CD40) are up-regulated to activate the adaptive immune response. (SALLUSTO et al., 1995 and CELLA et al, 1996).

SCHWACHA et al. (1998) observed that NK cells as a part of the innate immune system are important in the initiation of an immune response. The cytotoxic activity of NK cells is enhanced early after Salmonella infection and returns to basal levels 7 days after infection (SHAFER et al., 1992). NK cells are critical for T-lymphocyte-independent macrophage activation through the production of IFN-γ (SCHWACHA et al., 1998). NK cell derived IFN-γ activates bactericidal mechanisms in macrophages as well as the

release of proinflammatory cytokines such as IL-1, IL-6, IL-12 and IL-18. (SCHWACHA et al., 1998).

Bacterial products (e.g. LPS) and host components stimulate the immune response and may lead to a systemic production of proinflammatory cytokines and chemokines which help to recruit and activate immune cells to eliminate invading pathogens (DUNN et al., 1991). Although these cytokines are indispensable for efficient control of the growth and dissemination of pathogen, an excessive inflammatory response is potentially auto-destructive and may lead to microcirculatory dysfunctions causing tissue damage, septic shock and eventually death (SHAFER et al., 1992). Among these soluble factors is IL-12, which plays a crucial role in the development of cell-mediated immunity. This cytokine is produced by macrophages and dendritic cells in response to bacteria and parasites or bacterial components such as LPS (CHONG et al., 1996 and TRINCHIERI et al., 1995). It is composed of two subunits, designated p35 and p40. IL-12 promotes the development of Th1 cells, which secrete IFN-γ in response to antigen specific activation. TH1 cells act back through IFN-γ secretion to activate macrophages early in an immune response to the stimulating antigen (MUNDER et al., 1998), thereby limiting the replication and dissemination of S. typhimurium.

IL-18 was recently added to the list of relevant regulatory cytokines in several infection models. It is produced by activated macrophages and functions synergistically with IL-12 to induce the production of IFN-γ by T cells. (TAKEDA et al., 1998).

As already outlined, the clearance of facultative intracellular pathogens such as Salmonella species requires IFN-γ secreted from $CD4^+$ T cells or NK cells. The precise mechanisms linking the recognition of intracellular pathogens with the induction of IFN-γ producing T cells are still poorly understood (JOHN et al., 2002). IFN-γ is an obvious candidate as the mediator of macrophage activation, as IFN-γ potently enhances bacterial killing by these cells. In the early phase of an infection, IFN-γ leads to bactericidal responses, but in later stages bacteriostasis rather than bacterial killing was observed. A possible explanation for these contrasting effects at different times could be that changes have taken place in the bacteria. In vitro the early phase bacteria are easily killed by IFN-γ As Salmonella parasites develop in the macrophages in these in vitro systems, bacterial genes necessary for intracellular growth are switched on, making the bacteria less susceptible to IFN-γ killing, thus leading to bacteriostasis. An additional mechanism has been proposed, that macrophages operating at different

times are functionally different subtypes, with respect to their ability to kill intracellular bacteria (MUOTIALA et al., 1990).

The initiation of an antigen specific immune response by the adaptive immune system is a highly organized process. It involves antigen processing and presentation at the right location and at the appropriate times by specialized cells called antigen presenting cells (APC) such as macrophages, DCs and B cells (WICK et al., 1994).
In addition to their described role as an important cell in the first line defence against bacterial infections, macrophages have the capacity for antigen processing and presentation (HARDING 1993, UNANUE 1984). Macrophages degrade antigen into peptides that bind to major histocompatibility complex class II molecules (MHCII), and thereby present the peptides on the cell surface for recognition by T lymphocytes (HARDING 1993). Recognition of peptide: MHCII complexes activates T cell-mediated immunity, especially $CD4^+$ T helper cells, that induce and regulate humoral and cellular immune responses. $CD4^+$ cells, polarized by cytokines such as IL-12 into Th1 cells, secrete IFN-γ, which activates macrophages and thereby activates the effector mechanisms leading to pathogen destruction and elimination. Although the Th2 cells are classical helper cells and also provide the signals to B cells, but their role for antibody production in Salmonella specific immune responses is less clear, in particular since these cells are believed to be primarily associated with the clearance of extracellular pathogens (MITTRÜKER et al., 1993 and SCOTT et al., 1991).

The activation of $CD8^+$ MHC class I restricted cytotoxic T cells (CTLs) requires the cytoplasmic localization of foreign proteins for processing and subsequent presentation of peptides via MHC class I molecules (UNANUE et al., 1984). There is evidence suggesting that $CD8^+$ T cells participate in immunity against S. typhimurium, but the mechanism involved is poorly understood (WICK et al., 1994 and HARDING et al., 1993). TURNER et al. (1993) suggested that the induction of CTLs by S. typhimurium may result from the escape of the bacteria from the phagosome into the cytosol, and its subsequent presentation through the MHC class I pathway.

2.2 The immune response of chicken to S. typhimurium

The aim of vaccination of chickens with S. typhimurium vaccines is to increase the resistance of birds to Salmonella infection by activation of the specific immune

response. Primary goals are to reduce intestinal colonization and to prevent the dissemination of bacteria, in particular to the reproductive tract. Those Salmonella serotypes, which cause food poisoning in man, colonize the intestinal tract of birds, but rarely cause systemic disease unless, very young chicks which are infected with high doses of highly pathogenic strains, which may lead to gastroenteritis and intestinal lesions (KAISER et al., 2000). SMITH et al. (1980) and BARROW et al. (1988) have demonstrated that the main site of colonization within the intestine of chickens is the caecum and this may be due to the anatomical and structural location which allows the caecum to act as a blind sac with low content flow rate. Thus, some infected chickens become carriers of Salmonella for longer periods. These carrier chickens serve as nuclei for the proliferation of Salmonella within farms and for massive cross infection during transportation, which ultimately leads to a high carcass contamination rate in poultry processing plants.

Several approaches are taken to control Salmonella infection in commercial chicken farms. Vaccination has been implemented by law in Germany and is now widely used in EU member states to control S. typhimurium and S. enteritidis. Priority is given to live attenuated vaccines even though inactivated vaccines are on the market. Oral immunization of chickens with S. typhimurium has been shown to be effective (KANTELE et al., 1991), despite our limited knowledge of the immune effector mechanism. It is largely known, that the cell mediated immune response plays the major role in the chicken, an assumption that is derived from studies in the murine model. However, it is also widely accepted that a major goal of any immunization strategy is the induction of protective immunity at the site of mucosal entry of the pathogen (MCGHEE et al., 1992).

Rather little is known about the molecular mechanisms of Salmonella entry in the chicken gut, though the basic mechanisms of pathogenesis of S. typhimurium appear to be similar to a large extent to those in mammals. Invasion of S. typhimurium into the intestinal mucosa causes rapid inflammation and infiltrations of large numbers of heterophils, the avian equivalent of mammalian neutrophils, followed by attraction of macrophages. This influx of inflammatory cells in response to S. typhimurium results in intestinal lesions, which is in contrast to infections with host adapted S. pullorum. S. pullorum infection is not followed by rapid inflammation, and only small numbers of heterophils are found in association with the intestinal epithelium (KAISER et al., 2000). Clearly, a better understanding of how Salmonella interacts with the chicken host is

needed to understand the disease and colonization processes both in terms of animal and public health issues.

As known from the mouse models, attenuated S. typhimurium vaccines combine the advantages of inducing a strong mucosal, humoral and cell mediated immunity. These vaccines target the main organ of bacterial replication and invasion, the gut mucosa. Moreover, several attenuated vaccine strains of S. typhimurium with different properties exist, potentially allowing the optimisation of vaccination protocols.

The immune effector mechanisms elicited by S. typhimurium vaccines in the chicken have not been studied in great detail. Most work has focused on the induction of IgG type Salmonella specific antibodies, primarily because of the current lack of methods to measure cell mediated immunity in the chicken. Recently, progress was made in the field of chicken cytokine and chemokine research, and this knowledge was applied to better understand Salmonella infection. WITHANAGE et al. (2004) showed that interleukin 8 (IL-8), K60 (a CXC chemokine), macrophage inflammatory protein 1 beta and IL-1 beta levels were significantly up-regulated in the intestinal tissues and in the liver of infected birds. However, the spleen of infected birds shows little or no changes in the expression levels of these cytokines and chemokines. Importantly, increased expression of the proinflammatory cytokines and chemokines (up to several hundred-fold) correlated with the presence of inflammatory signs in those tissues and with the massive influx of heterophils. In addition, cytokine expression by heterophils correlates with the resistance and susceptibility of different chicken lines (SWAGGERTY et al., 2004). Since this study focused on the early stages of a S. typhimurium infection, no data have been reported on the induction of cytokine patterns during vaccination and anamnestic immune response. As a consequence, the role of T cells and T cell subsets in the immune response of chickens to S. typhimurium is not known (HASSAN et al., 1990 and BRITO et al., 1993). However, the availability of monoclonal antibodies recognizing avian T cell-associated antigens should facilitate the study of the cellular immune response of chickens. This is demonstrated by numerous studies characterizing the development of chicken T cells (CHEN et al., 1994) and the cytokines secreted in response to stimulation by mitogenes (STAEHELI et al., 2001).

One of the very few studies on T cell responses in Salmonella infection was published by BERNDT et al. (2001). The authors provided convincing evidence that $CD8^+$ TCR-1 cells may play a significant role during the early infection. They showed, that the proportions of TCR-1 cells significantly increased in the peripheral blood and spleen

after infection and immunization. Furthermore, the proportion of conventional $CD8^+$ α/β T cells was reduced. This finding is in agreement with previous observations from ARSTILA (1996). Detailed studies in mice have clearly demonstrated that γ/δ T cells are important cells during the early stages of a Salmonella infection (HIROMATSU et al., 1992). Interestingly, significant differences exist between species with regard to the frequency of γ/δ T cells. Chickens are known to have a larger γ/δ T cell population (up to 30% in blood) in comparison to mice and humans (about 5%) (COOPER et al., 1991; HEIN and MACKAY, 1991). Data supporting a role of γ/δ T cells in the adaptive immune response have been published (KASAHARA et al., 1993), and it was suggested that these cells may possess cytotoxic activity (SHARMA, 1997).

A major problem in avain immunology research is the lack of technologies to measure antigen specific T cell responses. Therefore, no data are available showing a specific response of α/β T cells to defined Salmonella antigens.

The local intestinal humoral immune response has been shown to be one of the major contributors to protection against enteric bacterial disease. Among the most important protective humoral immune factors at mucosal surfaces, where, most pathogens enter the host, are locally produced antibodies of the secretory IgA (sIgA) isotype, which act as the first line of defence against invading pathogen. This isotype accounts for more than 80 % of all antibodies produced in mucosal associated tissues (McGHEE et al., 1989). The sIgA is induced, transported and regulated by mechanisms that are remarkably distinct from those involved in systemic antibody response. In the gut antigen is frequently taken up into the gut associated lymphoid tissue (GALT), which is collectively represented by payer's patches (PP) and caecal tonsils. The PP contain dome regions enriched with lymphocytes, macrophages and some plasma cells. This dome area is covered by micro-fold (M) cells, which are specialized in the uptake and transport of luminal antigens in the follicles of the underlying lymphoid tissue, which contain germinal centers. Here, B cells switch to produce IgA and subsequently migrate to the lamina propria to secret this immunoglobulin isotype. IgA antibodies have been shown to inhibit microbial adherence and thereby prevent the absorption of antigens from mucosal surfaces. Interestingly, this inhibitory activity is not necessarily associated with an antigen specific antibody activity. It was shown, that the terminal mannose-containing oligosaccharide side chains on the heavy chains of the IgA_2 molecules of mice are recognized by mannose–specific lectins present on type I fimbrie of Salmonella. It was suggested that these carbohydrate- specific interactions represent

an important protective function of sIgA against an abroad spectrum of intestinal bacteria (MCGHEE et al., 1992).

In order to be successful, mucosal vaccines have to be evaluated for their ability to stimulate the local secretory immunoglobulin system at mucosal surfaces (Wendy, I et al 1998). The effectiveness of secretory IgA in protecting the mucosa from bacteria by way of immune exclusion in avian species was first described in a Salmonella model (PARRY et al., 1977; PARRY and PORTER, 1981) These authors showed that the onset of intestinal invasion is delayed in vaccinated birds compared with control birds. However, the immunization reduced but did not prevent intestinal colonization with S. typhimurium.

The aim of this work was to get further insights into the early and anamnestic immune responses of the chicken to S. typhimurium vaccination. In particular, the response of lymphocyte subsets in the spleen, their functional status and the induction of the IgA system were the focus of this work.

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Experimental birds

Specific pathogen free Valo eggs were obtained from “Lohman Tierzucht, Cuxhaven”, which were incubated and hatched in the Institute for Animal Physiology. The birds were kept in small groups and received commercial layer feed and water ad libidum.
This study was carried on 300 birds.

3.1.2 Cell culture media and reagents

445 ml RPMI 1640[1]
50 ml fetal calf serum (FCS)[3], inactivated at 56°C for 30 min.
5 ml penicillin-streptomycin solution[2] (100 IU/ml penicillin and 100 µg/ml streptomycin)
Ficoll – Paque[2]
Trypanblue[2]

3.1.3 Buffers and solutions

If not stated otherwise all chemicals were obtained from Merck, Darmstadt.

3.1.3.1 Enzyme linked immunosorbent assay (ELISA)

LPS solution
LPS from S. typhimurium (Sigma L6511) 1 mg /ml[4] was dissolved in PBS to obtain a final concentration of 10 µg /ml.

Skim milk powder solution 4%
4 g skim milk milk powder[1]were dissolved in 100 ml PBS

Phosphate buffered saline (PBS) pH 7.2
8,0 g NaCl[1]
0,2 g KCl[1]

1,4 g Na_2HPO_4 x $2H_2O$[1]

0,2 g KH_2PO_4[1]

Ad 1000 ml distilled water

PBS-T (0.05 % Tween 20)

0,5 ml Tween-201

Ad 1000 ml PBS

Tetramethylbenzidin solution

6 mg 3,3`, 5,5`-Tertramethylbenzidin (TMB)[6]

Ad 1 ml Dimethylsulfoxid (DMSO)[1]

TMB-Buffer

8,2 g Na-Acetate (CH^3COONa)[6]

3,15 g Citric acid – Monohydrat ($C_6H_8O_7xH_2O$)[6]

ad 1000 ml distilled water

TMB working solution

323-µl TMB solutions

10 ml TMB buffer 37°C

3,0 µl 30% H_2O_2[1]

Sulfuric acid (H_2SO_4), 1 M

472 ml distilled water

8 ml H_2SO_4 96%[6]

3.1.3.2 Immunofluorescence staining (Flow cytometry)

Fluorescence buffer

1 g Bovine Serum Albumin (BSA)[1]

0,05 g NaN_3[6]

ad 100 ml PBS

FACS buffer

100 mg NaN_3

ad 1000 ml PBS

Paraformaldehyd (PFA) 4 %

20 g Paraformaldehyd[6]

ad 450 ml distilled water

3.1.3.3 Immunohistochemical staining

Aceton [1]

Buffers

PBS 1 % BSA

0,1 M Tris buffer pH 8,2

Blocking solution

1ml PBS 1 % BS A + 15 µl serum of the species from which the secondary antibodies was provided

Antibodies

First antibodies (IgA: clone A-1, IgM: clone M-1;[7])

Second antibody (goat-anti-mouse immunoglobulin, dilution 1:100, containing 25% chicken serum)[8]

Enzyme

Peroxidase anti peroxidase compex (Sigma; dilution 1:300)

Substrate

3,3'- diaminobenzidine [9]

Mayer,s Haematoxylin [5]

1g Haematoxylin dissolved in 1 litter distilled water

Mounting

Canada balsam [10]

3.1.4 Monoclonal antibodies (mAb)

mAb	Specificity	Citation
CT4	CD4	Kindly provided by Dr.Chen-LoChen, University of Alabama, USA
CT8	CD8	Kindly provided by Dr.Chen-LoChen, University of Alabama, USA
TCR-1	TCR γ/δ	Kindly provided by Dr.Chen-LoChen, University of Alabama, USA
TCR-2	TCR α/ Vβ1	Kindly provided by Dr. Josef Cihak, University of Munich, Germany
TCR-3	TCR α/Vβ2	Kindly provided by Dr.Chen-LoChen, University of Alabama, USA
A1	IgA	ERHARD et al., 1992
L-chain	L-Chain	ERHARD et al., 1992
M1	IgM	ERHARD et al., 1992
G1	IgG	ERHARD et al., 1992
Anti-Mouse-IgG-FITC	IgG	Sigma Deisenhofer
IgA- POD	IgA	ERHARD et al., 1992

3.1.5 Bacterial Vaccine

Salmonella typhimurium live vaccine was obtained from Lohman Animal Health, Cuxhaven. The vaccine was diluted in 10 ml PBS to give a final concentration of 1x 10^{10} CFU / ml. This suspension was further diluted in PBS to obtain the final concentration of 2 x 10^{8} CFU / ml. 0.5 ml of this suspension was given into the crop using a blunt end buttom-hole canula.

3.2 METHODS

3.2.1 Detection of S. typhimurium specific IgA antibody titers by ELISA

Groups of chicken were vaccinated orally with Salmonella typhimurium live vaccine at dose of 2 x 10^8 / ml CFU by at the age of 1 day and/or at 6 weeks of age as the first booster immunization. Experimental details are described with the respective experiments.

3.2.1.1 Sample collection

Preparation of serum

Blood from both vaccinated and control birds was collected from the wing vein at different time points. The blood was collected into 2 ml Eppendorf cups and incubated for 1 hour at 37°C and subsequently centrifuged at 5000 x g for 10 minutes. The serum was collected and stored at –20°C until used.

Preparation of bile

The gall bladder was collected into 2 ml Eppendorf cups, opened with a pair of scissors and diluted in 1 ml PBS. This preparation was centrifuged at 14.000 x g for 10 minutes. The supernatant was collected and stored at –20°C until used in the ELISA.

3.2.1.2 ELISA procedure

Coating 96 -well ELISA plates (Nunc-Maxisorb Polystyren)[11] were coated with 100 µl LPS solution from S. typhimurium (10 µg/ml) per well, which was diluted in PBS and incubated over night at 4^0C. The plates were washed with PBS -T using an ELISA plate washer[12]

Blocking Skim milk powder was dissolved in PBS to a final concentration of 4 %, 200 µl were given in each well and the plates were incubated at 37°C for 1 hour. The plates were washed with PBS-T.

Samples Serum samples were two fold diluted in PBS. 100 µl of the dilutions were given to each well and plates were incubated for 1 h at 37°C. The plates

were washed with PBS-T.

Secondary Antibody Mouse-anti-chicken IgA peroxidase-conjugated (IgA-POD) was diluted in PBS-T, subsequently, 50 µl were added to each well and the plates were incubated at 37°C for 1 hour.

Substrate solutions TMB working solution was freshly prepared and 100µl were added to each well. Plates were incubated for 10 minutes in the dark at RT.

Stop reagent The reaction was stopped by the addition of 50 µl of 1 M H_2SO_4 solution.

3.2.3 Leucocytes isolation from spleen

The birds were killed and the organs (spleens and caecum) were removed aseptically from both vaccinated and control birds and placed into sterile ice cold PBS and kept on ice until further processed. To isolate the caecal tonsils (C.T), the caecum was opened longitudinally, the ceacal tonsils were dissected from the remaining tissue and put in 50 ml tubes which contained ice cold PBS. The C.T was cleaned from the intestinal content and mucus by shaking for 15 min. Spleen and caecal tonsil tissues were squeezed through a stainless steel sieve (66 mesh) and the resulting single cell suspension further separated by density gradient centrifugation. 10 ml of the cell suspension were layered on 10 ml of Ficoll-Paque in a 50 ml tube and centrifuged at 600x g for 12 min at RT. The interphase was collected and washed 2 times with cold PBS. Finally, the cell pellets were suspended in RPMI 1640 medium with 10 % FCS and cell counting was carried out by using Trypan blue. The cell concentration was adjusted to 5 x 10^6 cells / ml in medium with 10% FCS.

3.2.4 Staining the leucocytes for flow cytometric analysis (FACS)

Cell staining was performed in 96- well-round bottom plates. 5 x10^6 cells were given to each well and centrifuged at 716x g for 2 minutes. The resulting cell pellets were resuspended with in 50 µl of the respective monoclonal antibodies (CD4, CD8, TCR-1,

TCR-2, TCR- 3, L-chain, IgA, IgM, IgG) and incubated for 25 minutes on ice in a dark box. The cells were subsequently washed with 150-µl fluorescence buffer, centrifuged as described above. The cell pellets were resuspended with 35 µl of the working dilution of the secondary antibody (anti-Mouse–IgG-FITC)[5] conjugate (1:50 diluted in fluorescence buffer) and incubated for 20 minutes on ice and in a dark box. The cells were washed twice with 150-µl fluorescence buffers. For measurement, the cell pellets were resuspended with 300 µl fluorescence buffer and transfered into 5 ml tubes. Measurement was performed on a Fluorescence Activated Cell Scanner (FACScan, Becton Dickenson, Heidelberg) and the results were analysed by using Cell Quest Pro and Win MDI 2.8 programs. If the cells were not immediately measured, they were fixed in the tubes by the addition of 100 µl of a 4% PFA solution and stored at 4°C. Analysis was performed within 48 hours.

3.2.5 Lymphocyte proliferation Assays

96-well flat-bottomed cell cultur plates were coated with 100 µl of the mAb (TCR-1, TCR-2, F71D7) at a concentration of 10 µg / ml and incubated for 2 hours at 37°C. Plates were washed with sterile PBS and spleen cells from both control and vaccinated birds were prepared as described (chapter 3.2.2). 100 µl of the cell suspension containing 5 x 10^6 cells per ml were added to each well. Where indicated LPS or the lysates of S. typhimurium live vaccine were added to the cultures at a final concentration of 10 µg / ml instead of TCR coating. The plates were incubated for 72 hours at 37°C and pulsed 1 µCi ^{3}H-thymidine per well (10 µl of ^{3}H-thymidine per well) for another 16 hours. Cells were harvested on filters using a cell harvester and incorporation of radioactivity was determined on a Microplate Scintillation Counter (Canberra, Packard, Dreieich, Germany).

3.2.6 Immunohistochemical staining

IgA^+ and IgM^+ cells of spleens and caecal tonsils were stained immunohistochemically as described (Berndt and Methner, 2001). Briefly, frozen sections (7µm in thickness) were prepared and stored at –20°C until use. After actone-fixation, the tissue sections were sequentially incubated with the appropriate monoclonal antibodies (IgA: clone A-1, IgM: Clone M-1; Southern Biotechnology Associates, Eching, Germany), the secondary goat-anti mouse immunoglobulin (Sigma-Aldrich fine Chemicals, Taufkirchen, Germany;

dilution 1:100, containing 25 % chicken serum) and the peroxidase-antiperoxidase complex (Sigma; dilution 1:300). The enzyme-linked antibody was visualised by reaction with 3,3'-diaminobenzidine (DAB; Merk, Darmstadt, Germany; 1 mg/ml PBS) and hydrogen peroxide (0.02%) at room temperature for 10 min. For negative control, slides were incubated with normal mouse serum (dilution 1:500) instead of the primary monoclonal antibody. The sections were counter-stained with hematoxylin and mounted with Canada balsam (Riedel de Haen, Selze-Hannover, Germany)

Image analysis

Immunohistological tissue preparations were examined by light microscopic image analysis (analysis 3.0, soft Imaging System GmbH). All measurements were performed were performed at a 20 x magnification. Using the computer software, the region of interest for each tissue section was drawn as a rectangle on the screen. At least 3 reagions of interest for each tissue section and antibody were scanned, the percentages of antibody-stained areas determined and the mean value calculated.

3.2.7 Statistical analysis

The Student's *t*-test for the comparison of two independent samples was used for statistical evalution of differences between the groups.Values of $p \leq 0.05$ were considered as significant.

4 Results

4.1 Analysis of the lymphocytes response by flow cytometry

4.1.1 Splenic response one week after vaccination

In order to investigate the splenic immune response to S. typhimurium vaccination, birds were vaccinated with $1x10^8$ CFU live bacteria by oral application at the age of 1 day. Vaccines were applied in 0.5 ml PBS. The control birds received 0.5 ml PBS without antigen. The birds were killed one week after vaccination and spleens were removed and prepared as described (chapter 3.2.2.)

Spleen cells were stained with monoclonal antibodies to the CD4 and CD8 antigens and the α/vβ1, α/vβ2 and γ/δ Tcell receptor (TCR) as well as with an anti-L-chain antibody. The frequency of lymphocyte subsets was analysed by flow cytometry. Results were obtained from 5 birds per group.

In this trial birds clearly responded to vaccination with an increase in γ/δ -T cells. Thisdifference was statistically significant. In addition, the relative number of α/β -T cells were decreased in comparison with control birds. No significant differences were found for $CD4^+$ and $CD8^+$ T cells and B cells (table 1, annex 1, experiment 1).

	%CD4		%CD8		%TCR-1		%TCR-2		%TCR-3		%L-chain	
Control	24,0	6,1	22,6	3,8	8,2	2,1	35,1	7,7	14,3	6,7	17,2	6,7
Vaccinated	18,0	5,0	25,6	20,1	31,1**	9,4	22,0*	6,0	5,7	2,0	14,7	3,7

Table 1: Mean values and standard deviations of lymphocyte subsets in the spleens of control birds (n= 5) and vaccinated birds (n=5) in experiment 1. Birds were vaccinated at the age of 1 day with $1x10^8$ CFU of S. typhimurium live vaccine. Analysis was performed on day 7. Asterisk indicates mean for vaccinated chickens differs significantly from control value (*=p≤ 0.05, **=p≤ 0.01).

In order to confirm this observation, the experiment was repeated twice exactly in the same way. The birds were vaccinated orally with $1x10^8$ CFU live vaccine at the age of 1 day, control birds received 0.5 ml PBS. The birds were killed one week after vaccination, spleens were removed, prepared, stained and analysed by flow cytometry. The results are summarized in tables 2a and 2b and annex 1 (experiment 1).

Table 2a:

	%CD4		%CD8		%TCR-1		%TCR-2		%TCR-3		%L-chain	
Control	38,4	8,6	35,8	5,2	14,8	4,2	51,3	12,1	11,8	5,3	22,3	11,8
Vaccinated	27,5*	4,8	57,2**	9,7	37,5***	5,7	41,0	4,8	11,0	1,6	21,5	5,7

Table 2b:

	%CD4		%CD8		%TCR-1		%TCR-2		%TCR-3		%L-chain	
Control	32,4	2,6	28,0	0,6	14,2	3,9	39,2	6,1	8,6	1,9	19,9	6,1
Vaccinated	28,7*	1,1	49,5**	4,4	37,8***	5,2	39,2	5,7	8,9	1,5	14,3	3,2

Table 2: Mean values (MV) and standard deviation (SD) of lymphocyte subsets of two independent experiments (experiment 2 and 3), in the spleens of control birds (n= 5) and vaccinated birds (n=5) which were vaccinated at the age of 1 day with $1x10^8$ CFU of S. typhimurium live vaccine. Analysis was performed on day 7. Asterisk indicates mean for vaccinated chickens differs significantly from control value (*= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$).

As seen before, the relative numbers of γ/δ Tcells increased significantly in response to vaccination in both experiments. In addition, in these experiments $CD8^+$ cell numbers increased while the numbers of $CD4^+$ lymphocytes were significantly reduced both experiments. Moreover, no significant differences were observed for α/β T cells and B cells. Since no significant increase in $CD8^+$ cytotoxic lymphocytes was seen in experiment 1 while this cell population changed significantly in experiments 2 and 3, an explorative statistical analysis was performed. Values of all three experiments were pooled and subjected to the students T- test. The data are summarized in annex 1 and the results are shown in figure 1.

From this analysis it become apperent, that S. typhimurium live vaccine significantly influences the composition of splenic lymphocyte subpopulations. This is exemplified by the highly significant increase in $CD8^+$ cytotoxic T cells. More detailed studies showed that the relative numbers of γ/δ TCR positive T cells increased while classical α/β T cells remained largely unaffected. The same feature was observed for L – chain positive B lymphocytes.

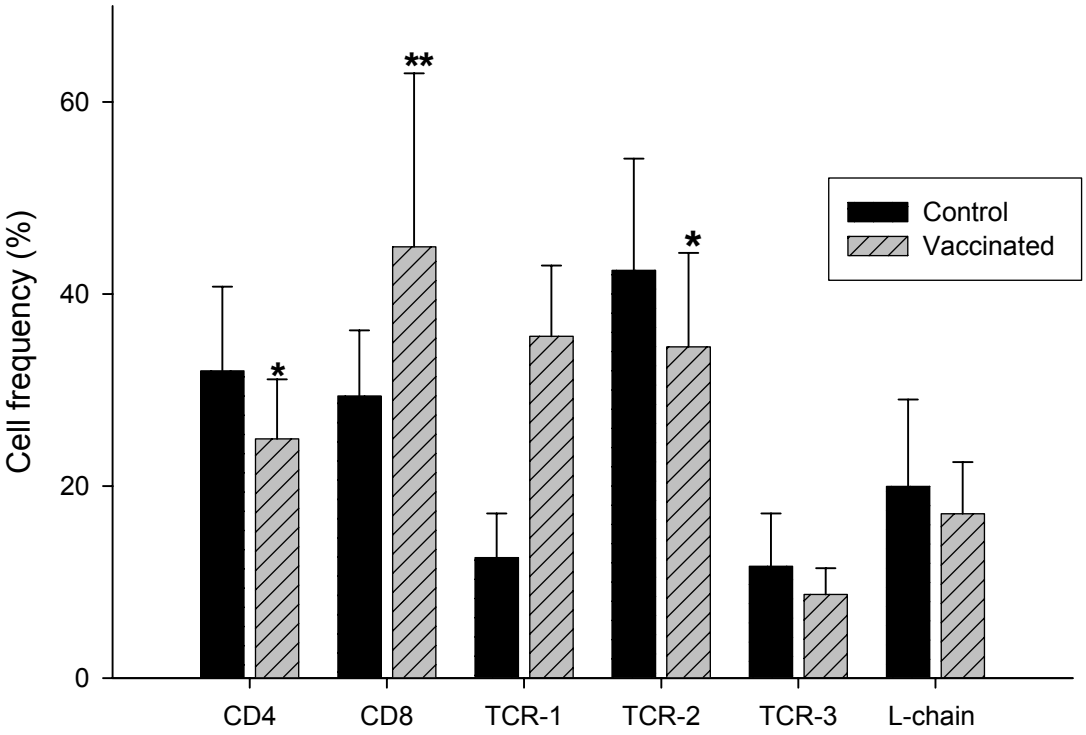

Figure 1: Frequency of lymphocyte subsets in the spleens of control birds (n= 16) and vaccinated birds (n=16) which were vaccinated at the age of 1 day with $1x10^8$ CFU of S. typhimurium live vaccine. Analysis was performed on day 7. Asterisk indicates mean for vaccinated chickens differs significantly from control value (*= $p \leq 0.05$, **= $p \leq 0.01$).

4.1.2 Splenic response two weeks after vaccination

In a second set of experiments (experiment 4-6), the effect of S. typhimurium live vaccine on the response of spleen cells two weeks after vaccination was investigated. 6 birds per group were vaccinated orally with $1x10^8$ CFU of S. typhimurium at the age of 1 day, which was applied in 0.5 ml PBS. Control birds received 0.5 ml PBS without antigen. The chickens were killed two weeks after vaccination and spleens were prepared and stained as described (chapter 3.2.2). In experiment 4 the birds showed a marked increase of γ/δ-T cells and B cells compared with the control birds. On the other hand both $CD4^+$ cells and the α/β T cell populations were significantly reduced table 3.

	%CD4		%CD8		%TCR-1		%TCR-2		%TCR- 3		%L- chain	
Control	44,0	7,2	42,9	3,9	10,7	2,6	63.0	5,5	14,8	2,0	9,1	3,1
Vaccinated	34,0*	4,0	46,1	8,3	24,9	4,0	46,1**	9,9	15,7	8,0	23,4**	4,5

Table 3: Mean values and standard deviations of lymphocyte subsets in the spleens of control birds (n= 6) and vaccinated birds (n=6) in experiment 4. Birds were vaccinated at the age of 1 day with $1x10^8$ CFU of S. typhimurium live vaccine. Analysis was performed on day 14. Asterisk indicates mean for vaccinated chickens differs significantly from control value. (*=p≤ 0.05, **=p≤ 0.01).

In order to confirm this observation, the experiment was repeated twice in the same way. The birds were vaccinated orally with $1x10^8$ CFU of live bacteria at the age of 1 day, control birds received PBS only. The birds were killed two weeks after vaccination and spleen cells were prepared, stained and analysed by flow cytometry. The results are summarized in tables 4a and 4b.

Unexpectedly there is no difference in the relative numbers of γ/δ T cells were seen in experiment 5 (table 4b). Instead the $CD8^+$ population increased significantly. In addition a shift was observed in the population of α/β T cells with reduced numbers of TCR-2^+ cells and increased numbers of TCR-3^+ cells.

None of these observations could be confirmed in experiment 6. Again, the common response pattern was found with a significant increase in γ/δ T cells and a not statistically significant reduction of $CD4^+$ cells.

Again the data sets of the three independent experiments numbers 4 to 6 were combined and analysed. The result is shown in figure 2 and the data sets are presented in annex 2. From this, it become clear that immunized birds still responded with an increase in splenic γ/δ T cells two weeks after vaccination.

Changes in the numbers of $CD4^+$ or $CD8^+$ T cells were not present any more. Instead, total B cell numbers started to increase at that time point in vaccinated chickens.

Table 4a :

	%CD4		%CD8		%TCR-1		%TCR-2		%TCR- 3		%L- chain	
Control	27,3	3,6	27,5	4,5	19,6	9,9	46,7	12,4	29,4	9,2	19,2	2,3
Vaccinated	29,5	4,3	40,9**	3,5	24,8	6,9	29,1*	6,7	47,0**	5,0	18,4	4,3

Table 4b:

	%CD4		%CD8		%TCR-1		%TCR-2		%TCR- 3		%L- chain	
Control	44,7	14,4	37,0	8,9	10,7	2,7	56,7	14,6	12,1	2,5	22,2	13
Vaccinated	35,1	2,4	39,8	5,2	21,4**	5,1	52,3	5,3	10,6	2,1	21,4	6,9

Table 4: Mean values (MV) and standard deviations (SD) of lymphocyte subsets of two independent experiments (experiment 5 and 6), in the spleens of control birds (n= 6) and vaccinated birds (n=6) which were vaccinated at the age of 1 day with $1x10^8$ CFU of S. typhimurium live vaccine. Analysis was performed on day 14. Asterisk indicate mean for vaccinated chickens differs significantly from control value (***=** $p \leq 0.05$, ****=** $p \leq 0.01$).

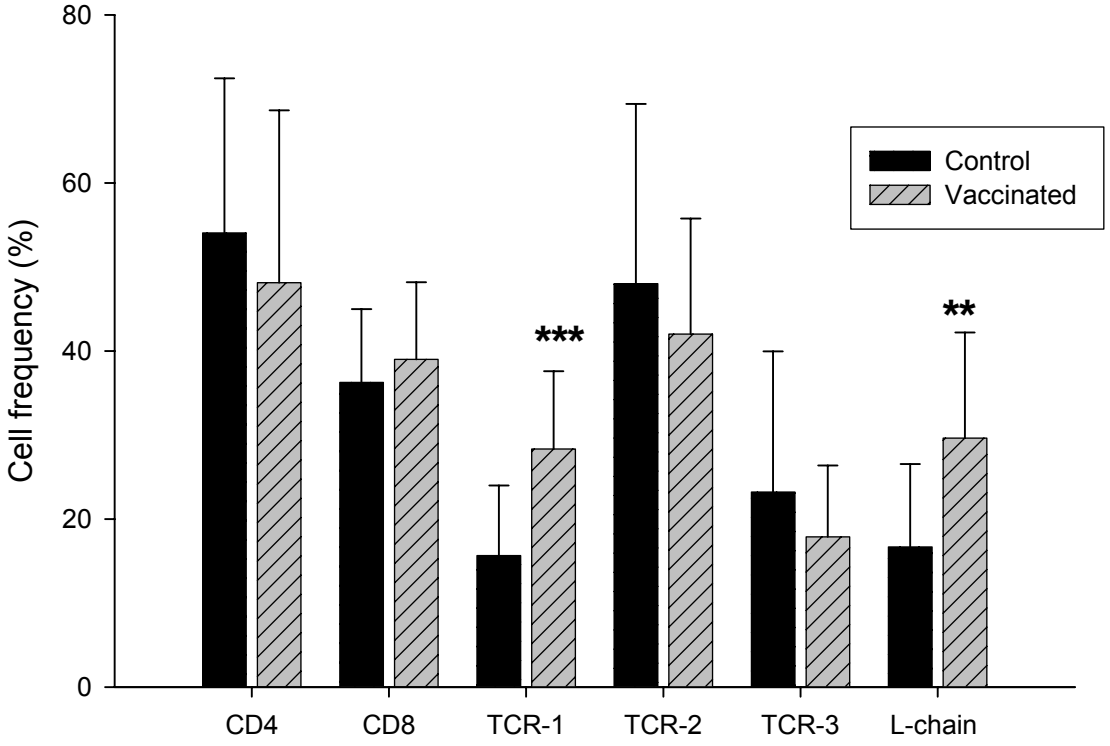

Figure 2: Frequency of lymphocyte subsets in the spleens of control birds (n= 17) and vaccinated birds (n=17) which were vaccinated at the age of 1 day with $1x10^8$ CFU of S. typhimurium live vaccine. Analysis was performed on day 14. Asterisk indicates mean for vaccinated chickens differs significantly from control value (**= p≤ 0.01, ***= p≤0.001).

4.1.3 Cellular responses in spleens and caecal tonsils to prime-boost vaccination.

Responses 1 week after booster vaccination

In a subsequent study, the effect of early vaccination in combination with a second immunization with S. typhimurium live vaccine (booster vaccination) on the local immune response in spleens and faecal tonsils was examined. Birds were vaccinated orally with $1x10^8$ CFU of live bacteria in 0.5 ml PBS on day one and again at the age of 6 weeks. Control birds received 0.5 ml PBS. The birds were killed 1 week after the booster immunization, spleens and caecal tonsils were removed and prepared as described (chapter 3.2.2). The cells from the spleens and caecal tonsils of vaccinated and control birds were stained with monoclonal antibodies to CD3, CD4, CD8, the α/Vß1 and α/Vß2 TCR and γ/δ TCR as well as the L-chain antigens.

The response of vaccinated birds one week after booster immunization was compared

with that of control chickens.

In this experiment, no significant differences were seen between the two groups in the spleen (table 5 and annex 3). However, a tendency toward a slight increase of $CD8^+$ and TCR-2^+ lymphocytes was observed indicating that the relative numbers of cytotoxic T lymphocytes increased. Similarly, a small increase in the numbers of B cells (L-$chain^+$) could be seen. Analysis of caecal tonsil lymphocytes was restricted to 3 birds per group. A relatively large variability was found for some parameters such as B cell frequencies and $CD4^+$ T cells populations. Thus, no significant differences in the cellular composition between the two groups were observed.

Antigens	**Spleen**				**Caecal tonsils**			
	Control (n=5)		Vaccinated (n=5)		Control (n=3)		Vaccinated (n=3)	
	MV	**SD**	**MV**	**SD**	**MV**	**SD**	**MV**	**SD**
%CD3	82,37	1,31	75,95	13,36	36,16	10,71	52,82	10,73
%CD4	27,47	4,39	24,52	6,91	15,57	15,70	24,42	5,19
%CD8	54,34	6,63	57,68	10,81	25,38	4,08	30,40	4,00
%TCR-1	25,84	3,44	19,67	5,83	8,80	2,57	13,40	0,20
%TCR-2	43,84	6,36	49,41	8,97	29,47	9,78	32,22	7,35
%TCR-3	15,08	2,66	13,34	4,25	9,14	4,25	13,47	0,87
%L-chain	18,94	2,25	23,32	8,15	29,94	22,24	38,59	13,24

Table 5: Lymphocyte subpopulations in spleens and caecal tonsils of 7 weeks old chickens. Vaccinated birds received $1x10^8$ CFU S. typhimurium live vaccine in PBS on day 1 and at the age of 6 weeks, while control birds received PBS only. Analysis was performed 1 week after the second vaccination.

4.1.4 Cellular responses in spleens and caecal tonsils to prime-boost vaccination

Responses 2 weeks after booster vaccination

The response of both T and B lymphocytes in spleens and caecal tonsils two weeks after booster immunization with live bacteria was evaluated. The birds were vaccinated orally at the age of 1 day and 6 weeks with $1x10^8$ CFU of S. typhimurium live vaccine in PBS. The control birds received PBS at the age of 1 day and 6 weeks

The birds were killed 2 weeks after the second vaccination, the spleen and caecal tonsils were removed and prepared as described (chapter 3.2.2). The cells from spleen and caecal tonsils were stained with monoclonal antibodies to CD3, CD4, CD8, the α/Vß1 and α/Vß2 TCR and γ/δ TCR as well as the L-chain, IgM, IgG and IgA antigens.

In this study, large variations were observed between the responses of lymphocytes in spleens and caecal tonsils and also between the two groups of birds (table 6 and annex 4). Regarding, the comparison of lymphocytes in the spleens between the two groups, there was a reduction in the relative numbers of L-chain$^+$ cells of vaccinated birds which was accompanied by a significant increase in CD4$^+$ and α/β TCR positive cells. Interestingly, the analysis of the lymphocytic response in the caecal tonsils, did not show significant changes in the T cell populations. However, an increase in IgA$^+$ cell numbers were observed. These results indicate that IgA secreting cells are activated in caecal tonsils in response to S. typhimurium vaccination.

Antigens	Spleen				Caecal tonsils			
	Control (n=9)		Vaccinated (n=9)		Control (n=3)		Vaccinated (n=3)	
	MV	**SD**	**MV**	**SD**	**MV**	**SD**	**MV**	**SD**
%CD3	73,62	5,30	76,63	6,64	42,16	3,00	61,6	10,60
%CD4	24,54	4,01	30,46*****	4,78	22,45	2,14	30,56	10,91
%CD8	45,02	5,17	49,54	6,18	20,21	3,21	26,72	4,30
%TCR-1	19,24	4,36	21,29	3,97	14,10	2,18	19,37	2,36
%TCR-2	44,30	4,79	50,98*****	7,38	33,45	4,29	32,02	4,06
%TCR-3	12,52	4,72	16,08	3,24	10,41	0,55	11,25	3,27
%L-chain	24,07	5,10	20,70	5,25	-	-	-	-
%IgG	1,70	0,96	2,53	1,65	3,08	0,76	3,64	2,09
%IgM	23,62	5,29	20,50	5,08	31,22	5,36	26,49	3,88
%IgA	2,58	1,63	4,83	2,88	4,34	3,04	21,65*****	4,21

Table 6: Lymphocyte responses in spleens and caecal tonsils of 8 weeks old chickens Vaccinated birds received 1x10^8 CFU of S. typhimurium live vaccine in PBS on day 1 and at the age of 6 weeks, while control birds received PBS only. Analysis was performed 2 week after the second vaccination. Asterisk indicates mean for vaccinated chickens differs significantly from control value (*****=p≤ 0.05).

4.1.5 Cellular responses in spleens and caecal tonsils of 7 and 8 weeks old chicken. Comparison of one and two immunizations.

Routine vaccination in poultry flocks is given on day 1 and at the age of 6 weeks. To further investigate the relevance of this prime – boost procedure, the following study was performed. Birds were vaccinated with 1x10^8 CFU of live Salmonella vaccine which was applied orally in PBS. Group 1 was vaccinated at the age of 6 weeks and group 2 was vaccinated at the age of 1 day and 6 weeks. Birds were killed at 7 and 8 weeks of age. Spleens and caecal tonsils were removed and prepared. The cells from spleens and caecal tonsils were stained with monoclonal antibodies as described for the previous experiments. The results are summarized in table 7 (and annex 5)

In agreement with the previous experiments, only few changes in the frequencies of T cell subsets were observed one week after booster vaccination in comparison with birds

receiving only one immunization. Again a tendency toward an increase in B cell numbers was seen. Importantly, B cell numbers showed a strong increase in the caecal tonsils which was mainly due to an increase in IgM^+ and IgA^+ cells, while the relative numbers of T lymphocytes were reduced.

Antigens	Spleen				Caecal tonsils	
	Group 1 (n=7)		Group 2 (n=7)		Group 1 (n=3)	Group 2 (n=3)
	MV	**SD**	**MV**	**SD**	**MV**	**MV**
%CD3	63,47	8,64	60,34	7,33	38,66	19,60
%CD4	29,84	5,72	26,31	2,46	29,71	12,15
%CD8	41,80	7,50	40,19	7,46	19,56	6,13
%TCR-1	20,21	6,09	21,0	7,77	8,94	7,95
%TCR-2	34,99	5,89	33,53	2,93	21,49	10,10
%L-chain	29,46	7,53	34,26	5,36	50,09	70,11
%IgG	1,06	0,58	1,24	0,36	0,95	1,73
%IgM	28,46	6,78	32,99	4,77	49,38	65,63
%IgA	0,98	0,63	1,29	0,55	0,90	4,06

Table 7: Lymphocyte responses in spleens and caecal tonsils in two groups of 7 weeks old chickens (1 week after booster immunization) which were vaccinated with $1x10^8$ CFU of S. typhimurium live vaccine. The first group was vaccinated at 6 weeks of age and the second group was vaccinated at 1 day and 6 weeks of age.

To obtain kinetic data on the anamestic response to vaccination, the responses were also analysed two weeks after the second immunization. The experimental design was as described in the previous experiment. In the spleen a significant increase in the frequency of α/β T cells were found in response to the booster immunization (table 8 and annex 6)). In contrast, γ/δ T cells remained largely unaffected and B cell numbers decreased slightly. The oposit picture was found in caecle tonsils. Here, a strong increase in the B cell frequencies was seen which was due to an increase in IgM^+ and IgA^+ lymphocytes. Compensatory, the T cell frequencies were reduced. No statistical comparison between the groups was performed since only three birds could be analysed per group.

Antigens	Spleen				Caecal tonsils	
	Group 1 (n=6)		Group 2 (n=6)		Group 1 (n=3)	Group 2 (n=3)
	MV	**SD**	**MV**	**SD**	**MV**	**MV**
%CD3	59,89	9,49	58,02	7,77	38,14	31,96
%CD4	33,05	6,83	33,62	7,11	20,30	14,24
%CD8	27,51	8,50	24,95	3,44	20,93	16,55
%TCR-1	12,51	2,80	12,77	1,65	10,03	9,70
%TCR-2	38,52	6,60	47,47*****	5,88	24,52	21,54
%L-chain	25,24	8,71	19,48	4,86	36,38	53,30
%IgG	0,78	0,21	1,17	0,53	2,39	3,00
%IgM	26,39	8,01	19,39	4,42	39,15	28,30
%IgA	0,79	0,21	0,64	0,31	5,48	22,90

Table (8): Lymphocyte responses in spleens and caecal tonsils in two groups of 8 weeks old chickens which were vaccinated with 1x10^8 CFU of S. typhimurium live vaccine. The first group was vaccinated only at 6 weeks of age and the second group was vaccinated at the age of one day and 6 weeks. Asterisk indicate mean for vaccinated chickens differs significantly from control value (*****=p≤ 0.05).

4.2 Lymphocyte Proliferation Assays

To investigate if oral vaccination with a live Salmonella vaccine induces a functional activation of T or B cells, lymphocyte proliferation assays were carried out. Birds were vaccinated orally at 1 day of age with 1x10^8 CFU of S. typhimurium live vaccine. Control birds received 0.5 ml PBS. The birds were killed at the age of 1 and 2 weeks and spleens were removed and prepared as described (chapter 3.2.2). Spleen cells were polyclonally stimulated through the α/β or γ/δ T cell receptors with the monoclonal antibodies TCR-2 and TCR-1, respectively. Control cultures were coated with mab F71D7 which does not bind to and activate lymphocytes. For S. typhimurium antigen specific stimulation LPS or a crude protein preparation of the S. typhimurium live vaccine strain (lysate) were added to the cell cultures. Cells were incubated at 37˚C for 72 h and pulsed with ^{3}H-thymidine for an additional 16 h. Two independend experiment were performed and the result are summarized in tables 9 and 10 and annex 7 and 8.

Stimulation		Control		Vaccinated	
		cpm		cpm	
		MV	SD	MV	SD
TCR-1	Experiment 1	1699	1329	2238	1157
	Experiment 2	4694	4073	7286	5253
TCR-2	Experiment 1	17057	16450	31462	28197
	Experiment 2	14337	8253	19714	11969
LPS	Experiment 1	1005	472	3086	1395
	Experiment 2	8227	8003	12325	7391
mAb F71D7	Experiment 1	1114	1215	3343	1161
	Experiment 2	2100	1271	4003	1868
Lysate		1674	911	886	851

Table 9: Proliferation assays of spleen cells of 1 week-old chicks, which were vaccinated at 1day of age with $1x10^8$ CFU of S. typhimurium live vaccine or were left unvaccinated. Mean values (MV) and standard deviations (SD) of counts per minute (cpm) of 6 birds per group in each of the two experiments are shown.

Polyclonal activation of spleen cells through the α/β T cell receptor (TCR-2 antibody) lead to a significant induction of T cell proliferation in both vaccinated and untreated birds. In contrast, crosslinking of the γ/δ TCR (TCR-1) had no effect on cell proliferation. A weak stimulation has observed in LPS treated cultures whereas the crude bacterial lysate had no proliferation inducing activity. When vaccinated and non-vaccinated birds were compared some differences were seen between these two groups, with a stronger response observed in cultures derived from vaccinated animals. However, it must be noted that even in unstimulated control cultures (F71D7 antibody treated) a nearly 2- to 3-fold higher background proliferation was seen in the vaccinated group. This observation indicates that cells from vaccinated birds taken one week after vaccination were most probably pre-activated *in vivo* and continued their proliferation program *in vitro*.

To analyse, if spleen cells may require more time to become activated and to responded to the bacteria, the same experiment was set up and analysed two weeks after vaccination (table 10 and annex 8). Again, no significant proliferative responses

were found, when cultures were activated with LPS, the crude bacterial lysate or through the γ/δ TCR (TCR-1). However, a very strong response was observed after stimulation of classical α/β T cells (TCR-2). Despite the large individual variability this difference was statistically significant. Collectively, these data show that the vaccine functionally activates splenic T cells and that the induction of this activation requires clearly more than 1 week from the application of the vaccine.

Stimulation	Control		Vaccinated	
	cpm		cpm	
	MV	**SD**	**MV**	**SD**
TCR-1	3141	1223	2516	978
TCR-2	4206	3914	85906*	46479
LPS	1648	1343	3099	422
Lysate	1123	349	1194	448
F71D7	4364	4078	4298	3712

Table 10: Proliferation assays of spleen cells of 2-weeks old chickens, which were vaccinated at 1day of age with $1x10^8$ CFU of S. typhimurium live vaccine or were left unvaccinated. Mean values (MV) and standard deviations (SD) of counts per minute (cpm) of 6 birds per group are shown. Asterisk indicates mean for vaccinated chickens differs significantly from control value (*=p≤ 0.05).

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To further evaluate the functional properties of lymphocytes in response to a second vaccination, birds received the S. typhimurium live vaccine at 1 day and 6 weeks of age or at 6 weeks only. Spleen cells were obtained one week after the vaccination and cell proliferation assays were performed as described before. The results are presented in table 11 (annex 9). No significant differences were found between the two groups in response to LPS or lysate treatment. Interestingly, a stronger response to TCR-2 crosslinking was seen in those birds, which received the vaccine for the first time. Since only 7 birds could be analysed per group in one experiment this difference could not be statistically confirmed.

Stimulation	Group 1		Group 2	
	cpm		cpm	
	MV	**SD**	**MV**	**SD**
TCR-2	83570	36129	48591	21951
LPS	1680	913	827	468
Lysate	1700	756	352	84
F71D7	12311	413	1135	664

Table 11: Proliferation assays of spleen cells of 7 weeks old chicks, which were vaccinated at 6 weeks of age (group 1) or at one day and at 6 weeks of age (group 2) with $1x10^8$ CFU of S. typhimurium live vaccine. Mean values (MV) and standard deviations (SD) of counts per minute (cpm) of 6 birds per group are shown

4.3 Investigation of the S. typhimurium LPS specific IgA response

Numerous studies have investigated the IgG response of chickens to Salmonella vaccination and infection. However, very little is known about the IgA response.

To measure the level of LPS specific IgA responses to S. typhimurium live vaccine, several experimental designs were set up. In the first experiment, birds were vaccinated orally at the age of 1 day with $1x10^8$ CFU of live bacteria and vaccinated again at 6 weeks of age. Control birds received 0.5 ml PBS without antigen. Serum samples was collected at 7, 8, 9, 10, 12, 14 and 16 weeks of age (1, 2, 3, 4, 6, 8.and 10 weeks after the second immunization). The results were obtained from 19 birds per group and the mean values and standard deviations calculated. The results are presented in figure 3.

A S. typhimurium LPS specific IgA response was clearly observed 1 week after the second immunization. The antibody titers increased to peak levels in the second and third week after booster vaccination and subsequently declined to control levels. Large differences were observed between individual birds. Despite this variability within the groups statistically significant differences were found between vaccinated and unvaccinated groups 1, 2 and 3 weeks after the second immunization. Antibody titers declined thereafter to background levels and remained low until the end of the experiment 10 weeks after the booster immunization.

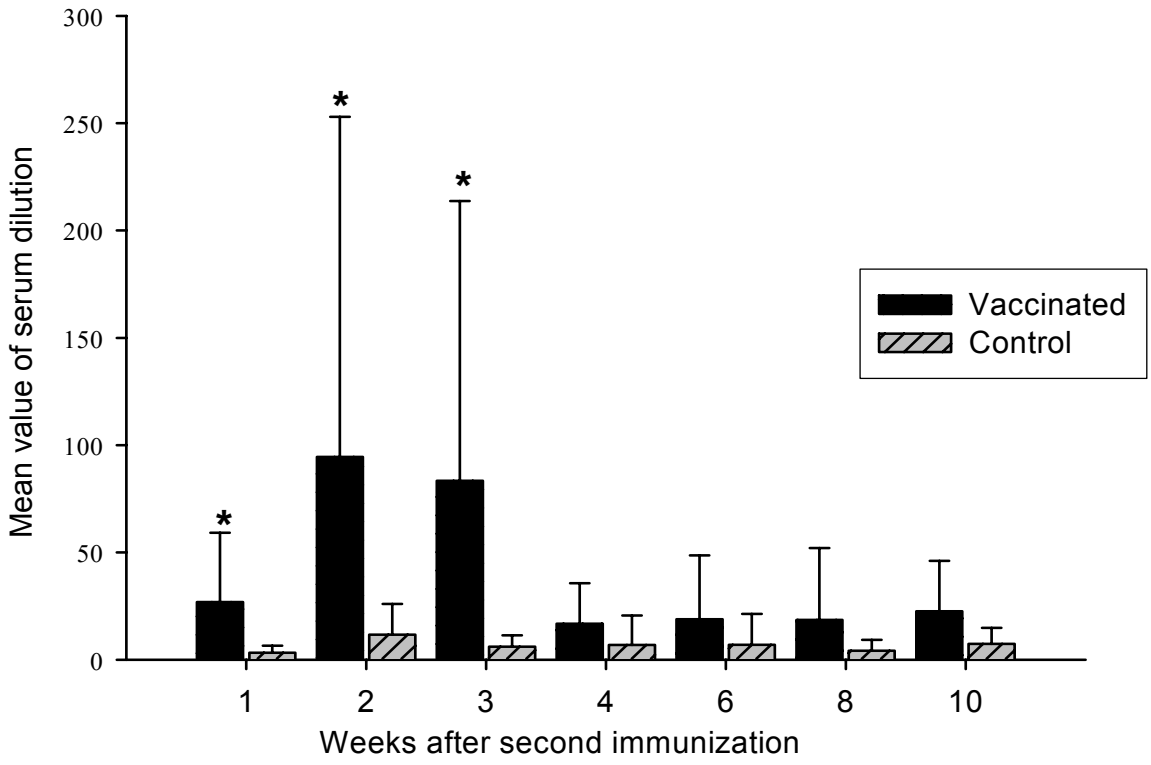

Figure 3: S. typhimurium LPS specific IgA response in the serum of vaccinated and control chickens between 7 to 16 weeks of age (1, 2, 3, 4, 6, 8 and 10 weeks after second immunization). 19 birds per group were either treated with the vaccine diluent only or vaccinated at 1 day and 6 weeks of age with $1x10^8$ CFU of live bacteria. Asterisk indicates mean for vaccinated chickens differs significantly from control value (* = $p \leq 0.05$)

To confirm this observation the experiment was repeated using the same vaccination protocol. Birds received $1x10^8$ CFU of live bacteria orally at the age of 1 day and 6 weeks. Control birds received 0.5 ml PBS without antigen. In this trial serum samples were collected from 10 birds per group one week and two weeks after the second immunization. After serum collection birds were killed and the bile was collected for ELISA analysis. The results are summarized in figures 4 and 5.

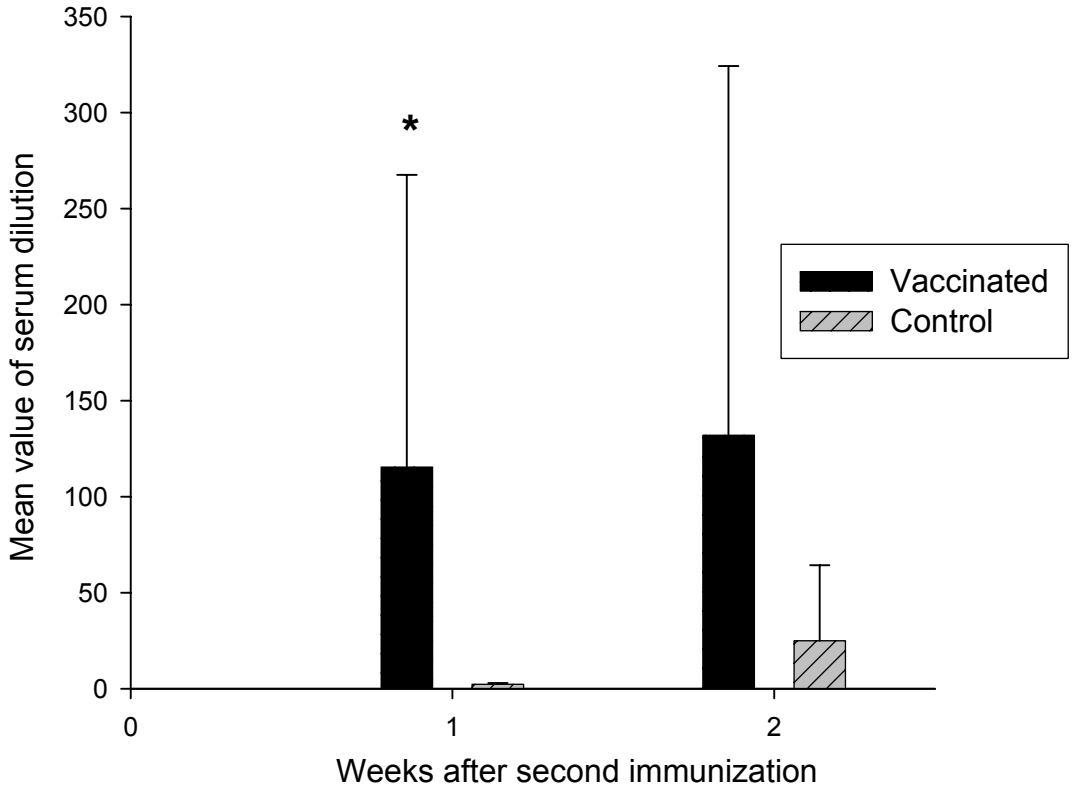

Figure 4: S. typhimurium LPS specific IgA response in the serum of vaccinated and control chickens at 7 and 8 weeks of age (1 and 2 weeks after second immunization). 10 birds per group were either treated with the vaccine diluent only or vaccinated at 1 day and 6 weeks of age with $1x10^8$ CFU of live bacteria. Asterisk indicates mean for vaccinated chickens differs significantly from control value (* = $p \leq 0.05$).

As expected, a significant increase in LPS specific IgA titres was found in serum samples 1 week after booster vaccination. In addition, birds of the vaccinated group showed much higher antibody titers 2 weeks after the second immunization. However, this differences was not significant due to the large individual variability.

A similar observation was made when LPS specific antibody titers in the bile from the same birds were analysed (figure 5). Again, marked differences were seen between vaccinated and non vaccinated birds one and two weeks after the second vaccination but these differences were not statistically significant as a result of the large differences between individual birds.

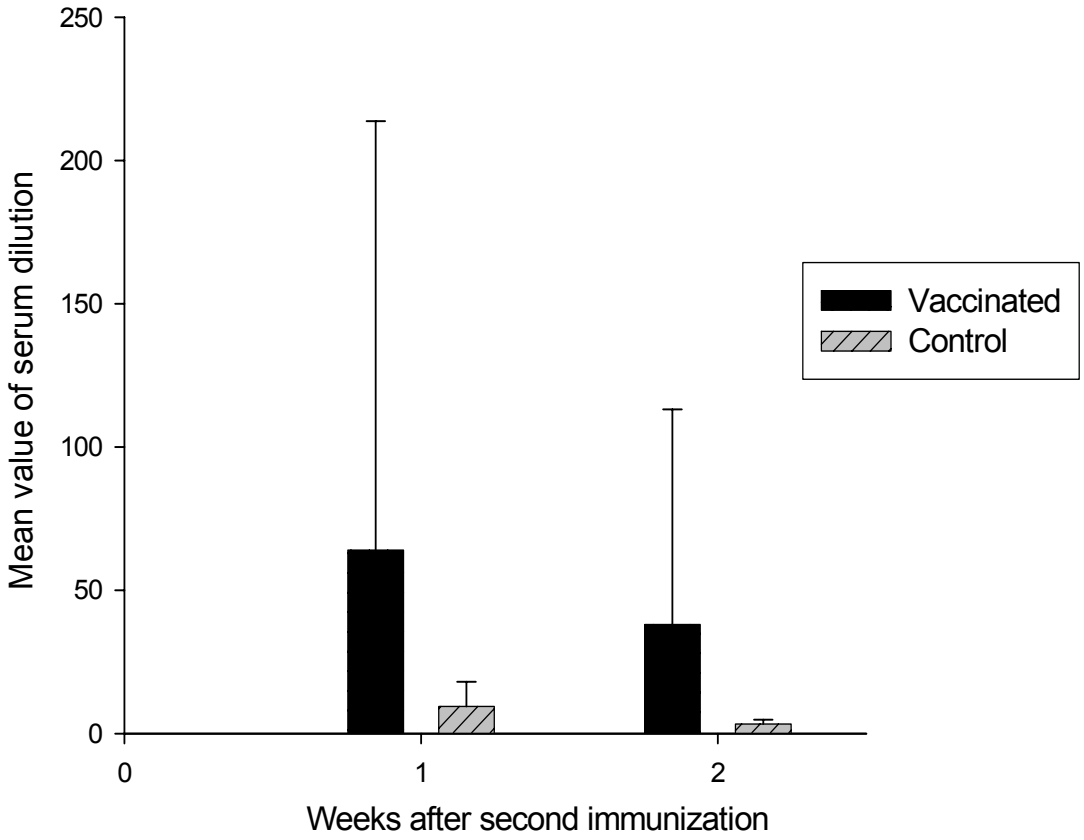

Figure 5: S. typhimurium LPS specific IgA response in the bile of vaccinated and control chickens at 7 and 8 weeks of age (1 and 2 weeks after second immunization). 10 birds per group were either treated with the vaccine diluent only or vaccinated at 1 day and 6 weeks of age with $1x10^8$ CFU of live bacteria.

To further investigate the relevance of the primary immunization on day one after hatch an experiment was designed in which one group of birds received the vaccine on day one and at the age of 6 weeks and a second group was vaccinated only at 6 weeks of age. Birds were immunized as described before. S. typhimurium LPS specific serum IgA levels were clearly higher in those birds which received two immunisations than in the second group which were vaccinated only once (figure 6). This difference was not significant at one week after the booster immunization but a statistically significant difference was found one week later. These results confirm the previous data and show that the booster immunization is critical for the induction of high IgA antibody levels. As before, antigen specific IgA antibody titers were also measured in the bile of these birds. The results obtained largely reflect those obtained from the serum analysis. Higher antigen specific IgA levels were found in birds vaccinated twice than in birds vaccinated only once (figure 7). The variability between individual birds and the small

number of test birds per group may account for the fact that these differences, even though obviouse, were not statistically significant.

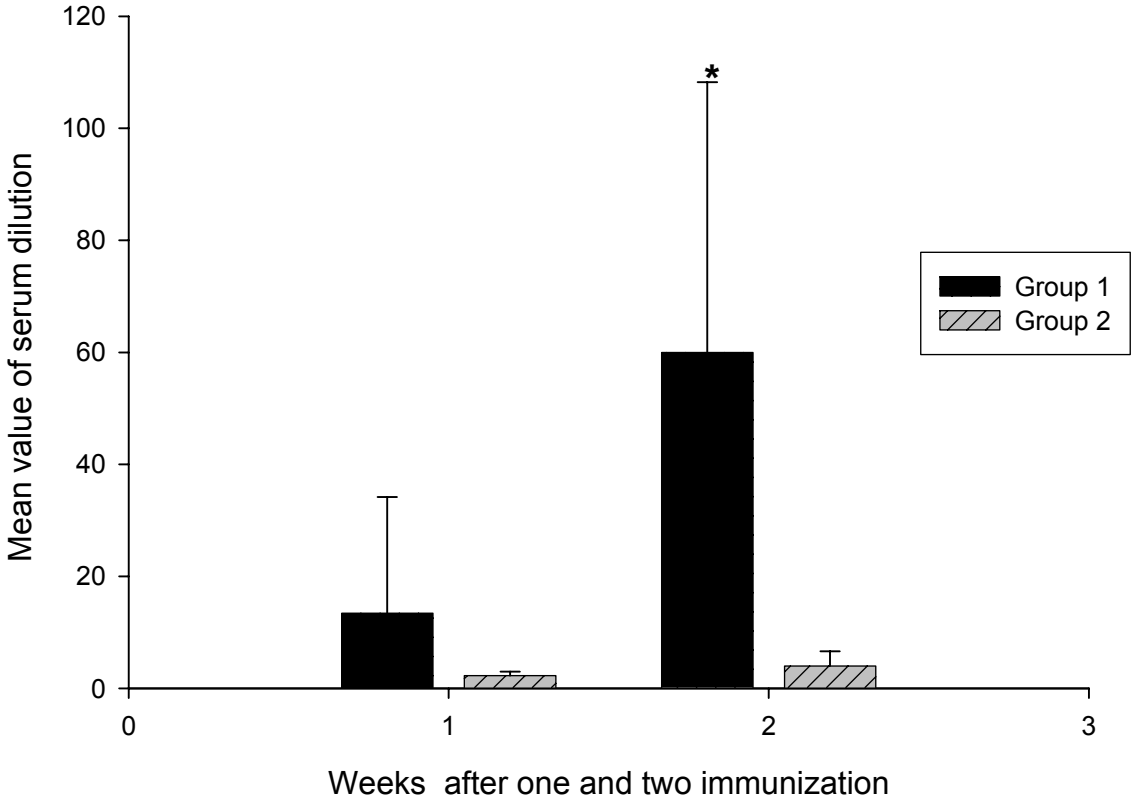

Figure 6 : S. typhimurium LPS specific IgA titers in the serum of birds immunized with $1x10^8$ CFU of S. typhimurium live vaccine either at day 1 and 6 weeks of age (group 1) or at 6 weeks of age only (group 2). Asterisk indicates mean for vaccinated chickens differs significantly from control value (*=p≤ 0.05).

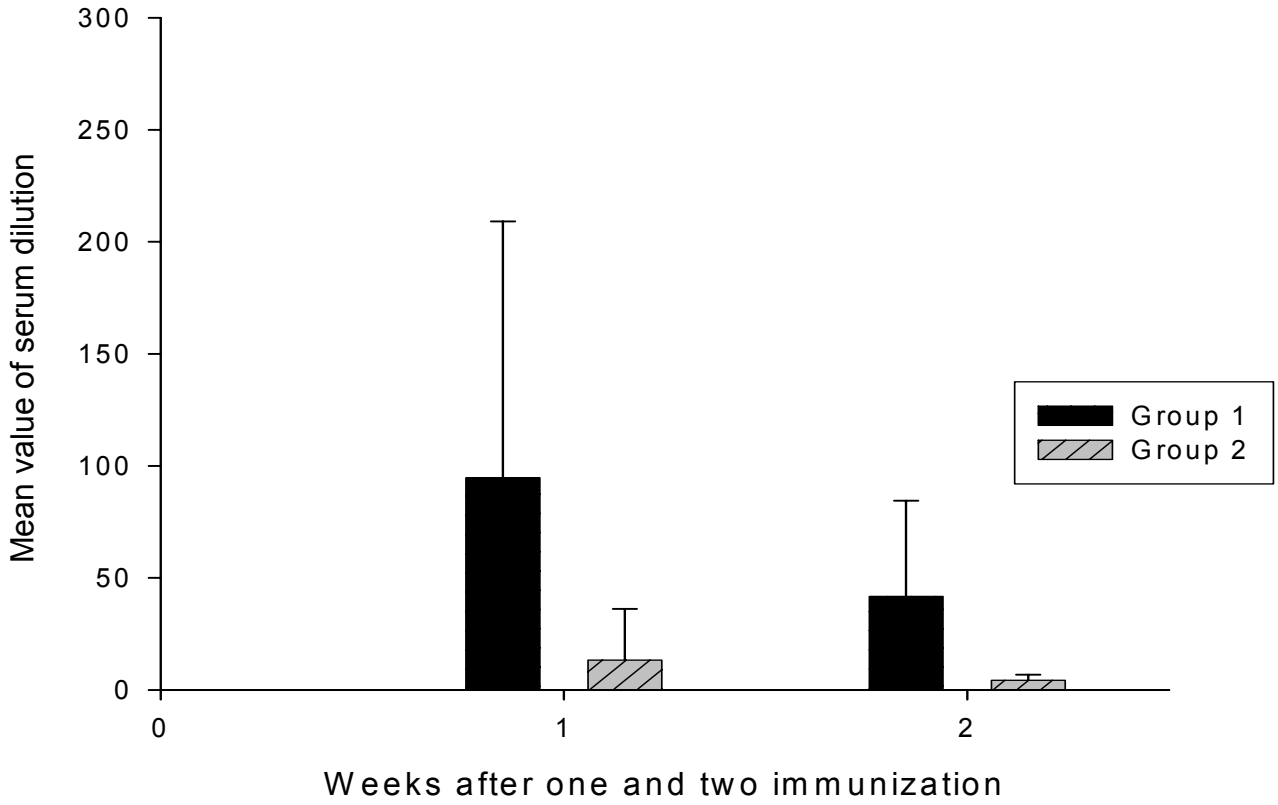

Figure 7 : S. typhimurium LPS specific IgA titers in the bile of birds immunized with $1x10^8$ CFU of S. typhimurium live vaccine either at day 1 and 6 weeks of age (group 1) or at 6 weeks of age only (group 2). Samples were obtained from the same birds as shown in figure 6.

4.4 Comparison of B cell frequencies in spleens and caecal tonsils after vaccination by immunohistology

The experiments described above showed that vaccination lead to a significant increase in S. typhimurium specific antibody levels. In order to better understand this response, spleens and caecl tonsils of immunized birds were subjected to immunohistological analysis. Organs of birds vaccinated at day 1 and at 6 weeks of age and birds vaccinated only at 6 weeks of age were compared. Semiquantitative analysis of either IgM positive or IgA positive surface areas was performed as described in the methode section (3.2.4).

In the spleens, a significant increase in the number of IgM^+ cells was observed one week after booster immunization. One week later IgM^+ cell numbers had declined to the levels found in control birds which had received only one immunization at 6 weeks of age. (figure 8). A similar response was observed for IgA^+ cells (figure 9). The IgA^+

surface area of the tissue sections increased nearly 9-fold one week after the second immunization in comparison with the control group. At two weeks after vaccination the IgA^+ surface area was smaller than at one week but still larger in birds with two immunizations than in those which were vaccinated only once.

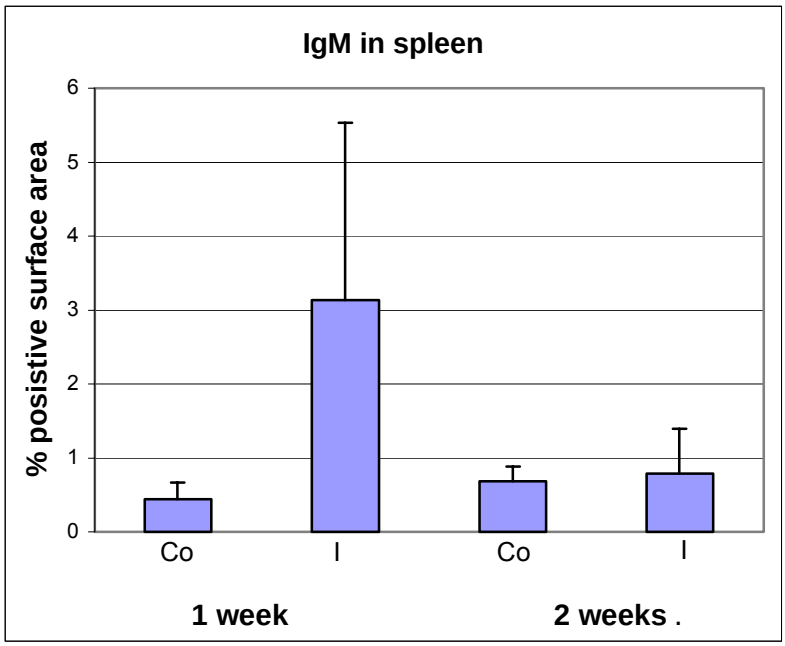

Figure 8 : Immunohistological analysis of IgM^+ B cell frequencies in spleens of birds immunized once at 6 weeks of age or twice at one day and 6 weeks of age with S. typhimurium live vaccine. Analysis was performed one and two weeks after the second vaccination (n = 3). (Co = control group and I = Immunized group).

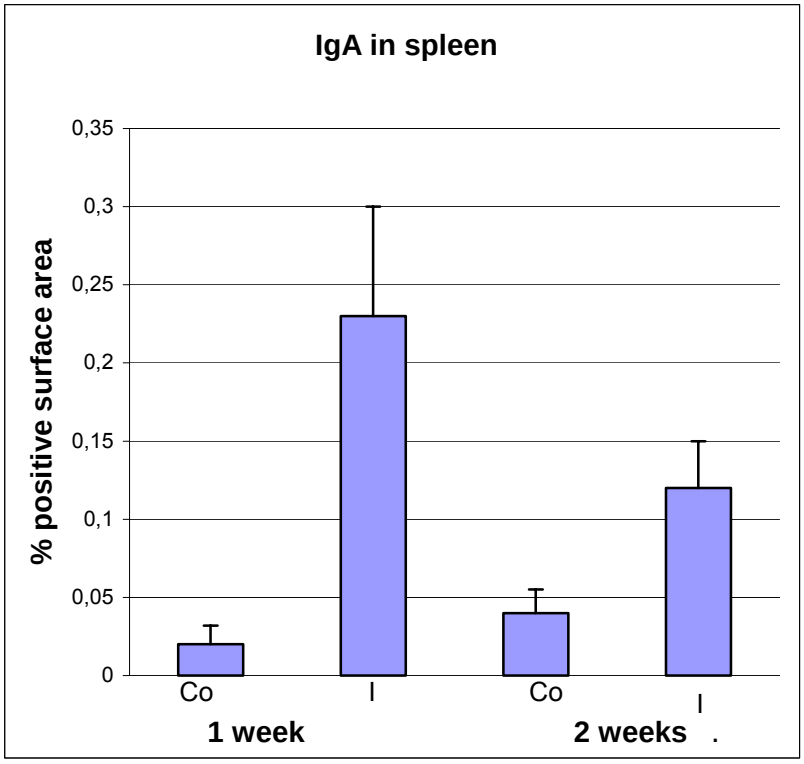

Figure 9 : Immunohistological analysis of IgA^+ B cell frequencies in spleens of birds immunised once at 6 weeks of age or twice at one day and 6 weeks of age with S. typhimurium live vaccine. Analysis was performed one and two weeks after the second vaccination. (n =3).
(Co = control & I= Immunized).

Comparative analysis were perferomed on caecle tonsil tissues from the same birds. The results are presented in figures 10 and 11. Again, an increase of IgM^+ cells was found one week after the second vaccination in comparison with birds immunised only once. Interstingly, no differences were seen one week later, indicating that immunisation with the live vaccine induced IgM^+ B cell proliferation or immigration to caecle tonsils. This effect was not obviouse after one week but clearly visible at two weeks.

A nearly identical effect was found if caecle tonsils were stained for IgA^+ B cells (figure 11). However, it should be noeted, that the percentage of the positive surface area was up to 10-fold higher for IgA^+ B cells than for IgM^+ B cells, demonstrating that IgA secreting cells represent the main B cell population in these lymphoid structures.

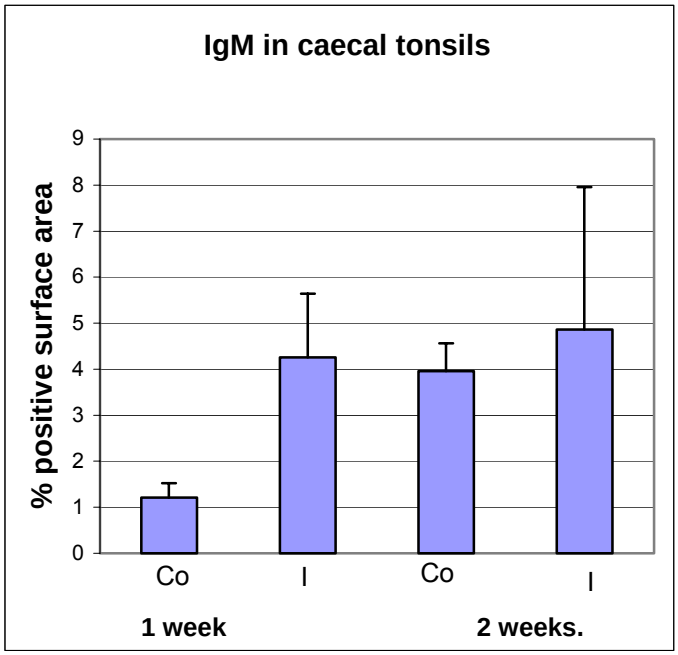

Figure 10 : Immunohistological analysis of IgM^+ B cell frequencies in caecle tonsils of birds immunized once at 6 weeks of age or twice at one day and 6 weeks of age with S. typhimurium live vaccine. Analysis was performed one and two weeks after the second vaccination. (n =3)

(Co = control group and I = Immunized group).

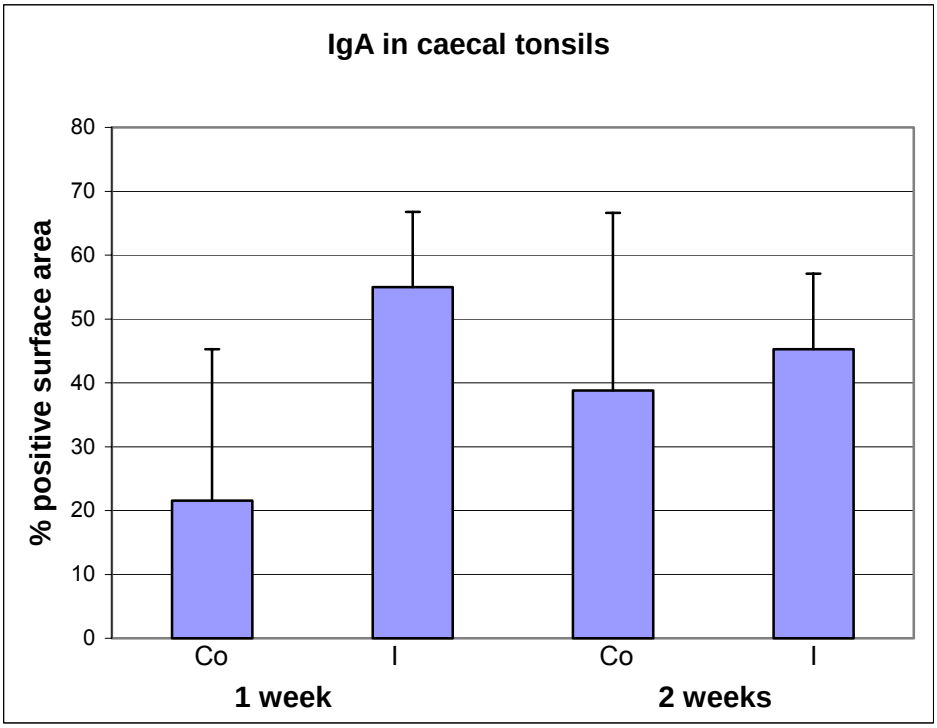

Figure 11 : Immunohistological analysis of IgA^+ B cell frequencies in caecle tonsils of birds immunized once at 6 weeks of age or twice at one day and 6 weeks of age with S. typhimurium live vaccine. Analysis was performed one and two weeks after the second vaccination. (n = 3)
(Co =control group and I = Immunized group).

5 DISCUSSION

Host-pathogen interactions during Salmonella infection have been studied most intensively in the mouse model (MITTRÜKER and KAUFMANN, 2000). This work lead to fundamental concepts describing bacterial invasion and dissemination, the role of innate and adaptive immune responses and immune evasion strategies used by Salmonella species. It has also been instrumental in the understanding of vaccine function and efficacy. It is believed that chickens have developed similar immune defence mechanisms to combat pathogens as described in mice. However, the avian immune system displays fundamental differences to the mammalian system. First, B cell development and the diversification of the antibody repertoire take place in a unique organ, the bursa of Fabricius (LANGMAN et al., 1993). Secondly, birds lack lymph nodes and therefore antigen uptake and handling differs from that in mammals. In addition, certain lymphocyte subsets such as γ/δ T cells show striking differences (SOWDER et al., 1988). Thus, the immune response to infection or vaccination may significantly differ from that of other domestic animals.

Vaccination to control Salmonella infections in chickens is now widely used in the poultry industry and in some countries, including Germany, implemented by law (MARTIN G., and MEYER H., 1994). Field studies have shown the efficacy of vaccination programs which are part of a general strategy to eliminate or at least to reduce Salmonella contamination of poultry products in the European Union. Live vaccines are believed to be highly effective in inducing an adaptive immune response leading to the development of memory B and T cells. However, very little experimental evidence is provided in the literature thus far. Therefore, it was the aim of this work to obtain initial information on the immune responses of B and T cells elicited by S. typhimurium vaccination.

In a first set of experiments flow cytometric analysis was applied to investigate changes in lymphocyte subpopulations after vaccination with an attenuated live vaccine.

Vaccination of birds one day after hatch induced marked changes in the lymphocyte subset composition in the spleen. The relative numbers of γ/δ T cells increased significantly within one week after vaccination. This observation was made in three independent experiments. In addition, the frequency of $CD8^+$ lymphocytes in spleens from vaccinated birds was significantly higher than in control animals. On the contrary,

a reduction was found in the $CD4^+$ T helpers cell population and to a minor extend in α/β T cells. Together, these data clearly show that the infiltration of the spleen by S. typhimurium induces a highly significant response of the immune system. Comparable results were obtained by Berndt et al. (2001) who used flow cytometry and immunohistology to analyze the lymphocyte composition in different organs after immunization and vaccination. In their experiments it was shown that the number of γ/δ T cells not only increased in the spleen, but also in the blood and in the ceca. Using double staining techniques the authors further showed that the increased numbers of γ/δ T cells and $CD8^+$ cells could be attributed to one single cell type, the $CD8^+$ and the γ/δ TCR expressing T cells. Based on this work it is highly likely that the concomitant increase in $CD8^+$ and γ/δ T cells observed in this study is caused by a single lymphocyte population, the $CD8^+/\ \gamma/\delta$ T cells.

To better understand the kinetics of this response, birds vaccinated in exactly the same way were analyzed two weeks after vaccination. At that time point, significantly increased numbers of γ/δ T cells were still observed. However, the proportion of γ/δ T cells was clearly reduced in comparison with that found one week after vaccination. Interestingly, the relative B cell numbers started to increase in the spleen of vaccinated birds in comparison to unvaccinated controls.

Numerous studies on the biology of this vaccine have shown that the attenuated vaccine strain is invasive and can be isolated from spleens 5-6 days after oral vaccination of day old chickens (ZHU et al., 2002). However, 8-9 days after vaccination no bacteria can be cultured from spleen or liver of immunized birds, indicating that the bacteria are successfully cleared or highly reduced in these organs. The γ/δ T cell response parallels this kinetic feature. 1-2 days after bacteria reach the spleen and start to replicate in this organ, γ/δ T cell are either attracted to the spleen or proliferate in the organ. As bacteria are eliminated from the organ the number of γ/δ T cells declines. This highly specific response pattern strongly suggests that γ/δ T cell play a significant role in the control of S. typhimurium infections. The precise role of this T cell subset is still unknown even though a large number of studies have been published (BERND A., and METHNER U., 2001). SHARMA et al. (1997) reported that chicken γ/δ T cell posses cytotoxic activities. If correct, this function may lead to the lysis of infected cells and thereby to the liberation of bacteria from their niche in the cytoplasm of macrophages. It can be speculated that liberated bacteria will be destroyed by professional phagocytes or immunoglobulin and complement mediated effector

mechanisms (KAUFMANN 1988). However, this theory is not supported by experimental evidence and a role of γ/δ T cells as cytotoxic effector cells is still questioned in both the murine and the chicken system. Recently, another function of γ/δ T cells has been proposed, which is strongly supported by experimental evidence in the mouse. According to this work, γ/δ T cell significantly contribute to immunological surveillance, in particular at mucosal membranes and in the skin (SANCHEZ-GARCIA and McCORMACK, 1996). Based on this observation, the γ/δ T cell response would not be important for the elimination of the parasite but would be critical in the initiation of an adequate immune response and in the activation of tissue repair mechanisms. Ultimately, these cells would by essential to maintain and rebuild the splenic architecture. As very little is known about the function of γ/δ T cells even in mice, most of this discussion is speculative. However, the chicken might be an excellent model system to further investigate γ/δ T cell functions, since chickens have comparatively large numbers of these cells.

The hallmark of vaccination is the induction of a memory response by the adaptive immune system. Even though critical for the early control of the pathogen and the initiation of an adaptive immune response, the experiments studied thus far primarily reflect the activation of the innate immune system. To investigate, if the adaptive immune system is already primed during the first week of live, when the first vaccination is given to chickens, prime-boost experiments were performed. Birds were either vaccinated on day one and at 6 weeks of age or left unvaccinated. Comparison of these two groups did not show any statistically significant changes in the lymphocyte subset composition in the spleen or the caecal tonsils one week after the second vaccination. However, slight changes were observed such as an increase of classical α/β T cell numbers in the spleen and an increase in the B cell numbers in caecal tonsils. A kinetic study confirmed this observation. Two weeks after the second immunization a significant increase in the α/β T cell population was found in the spleens. This was paralleled by an increase in the $CD4^+$ T cell frequency, although not significant, increase in the $CD8^+$ T cell numbers. In caecal tonsils no obvious changes were found in the T cell compartment. Since the analysis of the total B cell frequency had indicated an increase in the relative numbers one week after the booster vaccination, the B cell population was analyzed in greater detail at this time point. While the frequency of IgG^+ cells did not change, large changes were observed in the IgM^+ and IgA^+ compartment. The relative numbers of IgM^+ B cells decreased whereas the IgA^+ B cell numbers

increased dramatically from 4% to more than 20%. However, the size of this organ did not allow individual analysis and as a consequence pools of 6 caecal tonsils (from 3 birds) were prepared for cell isolation and subsequent cell staining. This approach allowed the use of a larger number of cell markers but prevented a statistical analysis of the results. However, the observations were confirmed in a subsequent experiment by which the role of two vaccinations was further analyzed. In this study one group of birds was immunized on day one and at 6 weeks of age and a second group only at 6 weeks of age. Again, only minor changes were found in 7 weeks old birds (one week after the one or two immunization). The most obvious finding was an increase in the B cells number in caecal tonsils of those birds, which had received two vaccinations. This was largely due to higher IgM^+ B cell frequencies. One week later, this increase in B cell numbers was still apparent. But at this time point, the frequency of IgM^+ B cells was decreased from the value found one week after vaccination in birds that had received two vaccinations, but IgA^+ B cell frequencies were strongly elevated. In the spleen significant changes were only observed in the α/β T cell compartment with an increase in double vaccinated chickens.

From this work a picture emerges that is in good agreement with the basic concept of vaccination induced immunity. Immunization at one day of age activates the adaptive immune system. As a consequence, re-immunization at 6 weeks of age activates memory cells. In the spleen these are classical α/β T cells, while in the mucosa associated immune system of the caecum B cells are primarily activated. In contrast, birds receiving only one immunization at 6 weeks of age respond weakly. The most striking observation is the strong increase of IgA^+ B cells in the caecal tonsils. While IgM^+ cells dominate in this organ, one week after booster immunization IgA^+ cells dramatically increase and the IgM^+ cell numbers decrease. This observation is best explained by the induction of immunoglobulin class switch events from IgM to IgA as a reaction to the vaccination.

To better understand the response to S. typhimurium vaccination, experiments were performed to investigate the functional activation of T and B lymphocytes in the spleen. T cell function was analyzed through their ability to proliferate in response to the activation (crosslinking) of the T cell receptor. Both, the α/β or the γ/δ TCRs were stimulated thereby inducing a polyclonal activation. These experiments were complemented by assays in which LPS or a crude lysate of S. typhimurium were used as more specific activation signals.

First, the early response of young chickens one and two weeks after primary vaccination was studied. Even though γ/δ T cell numbers consistently increased after vaccination, as shown by flow cytometric analysis, no significant functional activation of these cells could by demonstrated at both time points. One explanation for this discrepancy could be that γ/δ T cells are not activated appropriately by the stimulation protocol used. γ/δ T cells may require a more sophisticated activation signal than simple TCR crosslinking. However, these signals are still poorly understood in both the chicken and the murine system (BUCY et al., 1991). Future experiments should therefore use different stimulation protocols and different readout systems. However, since we still lack a comprehensive understanding of the role of γ/δ T cells in the innate and adaptive immune response, rational experimental protocols are difficult to establish. Thus future work must continue to use the available techniques to screen for functional properties of this unique T cell subset. As discussed above, the chicken with its high frequency of γ/δ T cells may provide a good model for this work. The observation that γ/δ T cells are the first cells responding to Salmonella infection strongly suggests this infection system as an experimental model for γ/δ T cell research.

The analysis of the α/β T cell activation level showed a different picture. At one week of age a slightly increased proliferative activity was found in vaccinated birds in comparison to unvaccinated animals. The response was even stronger at two weeks of age. Despite large individual variations this difference became statistically significant. To our knowledge, this is the first report showing that α/β T cells are functionally activated by S. typhimurium vaccination in the chicken. Interestingly, while γ/δ T cells increased in numbers shortly after immunization or infection, a functional activation could not be shown for these cells. On the contrary, no significant changes in the α/β T cell numbers were found but α/β T cells present in spleens of vaccinated birds clearly changed their functional phenotype. Since α/β T cells represent the most important T lymphocyte subset in the adaptive immune system this observation provides experimental support to the hypothesis that vaccination at the first day of hatch can induce specific immunity.

An interesting response pattern emerged when α/β T cell responses were compared in birds vaccinated twice at one day and at 6 weeks of age or at 6 weeks only. A stronger response was seen in the group which was vaccinated only once indicating that

immunization induced a strong primary response. On the contrary, α/β T cells from birds immunized twice showed a proliferative activity well above background levels but not as high as in the single immunization group. It may be speculated that these cells are of a memory phenotype and may help to boost the recall response to vaccination through their T helper cell activity which is not efficiently measured by simple proliferation assays. From these experiments it becomes clear that future work must be performed with larger groups of animals to allow statistical analysis in particular when a high individual variability is seen. In addition, new readout systems are urgently needed to investigate α/β T cell functions in more detail during primary and secondary immune responses. Recent developments in the field of avian immunology will help to solve this issue at least in part. As discussed in the literature review section, the type of T helper cell responses is of particular importance for the protection of mice and humans from Salmonella infection (OSORIO et al., 2002). T helper 1 (Th1) responses leading to the secretion of IFN-γ are critical for the resolution of infection (BAO et al., 2000), while Th2 responses and the secretion of IL-4 and IL-5 are considered to be related to susceptibility (MEDHAT et al., 1998). Through the work of Lambrecht and colleagues (LAWSON et al., 2001) it is now possible to measure the secretion of the T cell cytokine IFN-γ by an ELISA system and therefore the induction of a Th1 based immune response. Most recently, Kaiser's group at the Institute of Animal Health in England (KAISERet al., 2004) succeeded to identify the genes encoding the Th2 cytokine cluster. Once appropriate reagents to these cytokines are developed it should be feasible to characterize Th2 type immune responses in more detail and to obtain a better understanding of T cell responses to intracellular parasites such as Salmonella.

In addition to the TCR crosslinking studies experiments were performed to investigate lymphocyte responses to S. typhimurium antigens. LPS was used as the prototypic constituent of Salmonella. It is known to be a potent B cell mitogen in mice and humans (SCHARDER et al., 1975). However, no specific lymphocyte proliferative response could be demonstrated to LPS both after primary and secondary immunization. From previous studies it is known, that chickens posess a functional LPS receptor. Macrophages readily respond to LPS with an increased phagocytic activity (KEWAKI et al., 1993), the secretion of several cytokines including IL-6 (SCHNEIDER et al., 2001 Biochemistry) and the production of nitric oxide (KASPERS 1995). In contrast, B cell activation by LPS has not been shown convincingly in the chicken system. This may be

due to a lack of LPS receptors on B cells or to the rapid induction of apoptosis in LPS treated B cells.

In addition to the molecularly defined antigen S. typhimurium LPS, a crude bacterial lysate was used to activate lymphocytes *in vitro*. Similar studies were performed in the mouse model of S. typhimurium infection and have shown significant T cell responses to these antigen preparations (BRITO et al., 1993). In this study no such reactivity was found after primary and secondary immunization. No reactivity or even a reduction below background proliferation was observed if lysates were added to the cultures. This observation may indicate that crude bacterial lysates are toxic to chicken T cells and require additional treatment prior to the application to T cell cultures. Since antigen specific T cell activation is very difficult to be induced in chicken T cells the results may also be interpreted as another example for the problems associated with immunological studies in other systems than mouse and man. Clearly, this aspect deserves a major effort to provide avian immunologists with the technologies and tools to perform antigen specific functional assays.

The work discussed thus far has focused on the T cell system. However, striking responses to booster vaccination were seen in the B cell compartment in particular by IgA^+ B cells as described before. Consequently, the antigen specific IgA response to S. typhimurium vaccination was investigated in detail. The basic knowledge for this type of investigation has been available since the pioneering work by ROSE and ORLANS (1981). Additional tools such as monoclonal antibodies were developed subsequently by several groups (ERHARD et al., 1992) but few studies have been published describing IgA recall responses to defined antigens in the chicken. Most work was performed in the coccidia model (DOLLOUL et al., 2003) as this parasite replicates in the intestinal mucosa and therefore at a location were IgA responses are believed to be of primary relevance (MICHETTI et al., 1994)). Because S. typhimurium replicates in the caecum and invades through the gut mucosa IgA production may be an important effector mechanism in the control of Salmonella infection (MITTRÜCKER and KAUFMAN 2000 and CHEN et al., 2002).

A set of experiments was performed to investigate the IgA response to the S. typhimurium vaccine. From earlier work it is known that IgA antibodies can first be detected at very low levels in the serum of 8–10 days old birds (Kaspers 1989). An antigen specific IgA response at this early stage has not been described thus far. Therefore, this work focused on the recall response to vaccination in older birds with a

mature IgA system. Birds vaccinated right after hatch and re-immunized at 6 weeks of age showed a strong S. typhimurium specific IgA response One, two and three weeks after vaccination. IgA antibody titers were significantly higher in vaccinated than in unvaccinated birds clearly showing that this live vaccine induces a mucosal immune response. Comparable results were obtained in subsequent experiments. Interestingly, four weeks after vaccination IgA antibody titers declined to background values and remained at low levels. In the first experiment groups of vaccinated birds were compared with unvaccinated animals. To investigate the relevance of the primary vaccination on day one after hatch, an experiment was performed in which birds received either two immunizations (day 1 and at 6 weeks) or only one vaccination at 6 weeks of age. This study confirmed the previous observation that birds vaccinated twice exhibit a strong serum IgA response. In striking contrast LPS specific IgA titers remained low in birds vaccinated at 6 weeks of age for the first time. The difference between these two groups was clearly seen one week after the second vaccination and became statistically significant one week later.

Collectively, these results show that birds develop a memory immune response to immunization with a S. typhimurium live vaccine. This vaccine not only activates the innate immune system in neonatal chicks but also leads to the priming of antigen specific lymphocytes. Booster vaccination then activates primed cells from the memory B cell pool to differentiate into IgA secreting plasma cells. As a result antigen, specific antibody titers increase in the circulation. It is important to note that the flow cytometric data are in agreement with this interpretation. As discussed before, the frequency of B cells in caecal tonsils increased in vaccinated birds one week after booster immunization. At that time point the increase was largely due to IgM^+ B cells. However one week later a strong increase of IgA^+ B cells was found in caecal tonsils and paralleled by a decrease in IgM^+ B cell numbers. Additional immuno-histological studies performed in cooperation with Dr. A. Berndt at the National Institute for Animal Health in Jena showed that the frequency of IgM^+ B cells increased in vaccinated birds one week after the second immunization in both spleen and ceacal tonsils. Interestingly, while the numbers of IgM^+ cells decreased one week later, the frequency of IgA^+ cells increased. Together these data strongly support the concept of a vaccination induced immunoglobulin class switch from IgM to IgA with the subsequent secretion of antigen specific IgA antibodies. IgA secreting cells are most probably located in the ceacal tonsils as suggested by the flow cytometric data and to certain extend in the spleen as revealed by immuno-histology.

The primary function of IgA antibodies is to protect mucosal surfaces from invading pathogens (MUIR et al., 1998). IgA is particularly suited to meet this task since the association with the poly-Ig receptor on the baso-lateral surface of the mucosal epithelium allows the transport of IgA from serum through the epithelial cell to the luminal surface (SHELDRAKE et al., 1984). This process also provides the association of the IgA molecule with the secretory component which protects the molecule from proteolytic digestion in the intestinal tract (HOLMGREN et al., 1992). The chicken is known to secret large amounts of IgA through the bile (ROSE and ORLANS 1981). This IgA will ultimately reach the gastro-intestinal tract.

While high IgA titers in the serum are a clear indication for a memory immune response they may not be of significant functional relevance in the circulation. Therefore, the presence of antigen specific IgA antibodies in the bile was investigated. High IgA titers were found in two independent experiments. These titers clearly exceeded those of unvaccinated birds or birds vaccinated only once at 6 weeks of age. In these studies large differences were found between individual birds leading to large standard deviations. As a consequence these differences were not statistically significant. However, the work provides strong evidence that antigen specific serum IgA is indeed secreted through the bile into the gut. Subsequent experiments must take this problem into account by using larger numbers of animals to allow statistical analysis.

This work has shown that S. typhimurium live vaccines induce a strong innate immune response in neonatal chickens which, as known from other studies (METHNER et al., 2004), leads to the elimination of the bacteria. In addition, the vaccine strain induces a strong memory response which is best demonstrated by the induction of high antigen specific IgA antibody titers in response to the booster immunization. As immunoglobulin class switch and IgA secretion require the help by Th2 type T cells this work also indicates that a memory T cell response is induced by the vaccine.

Future work in this system has to address a number of questions. The functional relevance of LPS specific IgA is not clear thus far. The working hypothesis would be that luminal IgA prevents the adhesion and invasion of bacteria and thus helps to limit the systemic Salmonella infection. However, this work requires established intestinal epithelial cell lines which are currently not available in the chicken system. Therefore, intestinal loop models may be the first choice for this type of functional studies.

Furthermore, the role of T cells is far from resolved and clearly requires additional work. As outlined, the recent progress made in avian cytokine research should help to address this issue on a functional level.

6 SUMMARY

Investigation of the humoral and cellular immune response of chickens to Salmonella typhimurium live vaccine

Immunization of chickens with live attenuated Salmonella vaccines is widely used to control the contamination of poultry products with Salmonella enterica serovars. In this study the immune response to vaccination with a live S. typhimurium vaccine strain was investigated. The vaccine was given on day one after hatch and at 6 weeks of age by crop instillation. Flow cytometry was used to quantify the response of lymphocytes to the vaccine in single cell suspensions from spleens and caecal tonsils. One week after the first immunization a significant increase in the frequency of γ/δ T cells was seen in vaccinated birds in comparison with unvaccinated animals. This increase was paralleled by an increase in the CD8 positive lymphocyte population and a compensatory decrease in the α/β T cell frequency. One week later the γ/δ T cell numbers were still elevated in comparison with control birds. At this time point a slight increase in the B cell frequency was also observed. Birds vaccinated at day one and at 6 weeks of age showed no significant differences of lymphocyte frequencies in spleens and caecal tonsils one week after the second immunization in comparison with unvaccinated birds. However, one week later α/β T cell numbers significantly increased in the spleen and the relative numbers of IgA^+ B cells were strongly elevated in the caecal tonsils. The same observation was made if birds vaccinated twice were compared with birds that had received only one immunization at 6 weeks of age. These studies clearly show that the vaccine induced an early innate immune response in spleens of neonatal birds while a prime-boost protocol leads to the activation of classical α/β T cells. T cell proliferation assays showed that no significantly different proliferative activity to T cell receptor crosslinking was observed one week after vaccination in neonatal birds. One week later splenic α/β T cells strongly proliferated to this stimulus. No such response could be demonstrated in spleens of 7 weeks old birds that had received two vaccinations. The polyclonal T cell activation did not differ significantly from that of birds vaccinated only once.

To further analyse if the vaccine induced increase in IgA^+ B cells in caecal tonsils, which lead to an increased IgA response, serum levels of vaccinated birds (day 1 and

at 6 weeks of age) and unvaccinated birds were compared. One, two and three weeks after the booster immunization significant ($p \leq 0.05$) increases in S. typhimurium LPS specific IgA titers were observed and were paralleled by a clear increase of IgA titers in the bile. The same observation was made if birds receiving two immunizations were compared with animals vaccinated only at 6 weeks of age. From this work it can be concluded that immunization with a live S. typhimurium vaccine leads to the induction of a memory immune response and to the activation of the secretory IgA system.

7 ZUSAMMENFASSUNG

Untersuchungen zur humoralen und zellulären Immunantwort auf eine Salmonella typhimurium Lebendvakzine beim Huhn

Die Immunisierung von Hühnern mit attenuierten Salmonella-Lebendimpfstoffen wird weit verbreitet eingesetzt, um die Kontamination von Geflügelprodukten mit Salmonella enterica Serovaren zu kontrollieren. In der hier vorgelegten Studie wurde die Immunreaktion auf die Impfung mit einem Salmonella typhimirium Lebendimpfstoff untersucht.

Die Impfung wurde einen Tag nach dem Schlupf und im Alter von 6 Wochen durch Kropfinstillation verabreicht. Mit Hilfe der Durchflusszytometrie wurde die Reaktion der Lymphozyten auf die Impfung in Einzelzellsuspensionen aus Milz und Caecaltonsillen quantifizieren. Eine Woche nach der ersten Immunisierung wurde ein signifikanter Anstieg der Frequenz von γ/δ T−Zellen bei den geimpften Vögeln im Gegensatz zu den nicht geimpften Tieren beobachtet. Dieser Anstieg ging mit einer Steigerung der $CD8^+$ Lymphozyten-Population und einer kompensatorischen Abnahme der α/β T-Zell-Frequenz einher. Auch eine Woche später war die Anzahl der γ/δ T-Zellen bei den immunisierten Tieren im Vergleich zu den Kontrolltieren erhöht. Zu diesem Zeitpunkt wurde ebenso ein leichter Anstieg der B-Zell-Frequenz beobachtet. Die Vögel, welche am ersten Tag nach dem Schlupf und mit 6 Wochen geimpft worden waren, zeigten eine Woche nach der zweiten Immunisierung im Vergleich zu den nicht geimpften Tieren keine signifikanten Unterschiede in der Frequenz der Lymphozyten in Milz und Caecaltonsillen. Eine Woche später wurde dagegen eine vermehrte Anzahl an α/β T-Zellen in der Milz und ein deutlicher Anstieg der IgA^+ B-Zellen in den Caecaltonsillen nachgewiesen. Die selbe Reaktion wurde beobachtet, wenn Vögel, welche zweimal geimpft worden waren, mit Hühnern verglichen wurden, die nur eine Immunisierung mit 6 Wochen erhalten hatten.

Diese Studien zeigen deutlich, dass die Impfung eine frühzeitige unspezifische Immunreaktion in der Milz der Küken induzierte, während die Reimmunisierung mit 6 Wochen zu einer Aktivierung der klassischen α/β T-Zellen führt.

T-Zell Proliferations-Assays zeigten eine Woche nach der Impfung von frisch geschlüpften Küken keine signifikanten Unterschiede in der proliferativen Aktivität von Zellen, die durch Quervernetzung der T-Zellenrezeptors aktiviert worden waren. Eine

Woche später zeigte sich dagegen eine signifikant erhöhte Proliferation der α/β T–Zellen aus der Milz der immunisierte Tiere. Eine solche Reaktion konnte nicht in den Milzen 6 Wochen alter Vögel gezeigt werden, welche zwei Impfungen erhalten hatten. Deren polyklonale T-Zellreaktion wich nicht signifikant von der der Vögel ab, die nur eine Impfung erhalten.

Um zu prüfen, ob die beobachtete Erhöhung der IgA^+ B-Zellfrequenzen in den Caecaltonsillen immunisierter Tiere mit einer deutlichen IgA-Antikörperproduktion einhergeht, wurden Seren von geimpften Vögeln (im Alter von einem Tag und 6 Wochen) und ungeimpften Tieren verglichen. Jeweils eine, zwei und drei Wochen nach der Reimmunisierung wurde ein signifikanter Anstieg von S. typhimurium LPS spezifischen IgA-Titern ($p < 0,05$) nachweisbar und parallel dazu eine klare Zunahme von IgA-Titern in der Galle deutlich. Die selbe Beobachtung wurde gemacht, wenn Gruppen von Tieren verglichen wurden, die zwei Immunisierungen an Tag 1 und mit 6 Wochen oder nur eine Immunisierung mit 6 Wochen erhalten hatten.

Aus diesen Untersuchungen kann gefolgert werden, dass die Immunisierung mit einem Salmonella typhimurium Lebendimpfstoff zur Induktion einer immunologischen Gedächtnisreaktion und zur Aktivierung des sekretorischen IgA-Systems führt.

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9 ANNEXES

Annex 1

Summary of the results obtained in experiment 1-3. For details see 4.1.1.

Control birds

	%CD4	%CD8	%TCR-1	%TCR-2	%TCR-3	L-chain
Experiment 1	20,73	17,33	10,33	27,32	13,44	17,43
	28,72	26,91	9,76	41,73	9,08	12,29
	26,74	25,52	9,5	38,55	11,04	14,78
	30,02	24,42	5,00	43,28	27,33	11,36
	13,66	18,91	6,56	24,42	10,61	29,94
Experiment 2	28,67	32,19	12,97	47,81	9,39	11,31
	52,31	41,93	9,43	74,39	22,61	10,92
	40,08	31,83	20,27	45,86	10,65	22,35
	30,02	38,62	13,83	41,13	9,01	30,14
	33,44	41,76	11,75	39,44	6,03	43,87
	45,9	28,71	20,46	59,33	13,10	15,08
Experiment 3	32,57	32,56	10,29	43,49	10,77	9,10
	28,35	25,47	11,92	32,39	8,87	23,4
	34,27	29,76	15,20	47,17	8,83	27,14
	35,75	26,42	12,11	31,85	5,34	18,48
	30,79	27,53	21,25	41,07	9,94	21,73
MV	**32,00**	**29,37**	**12,54**	**42,45**	**11,63**	**19,96**
SD	**8,76**	**6,84**	**4,59**	**11,63**	**5,50**	**9,06**

Vaccinated birds

	%CD4	%CD8	%TCR-1	%TCR-2	%TCR-3	% L-chain
Experiment 1	25,33	9,65	16,29	30,78	5,91	10,58
	15,44	64,92	38,63	16,28	2,35	13,27
	10,90	17,02	35,65	15,23	8,78	21,53
	16,85	14,03	40,92	20,99	5,70	13,59
	21,63	22,58	24,17	26,66	5,77	14,47
Experiment 2	28,68	63,86	32,14	38,94	11,49	22,76
	21,24	75,18	48,72	37,63	7,71	11,25
	21,99	55,6	37,88	37,94	12,38	29,21
	26,84	50,93	39,6	35,95	10,72	25,42
	32,01	51,19	32,76	47,61	12,43	21,93
	34,21	46,18	33,69	47,8	11,34	18,33
Experiment 3	27,27	53,34	31,00	45,78	6,62	11,45
	29,43	51,31	41,19	39,78	11,11	15,91
	28,00	41,91	37,55	42,65	9,73	19,41
	30,47	47,21	33,73	38,85	9,37	14,11
	28,29	53,72	45,69	28,96	8,03	10,65
MV	**24,91**	**44,91**	**35,60**	**34,49**	**8,72**	**17,12**
SD	**6,21**	**18,06**	**7,35**	**9,78**	**2,71**	**5,38**
T-Test	**0,0200**	**0,0100**	**0,0600**	**0,0500**	**0,0800**	**0,3100**

Annex 2

Summary of the results obtained in experiment 4-6. For details see 4.1.2.

Control birds

	%CD4	%CD8	%TCR-1	%TCR-2	% TCR-3	L-chain
Experiment 4	83,29	29,90	24,90	36,37	37,93	20,92
	83,08	22,19	22,73	13,99	36,79	22,87
	75,86	31,14	24,31	25,35	67,19	18,20
	80,94	23,79	31,10	11,28	36,41	16,87
	53,16	29,70	34,45	10,96	55,14	17,06
Experiment 5	45,03	45,96	11,36	64,25	12,58	10,71
	50,32	38,47	7,67	65,75	18,16	7,00
	53,66	43,05	9,33	70,84	16,13	5,00
	33,35	44,53	12,76	65,55	12,85	14,67
	42,11	48,14	8,31	57,15	13,92	9,07
	40,62	37,26	15,00	54,69	15,19	8,20
Experiment 6	28,58	35,08	14,38	39,6	8,90	33,16
	29,17	50,02	7,51	42,22	14,12	41,64
	51,61	47,42	11,32	64,89	15,53	12,85
	67,98	25,18	7,07	75,87	10,98	8,72
	37,08	32,01	13,43	45,89	9,30	28,74
	54,01	32,5	10,4	71,55	13,62	7,92
MV	**54,05**	**36,26**	**15,65**	**48,01**	**23,22**	**16,68**
SD	**18,40**	**8,71**	**8,33**	**21,40**	**16,73**	**9,86**

Vaccinated birds

	%CD4	%CD8	%TCR-1	%TCR-2	%TCR-3	%L-chain
Experiment 4	71,79	30,37	38,85	14,19	20,77	42,1
	79,14	36,05	42,64	19,00	24,89	56,00
	85,15	24,35	42,37	31,48	29,21	42,96
	72,09	31,48	35,08	30,08	40,83	48,63
	81,60	25,35	45,16	28,98	29,77	45,40
Experiment 5	30,03	45,64	27,95	34,87	14,75	31,18
	37,31	55,32	25,26	45,03	17,39	22,15
	33,25	41,80	28,34	39,52	15,8	22,32
	29,51	42,79	27,97	49,21	15,68	17,31
	33.31	33,36	22,77	42,34	15,43	26,84
	39,68	57,84	17,07	65,88	15,31	20,56
Experiment 6	35,01	43,30	14,67	56,13	12,82	18,54
	40,03	44,02	16,82	61,64	11,92	12,80
	34,08	45,77	25,79	52,19	9,31	16,92
	35,52	38,04	28,06	49,65	10,05	19,71
	32,77	37,20	24,89	46,34	12,66	26,25
	33,05	30,42	17,93	47,71	7,01	33,97
MV	**48,13**	**39,01**	**28,33**	**42,01**	**17,86**	**29,63**
SD	**20,51**	**9,16**	**9,25**	**13,75**	**8,50**	**12,58**
T-Test	**0,4434**	**0,3906**	**0,0014**	**0,3539**	**0,2644**	**0,0029**

Annex 3

Summery of the results obtained in experiment 7. For details see 4.1.3

Control birds

Organs	Bird No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	%TCR-3	% L-chain
Spleen	1	84,05	35,85	60,74	23,38	55,10	12,61	22,95
	2	83,71	23,30	59,51	26,55	35,69	12,85	18,49
	3	80,60	26,18	42,29	30,09	41,12	19,27	16,86
	4	81,51	27,21	52,76	28,55	44,60	13,51	19,55
	5	81,98	24,81	56,38	20,62	42,70	17,16	16,86
	MW	82,37	27,47	54,34	25,84	43,84	15,08	18,94
	SD	1,31	4,39	6,63	3,44	6,36	2,66	2,25
Caecal Tonsils	1	48,07	33,11	29,44	10,60	37,30	12,41	21,58
	2	33,12	10,76	21,29	9,95	18,50	4,34	13,09
	3	27,30	2,83	25,41	5,86	32,60	10,66	55,15
	MV	**36,16**	**15,57**	**25,38**	**8,80**	**29,47**	**9,14**	**29,94**
	SD	**10,71**	**15,70**	**4,08**	**2,57**	**9,78**	**4,25**	**22,24**

Vaccinated birds

Organs	Bird No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	%TCR-3	% L-chain
Spleen	1	83,42	32,89	67,30	25,50	48,98	17,38	11,28
	2	82,09	26,23	56,62	24,44	59,63	17,04	25,35
	3	80,57	20,67	61,91	13,93	52,94	14,40	24,78
	4	52,13	14,92	39,58	13,08	35,13	7,79	33,84
	5	81,54	27,87	62,98	21,40	50,36	10,11	21,34
	MV	75,95	24,52	57,68	19,67	49,41	13,34	23,32
	SD	13,36	6,91	10,81	5,83	8,97	4,25	8,15
Caecal Tonsils	1	40,49	18,95	29,88	13,26	23,85	12,50	53,65
	2	57,94	29,28	26,68	13,62	37,65	13,75	33,34
	3	60,04	25,04	34,63	13,31	35,15	14,17	28,77
	MV	**52,82**	**24,42**	**30,40**	**13,40**	**32,22**	**13,47**	**38,59**
	SD	**10,73**	**5,19**	**4,00**	**0,20**	**7,35**	**0,87**	**13,24**
	T –Test (spleen)	**0,3442**	**0,4608**	**0,5861**	**0,0894**	**0,3103**	**0,4784**	**0,3058**

Annex 4; part 1

Summary of the results obtained in experiment 8. For details see 4.1.4

Control birds

Organs	Bird No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	%TCR-3	% L-chain	%IgG	%IgM	%IgA
Spleen	1	75,00	19,70	37,88	24,70	44,35	5,88	18,87	0,34	17,00	1,11
	2	71,49	20,07	51,91	26,75	40,53	15,35	24,14	0,93	24,35	3,97
	3	71,89	28,65	42,27	19,41	43,79	14,56	23,65	1,13	23,65	5,70
	4	79,70	32,63	47,22	12,61	52,53	6,76	20,19	0,81	19,14	1,72
	5	80,25	23,30	52,63	18,23	43,48	18,08	20,47	1,23	20,94	1,12
	6	75,60	27,47	47,52	18,50	49,04	15,44	24,20	2,30	24,19	4,47
	7	63,02	24,05	37,08	13,30	42,12	5,96	33,24	2,57	33,68	0,82
	8	68,08	22,14	43,39	18,61	35,13	12,80	32,59	2,96	30,98	2,36
	9	77,59	22,86	45,32	21,06	47,76	17,85	19,32	3,07	18,69	1,94
	MV	**73,62**	**24,54**	**45,02**	**19,24**	**44,30**	**12,52**	**24,07**	**1,70**	**23,62**	**2,58**
	SD	**5,30**	**4,01**	**5,17**	**4,36**	**4,79**	**4,72**	**5,10**	**0,96**	**5,29**	**1,63**
Caecal Tonsils	1	41,92	22,11	21,73	13,69	27,38	9,69	-	2,02	24,49	1,17
	2	38,62	25,22	15,75	11,66	36,45	11,04	-	3,74	31,57	8,44
	3	45,95	20,02	23,15	16,96	36,53	10,50	-	3,48	37,61	3,42
	MV	**42,16**	**22,45**	**20,21**	**14,10**	**33,45**	**10,41**	**-**	**3,08**	**31,22**	**4,34**
	SD	**3,00**	**2,14**	**3,21**	**2,18**	**4,29**	**0,55**	**-**	**0,76**	**5,36**	**3,04**

Annex 4 ; part 2

Vaccinated birds

Organs	Birds No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	%TCR-3	% L-chain	%IgG	%IgM	%IgA
Spleen	1	87,10	35,94	53,77	17,16	61,27	21,27	11,18	0,61	11,75	1,36
	2	66,75	30,19	38,30	20,83	42,73	15,95	29,52	0,41	29,04	2,42
	3	89,97	29,94	60,01	15,25	61,89	20,58	19,01	1,64	18,33	0,98
	4	75,36	26,89	51,14	18,31	50,04	13,84	25,20	5,48	25,86	4,96
	5	80,42	31,43	47,41	25,76	44,71	15,67	20,34	1,67	20,28	7,52
	6	82,02	28,79	54,30	19,45	59,45	12,30	14,54	3,23	14,82	4,20
	7	76,03	32,21	43,23	23,81	47,10	18,36	24,28	2,47	24,21	4,12
	8	83,45	38,12	45,75	28,17	48,72	15,36	19,54	2,42	18,80	8,90
	9	75,56	20,60	51,93	22,84	42,92	11,38	22,73	4,88	21,39	9,01
	MV	79,63	30,46	49,54	21,29	50,98	16,08	20,70	2,53	20,50	4,83
	SD	6,64	4,78	6,18	3,97	7,38	3,24	5,25	1,65	5,08	2,88
C.T	1	76,06	45,96	20,83	22,15	27,23	15,66	-	6,59	30,23	15,73
C.T	2	54,74	22,04	28,37	16,39	37,16	7,83	-	2,06	28,10	25,16
C.T	3	52,57	23,68	30,97	19,58	31,64	10,27	-	2,26	21,14	24,06
	MV	**61,12**	**30,56**	**26,72**	**19,37**	**32,01**	**11,25**	**-**	**3,64**	**26,49**	**21,65**
	SD	**10,60**	**10,91**	**4,30**	**2,36**	**4,06**	**3,27**	**-**	**2,09**	**3,88**	**4,21**
	T -Test (spleen)	**0,0639**	**0,0167**	**0,1333**	**0,3412**	**0,0500**	**0,1002**	**0,2114**	**0,2408**	**0,2455**	**0,0774**

Annex 5

Summary of the results obtained in experiment 9. For details see 4.1.5

Birds vaccinated at 6 weeks of age (group1)

Organs	Bird No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	% L-chain	%IgG	%IgM	%IgA
Spleen	1	72,93	31,8	51,93	31,64	36,33	21,49	2,19	21,01	2,4
	2	74,09	24,82	55,48	26,57	35,86	20,87	0,81	19,8	0,89
	3	75,71	37,88	37,53	15,44	49,75	19,67	0,93	19,88	0,58
	4	74,54	28,75	50,66	26,48	37,87	18,3	1,19	18,48	0,95
	5	55,71	23,7	46,38	20,14	31,25	36,96	1,5	35,69	1,22
	6	60,96	37,88	36,63	13,7	37,04	34,07	0,36	29,76	0,44
	7	55,15	24,2	36,25	18,73	30,31	34,23	0,55	33,67	0,45
	MV	**63,47**	**29,84**	**41,80**	**20,21**	**34,99**	**29,46**	**1,06**	**28,46**	**0,98**
	SD	**8,64**	**5,72**	**7,50**	**6,09**	**5,89**	**7,53**	**0,58**	**6,78**	**0,63**
C.T Pool		**38,66**	**29,71**	**19,56**	**8,94**	**21,49**	**50,09**	**0,95**	**49,38**	**0,9**

Birds vaccinated at 1 day and 6 weeks of age (group 2)

Organs	Bird No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	% L-chain	%IgG	%IgM	%IgA
Spleen	1	68,19	23,06	47,73	26,63	37,61	26,32	1,53	25,52	1,04
	2	63,07	30,68	47,93	13	39,62	31,5	0,9	31,64	0,76
	3	69,48	30,26	42,79	30,35	33,35	24,01	1,03	23,45	0,42
	4	81,16	29,08	59,16	34,25	40,35	20,38	1,67	20,56	2,17
	5	58,62	28,05	43,07	14,09	35,32	32,87	1,51	32,45	0,58
	6	57,94	27,07	32,48	25,36	32,51	37,11	0,76	34,25	0,56
	7	64,62	30,12	42,19	16,37	39,34	31,79	0,81	30,4	0,69
	MV	**60,34**	**26,31**	**40,19**	**21,00**	**33,53**	**34,26**	**1,24**	**32,99**	**1,29**
	SD	**7,33**	**2,46**	**7,46**	**7,77**	**2,93**	**5,36**	**0,36**	**4,77**	**0,55**
C.T Pool		**19,60**	**12,15**	**6,13**	**7,95**	**10,10**	**70,11**	**1,73**	**65,63**	**4,06**
	T –Test (spleen)	**0,8560**	**0,5637**	**0,9873**	**0,7991**	**0,9872**	**0,5010**	**0,7322**	**0,4176**	**0,7730**

Annex 6

Summary of the results obtained in experiment 10. For details see 4.1.5

Birds vaccinated at 6 weeks of age (group 1)

Organs	Bird No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	%L-chain	%IgG	%IgM	%IgA
Spleen	1	73,19	30,26	44,19	8,29	43,92	19,50	0,95	22,89	0,97
	2	53,44	24,72	25,76	14,30	33,21	34,20	1,00	34,65	0,45
	3	65,49	42,75	24,04	10,63	39,34	32,48	0,41	31,91	1,00
	4	54,55	33,38	23,16	13,91	41,90	12,07	0,69	13,91	0,75
	5	64,88	39,14	27,38	11,87	44,72	30,86	0,83	32,55	0,90
	6	47,78	28,07	20,51	16,03	28,05	22,31	0,79	22,40	0,64
	MV	**59,89**	**33,05**	**27,51**	**12,51**	**38,52**	**25,24**	**0,78**	**26,39**	**0,79**
	SD	**9,49**	**6,83**	**8,50**	**2,80**	**6,60**	**8,71**	**0,21**	**8,01**	**0,21**
C.T Pool		**38,14**	**20,30**	**20,93**	**10,03**	**24,52**	**36,38**	**2,39**	**39,15**	**5,48**

Birds vaccinated at 1 day and 6 weeks of age (group 2)

Organs	Bird No.	%CD3	%CD4	%CD8	%TCR-1	%TCR-2	% L-chain	%IgG	%IgM	%IgA
Spleen	1	51,68	32,49	21,38	10,47	47,26	22,87	0,79	21,34	0,39
	2	58,98	29,47	28,85	12,37	48,86	19,53	1,13	19,28	0,39
	3	71,57	44,80	25,73	14,02	56,86	16,47	0,91	16,07	0,73
	4	53,65	30,17	23,21	11,62	46,31	26,43	2,23	26,33	1,17
	5	61,15	39,24	21,57	15,02	47,05	12,46	0,96	13,52	0,43
	6	51,06	25,52	28,97	13,14	38,45	19,09	0,97	19,82	0,73
	MV	**58,02**	**33,62**	**24,95**	**12,77**	**47,47**	**19,48**	**1,17**	**19,39**	**0,64**
	SD	**7,77**	**7,11**	**3,44**	**1,65**	**5,88**	**4,86**	**0,53**	**4,42**	**0,31**
C.T Pool		**31,96**	**14,24**	**16,55**	**9,70**	**21,54**	**53,30**	**3,00**	**28,30**	**22,90**
	T -Test (sleen)	**0,7164**	**0,8918**	**0,5182**	**0,8448**	**0,0329**	**0,1956**	**0,1458**	**0,0990**	**0,3656**

Annex 7

Summary of the results obtained in experiment 11. For details see 4.2

Control birds

	Bird No.	F71D7	TCR-1	TCR-2	LPS	Lysate
Experiment 1	**1**	4350	1884	16110	1062	2539
	2	3288	4002	51264	1911	1862
	3	1498	2259	19736	1155	1760
	4	1482	749	8034	863	2819
	5	908	459	4522	563	162
	6	1074	838	2674	478	901
	MV	**2100**	**1699**	**17057**	**1005**	**1674**
	SD	**1271**	**1329**	**16450**	**472**	**911**
Experiment 2	**1**	491	2057	28999	2923	
	2	697	10963	15597	23963	
	3	756	9741	15178	11280	
	4	156	498	2429	2375	
	5	3789	2748	16260	179	
	6	797	2157	7560	8644	
	MV	**1114**	**4694**	**14337**	**8227**	
	SD	**1215**	**4073**	**8253**	**8003**	

Vaccinated birds

	Bird No.	F71D7	TCR-1	TCR-2	LPS	Lysate
Experiment 1	**1**	2477	1245	18495	1777	418
	2	1265	917	44552	1630	2727
	3	7167	3550	86833	5145	890
	4	4779	2363	6373	3323	256
	5	3697	3635	4571	3863	331
	6	4633	1719	27949	1317	696
	MV	**4003**	**2238**	**31462**	**3086**	**886**
	SD	**1868**	**1157**	**28197**	**1395**	**851**
Experiment 2	**1**	4718	15948	18620	10316	
	2	2316	12897	28545	19855	
	3	3489	4395	40949	10512	
	4	2632	1806	6376	1444	
	5	1937	3059	8062	23646	
	6	4968	5611	15732	8179	
	MV	**3343**	**7286**	**19714**	**12325**	
	SD	**1161**	**5253**	**11970**	**7391**	
	T -Test	**0,0691**	**0,3686**	**0,1368**	**0,2980**	**0,1885**

Annex 8

Summary of the results obtained in experiment 12. For details see 4.2

Control

Bird No	F71D7	TCR-1	TCR-2	LPS	Lysate
1	3883	4053	3098	4562	1414
2	5824	3027	5824	955	955
3	859	1411	859	604	604
4	859	2211	1328	868	868
5	2151	2944	1952	1638	1638
6	12609	5198	12174	1258	1258
MV	**4364**	**3141**	**4206**	**1648**	**1123**
SD	**4078**	**1223**	**3914**	**1343**	**349**

Vaccinated

Bird No.	F71D7	TCR-1	TCR-2	LPS	Lysate
1	12321	985	44337	3865	1311
2	3976	2254	113269	3027	666
3	1872	1760	92260	3112	951
4	3442	3354	111279	3275	2019
5	3034	3916	146599	2494	828
6	1141	2824	7692	2820	1388
MV	**4298**	**2516**	**85906**	**3099**	**1194**
SD	**3712**	**978**	**46479**	**422**	**448**
T -Test	**0,9790**	**0,3940**	**0,0109**	**0,0608**	**0,7858**

Annex 9

Summary of the results obtained in experiment 13. For details see 4.2

Birds vaccinated at 6 weeks of age (group 1)

Bird No.	F71D7	TCR-2	LPS	Lysate
1	1013	90306	2087	1629
2	912	120407	1370	3080
3	910	87236	1333	1315
4	898	64133	812	1873
5	2118	119097	989	1139
6	1385	97048	1447	2285
7	1377	6761	3723	582
MV	**12311**	**83570**	**1680**	**1700**
SD	**413**	**36129**	**913**	**756**

Birds vaccinated at 1 day and 6 weeks of age (group 2)

Bird No.	F71D7	TCR-2	LPS	Lysate
1	804	21579	667	403
2	406	49577	412	190
3	1273	84248	1435	320
4	337	22241	490	346
5	2280	37794	937	401
6	1815	71875	1565	479
7	1027	52822	284	323
MV	**1135**	**48591**	**827**	**352**
SD	**664**	**21951**	**468**	**84**
T -Test	**0,7699**	**0,0705**	**0,0724**	

Used chemicals

1 AppliChem, Darmstadt

2 Amersham Pharmacia, Freiburg

3 Ratioharm, Ulm

4 Stigma, Deisenhofer, S. typhimurium Nr. (L.3216)

5 Stigma, Deisenhofer

6 Merck, Darmstadt

7Southern Biotechnology Associates, Echoing Germany

8 Sigma-Aldrich FineChemikalss, Taufkirchen, Germany

9 DAB; Merk, Darmstadt, Germany

10 Riedel de Haen, Selze-Hannover, Germany

11 Nunc, Wiesenbaden

12 Tecan M8/4R Columbus Plus

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