Immunological characterisation of two chicken lines

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Immunological characterisation of two chicken lines

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Two chicken lines were divergently selected for antibody response to sheep red blood cells (SRBC). Several experiments were conducted to investigate what mechanisms the immune system of the chickens uses to realize either the high (H) or the low (L) antibody production. The lines did not differ in phagocytic activity and there were no indications for line differences in antigen presentation. Selection affected both the T cell and the B cell compartment differently in the two lines. T cell activity was presently found to be higher in the L line. A relative higher number of B cells was found in the H line, which might attribute to a higher antibody response in this line. The lines also differed in cellular organization of the spleen. The organization in the H line spleen might favour antibody response. On the other hand, higher percentages $CD8^+$ cells were found in the L line and these might suppress antibody response. The percentages of $CD4^+$ cells differ also between the lines. $CD4^+$ cells are able to stimulate immune responses. Which response is stimulated - cellular or humoral - depends on the cytokines released by the immune cells.
When compared with the random bred control line, the high antibody response was found to be largely SRBC specific. The low antibody response in the L line seems to be less antigen specific. Selection lines can be readily used as *in vivo* models when the immune repertoire is genetically fixed and fully characterized.

Voor mijn ouders,
Alfred, Dian, Ella en Mike.

Voorwoord

Eindelijk ben ik dan aangeland bij het laatste deel van mijn proefschrift. Een hele opluchting, ook voor allen die meegewerkt hebben aan de totstandkoming van dit geheel en vooral ook voor het 'thuisfront'. Het thuisfront, Alfred, Dian, Ella en Mike, zijn dan ook de eersten die ik wil bedanken voor hun steun (vooral van Alfred) en zelfs begrip (ook van Alfred) en in ieder geval veel afleiding (door alle vier). Deze afleiding heeft natuurlijk wel wat vertragend gewerkt op de afronding van dit boekje, zodat er steeds voorzichtiger werd gevraagd "Hoe is't met het boekje?" Het vorderde gestaag, en inderdaad, het is afgekomen.

Toen ik in 1986 bij Veehouderij 'wat onderzoek ging doen aan de selectie-lijnen' was er geen sprake van een promotie-onderzoek. Echter, toen mijn eerste part-time contract na ruim 3 jaar afliep, zag Mevr. dr. ir. A.J. van der Zijpp, wel mogelijkheden om er op te promoveren. Nog een jaartje full-time onderzoek en dan moest het kunnen. Beste Akke, dat jaartje onderzoek is wel iets uitgelopen en full-time is het nooit geworden. Ik weet dat je eraan getwijfeld hebt of dit boekje er ooit wel zou komen en je was niet de enige. Zonder jouw hulp was het er ook nooit gekomen. De begeleiding die ik van je kreeg, met name tijdens het schrijven van mijn eerste artikelen zijn zeer belangrijk geweest voor mijn 'wetenschappelijke vorming'. Hiervoor, maar ook voor het voorwerk en het denkwerk dat je verricht hebt en de sturende discussies ben ik je bijzonder erkentelijk. Het is jammer dat je waarschijnlijk niet bij de verdediging van dit proefschrift aanwezig zal zijn, omdat je nieuwe baan in Ethiopië en Kenya dit onmogelijk maakt. Ik hoop echter dat je dit proefschrift met enige voldoening zal lezen.

Toen eenmaal het idee van 'promoveren op de selectie-lijnen' geboren was, bleek een promotor snel gevonden: Professor dr. J.P.T.M. Noordhuizen. Beste Jos, onze gesprekken vonden zelden na afspraak plaats. De vragende wenkbrauw als je mij tegen kwam achter de pc op het secretariaat van Veehouderij, zorgde er meestal wel voor dat daar het een en ander betreffende het proefschrift, financiën, projectvoorstellen etc. besproken werd. Aan het einde van mijn pc-dag kon je dan meestal weer wat leesvoer in je postvak vinden. De snelheid waarmee je dat leesvoer corrigeerde en de berustendheid waarmee je de tijd zag verstrijken zonder dat de afronding van het proefschrift in zicht kwam bewonder ik. Maar ik ben blij dat je vertrouwen hield in een goede afloop.

In Dr. ir. H.K. Parmentier, mijn co-promotor, had ik een zeer goede begeleider tijdens het laatste deel van mijn promotie-onderzoek en de schrijffase van dit boekje. Je begrijpelijke uitleg van de immunologische gebeurtenissen binnen een dier en je nieuwsgierigheid naar het 'hoe en wat in de kip' zijn kenmerken van de ware universitaire docent. Voor mijn onderzoek was je vooral belangrijk als praatpaal (of was het andersom?) en als iemand die verstand heeft van Immunologie. Henk, bedankt.

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nog vaak zullen voeren.

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Er zijn heel wat mensen die bijgedragen hebben aan dit proefschrift. Daarvan zal ik er hier slechts een paar noemen. Ineke Puls, mijn kamergenoot op Zodiac, heeft als vertaler of thesaurus, dierenarts (praktisch zowel als theoretisch), koffiehaler (voor ons of bij ons) en gesprekspartner over de meest uiteenlopende onderwerpen een veelzijdige invloed op dit proefschrift gehad. Zonder de hulp van Nanette van Hapert en Truus Gijsbertse (de bewoners van het secretariaat) had dit proefschrift nog heel lang op zich laten wachten, want er zijn heel wat dingen die zij wel konden en ik niet (met name op het gebied van printers, papier en floppy's). Naast collega's waren er ook nog studenten (met inbegrip van stagiaires) die een zeker stempel op dit proefschrift hebben gedrukt. Dankzij (soms ook ondanks) hun veelal praktische hulp bestaat dit proefschrift uit 8 hoofdstukken. Een van de studenten, Natasja Gianotten, wil ik hier met name noemen. Met veel enthousiasme heeft zij zich op het onderzoek geworpen, uit de co-auteurschappen mag wel blijken dat dit zeer gewaardeerd is.

Het is natuurlijk niet zo dat alleen (ex-)medewerkers van de vakgroep Veehouderij positieve bijdragen hebben geleverd aan dit proefschrift. De discussies met Suzan Jeurissen en Guus Koch (ID-DLO) over 'kippen-immunologie' en onze lijnen waren zeer nuttig. Tijdens deze discussies heb ik heel wat nieuwe ideeën over het bestuderen van 'immunologische pathways' opgedaan en/of verworpen. Ook van mogelijke andere interpretaties van mijn onderzoeksresultaten heb ik dankbaar gebruik gemaakt.

Om op de hoogte te blijven van relevant werk door anderen, heb ik heel wat bezoekjes aan de bibliotheek gebracht. Daar wist Annemarie Zijlmans altijd dat ene uiterst belangrijke artikel ergens vandaan te halen. En natuurlijk waren de ritjes in het karretje ook onvergetelijk.

Op deze plaats wil ik ook nog even mijn 'onbezoldigd promovenda-schap' onder de aandacht brengen. Hoewel dit voor de promovenda niet 'gratis promoveren' betekent, heeft het mij wel recht op faciliteiten en steun gegeven die ik bij de afronding van het proefschrift goed kon gebruiken.

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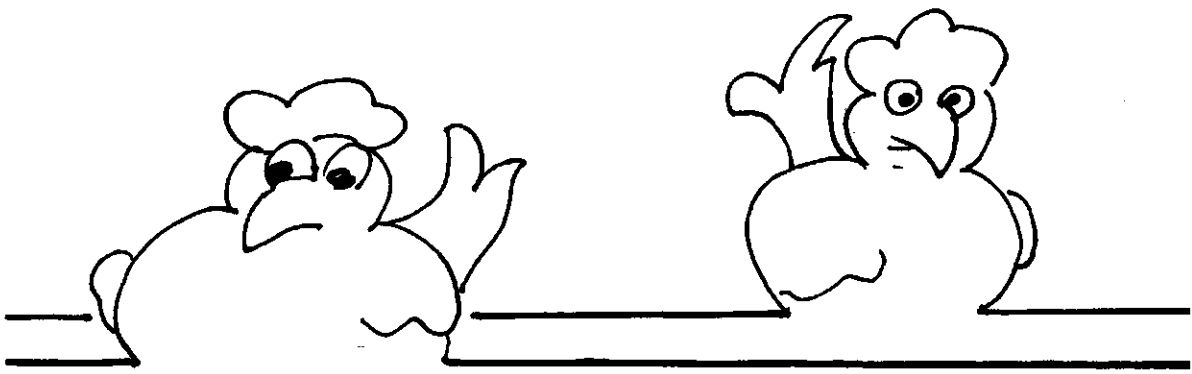
bedankt, Marijke



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chapter 1

GENERAL INTRODUCTION

INTRODUCTION

The production of poultry meat and eggs has been intensified further in the last decennia, not in the least because of decreasing margins between costs and income. This has resulted in a high animal density, which has enlarged the risks of infectious diseases. Unfortunately, the currently used measures like increased hygiene and the programs for vaccination, medication and eradication, are not sufficient to prevent outbreaks of diseases, considering the cases of Marek's disease (MD) and Newcastle disease (NCD) in 1992 in the Netherlands. Attempts to improve genetic resistance to particular pathogens seem to be successful. However, it seems economically more favourable to simultaneously enhance the resistance to all major pathogens to reach 'general disease resistance'.

In the view of a producer, an animal is considered resistant to a particular disease when an invading pathogen has no negative effect on the production (i.e. growth, eggs, etc.). The immune system plays an important role in the resistance to infectious diseases. And if one aims for improved general disease resistance, it is important to understand the genetic aspects of immune responsiveness. These genetics can be studied by using inbred strains with known immune deficiencies or using so-called 'marker genes', which are easy to detect (in contrast to 'resistance') and inherit in the same fashion as the resistance under selection (e.g. MHC). Another approach is the use of lines selected for high or low immune responsiveness. The advantage of the latter is that the phenotypic result (high or low immune responsiveness) is caused by accumulation of genes located at independent segregating loci and thus extremes of the naturally occurring populations can be studied (Mouton *et al.*, 1988).

Several research groups have selected lines of animals for immune responsiveness to investigate the possibilities of genetically increased disease resistance. These lines are widely used to study the genes regulating immune responses. But also the immune system itself is studied, as selection will alter the cascade of events that occur at the cell level. This cascade will finally result in the response selected for. Although much progress has been achieved in recent years, knowledge of how the immune system protects the body against pathogens is still elementary. The selection lines can be a great help in understanding how particular pathogens and the immune system can interact with each other. During selection, the alleles responsible for the response under selection will become fixed in the line. As a consequence of these fixed alleles, the selection line has only a limited repertoire of possible reactions to an antigen. These reactions (e.g. high or low antibody titer) will be controlled by several genes, all controlling a part of the cascade of cell reactions triggered by the entering of the antigen. When the complete cascade is disentangled in a selection line, one knows to a large extent how the immune system of this selection line will react on the pathogen,

as was demonstrated in selection lines of mice (Biozzi *et al.*, , 1984). Comparison of mortality and morbidity between two divergently selected lines, which are immunologically characterized, will give clues on how the immune system can protect the organism against the pathogen it is challenged with.

Selection for either high or low humoral immune response after immunization with sheep red blood cells (SRBC) has effects on cell interactions within the immune system. In this thesis, the effects are studied. SRBC are non-pathogenic antigens and because of their multiple antigenic sites, it is expected that a wide range of immune cells is stimulated after immunization with SRBC. Because SRBC are naturally derived, also parallels with determinants on pathogens might be expected. Therefore, effects of selection on disease resistance might be expected.

The immune system of chickens and mammals are considerably homologous, although, there are some differences. In mammals as well as in aves, the body has developed sophisticated defense systems, which reactions depend specifically on the kind of antigen and on the route of entrance. The strategies followed by this antigen-specific immune system to make the pathogen harmless are optimized and stored in so called 'memory cells'. This memory results in a faster and also more specific reaction at the next contact with the same antigen. In contrast, the innate immune system, or antigen non-specific immune system, has no memory and reacts, when triggered, always in the same fashion. However, often the pathogen is prevented from invading the body by the antigen non-specific system and thus a response of the antigen specific system is not necessary. The specific immune system traditionally, can be divided in two compartments, the humoral and the cellular immune response. However, both these specific responses work together to protect the body, although usually one of them is more profound.

A brief summary of the avian immune system, the avian Major Histocompatibility Complex (MHC) and the cell interactions in the humoral response will be given in the next sections. This is followed by a review considering the effects of genetic selection for immune responsiveness, including the selection for disease resistance to particular pathogens in chickens. Special attention will be given to the differences in the cell interactions used by the chicken lines, which are supposed to be the result of the selection conducted. In mice, a bidirectional selection experiment was started by Biozzi and associates in the 50's. Their lines selected for antibody response to SRBC, have been extensively studied and the results have given great impulses to the studies in other species. Therefore, results of the Biozzi mice are also included.

THE AVIAN IMMUNE SYSTEM

The Antigen Non-specific Immune System

An antigen can only be harmful for the body when it has circumvented the body's 'first line of defence'. This defence is antigen non-specific, and therefore independent of the type of antigen trying to enter. The structure of the skin and the low pH of the stomach are examples of this system. When an antigen has managed to enter the body, other non-specific immune reactions are triggered. For instance, certain types of bacteria can be destructed by lytic enzymes (i.e. lysozyme, phosphatase) found in the body fluids, or by the products of the complement cascade. Virally infected cells produce interferons, which induce a state of viral resistance in uninfected cells.

In the chicken, macrophages, heterophils and thrombocytes are phagocytic. This means they are able to internalize and destruct antigens intracellularly in a non-specific manner. During inflammation, chemotactic factors are released by damaged cells. These factors attract the heterophils and monocytes circulating in the blood. The monocytes differentiate into phagocytic macrophages when they arrive in tissue and, therefore, they are important in the destruction of pathogens at infection sites. The lungs are protected from antigens entering with inhalation by roaming macrophages. The phagocytic activity is also important in tissues with a special function in the clearance of the body, like the liver and spleen. In chickens, the capacity of heterophils and macrophages to clear the body from antigens was found to be under genetic control (Qureshi *et al.*, 1993; Puzzi *et al.*, 1990[a,b]).

The Cellular Immune System

Lymphocytes are the cells responsible for the specific immune system. As in mammals, precursor lymphocytes mature and differentiate in the thymus and are therefore called 'thymus derived lymphocytic cells' or, in short, T cells. These T cells mediate the cellular immune responses. The avian T cell development, recently reviewed by Chen *et al.* (1994), is found to be very similar to the mammalian system. All chicken T cells can be identified by the expression of the CD3 molecule on their surface (Chen *et al.*, 1986; Char *et al.*, 1990), which is associated with one T cell receptor (TCR) for signal transduction. In the chicken, three types of TCR can be found: TCR1, TCR2 and TCR3, originally named in accordance with the ontogenetic order observed. The TCR1 is of the γ/δ type and the TCR2 and TCR3 are two subtypes of the α/β receptors. T cells can also be characterized by the presence of the accessory molecules CD4 and CD8. $CD4^+$ T cells function as helper cells in immune responses, while the $CD8^+$ phenotype is either a cytotoxic or a suppressor cell. The tissue distribution and function of the TCR/CD3, CD4 and CD8 molecules are very similar to their mammalian counterparts (Cooper *et al.*, 1991). However, the chicken has,

compared to mammals, a relative large subset of γ/δ TCR1 lymphocytes in circulation.

TCR1 cells are found mostly in the splenic red pulp and intestinal epithelial area. Approximately two-third of these TCR1 cells express CD8 molecules on their surface (Bucy *et al.*, 1988; Chen *et al.*, 1988). The $TCR1^+CD8^+$ cells appear capable to suppress plasma cell-forming (Quere *et al.*, 1990[b]) and probably therefore, they are rarely found in the germinal centres of the spleen. TCR1 cells can exhibit cytotoxic activity (Chen *et al.*, 1994), but lack the capacity to initiate Graft-versus-host (GVH) attack. This might be due to the lower association with the CD4 molecule, because GVH response appeared to depend more on $CD4^+$ cells than on $CD8^+$ cells. Also, the TCR1 cells show a low mitogen response in the absence of TCR2 and TCR3 cells (Arstila *et al.*, 1993; Kasahara *et al.*, 1993). This suggests that chicken γ/δ cells depend on α/β T cells for growth (Chen *et al.*, 1994).

TCR2 cells home to the periarteriolar lymphatic sheaths in the spleen, where they tend to form dense aggregates. Less TCR2 cells are present in the red pulp region, where mostly $CD8^+$ cells are found regardless of their TCR type. In the intestines, TCR2 cells are located within the lamina propria, however, approximately 30 %, mainly $CD8^+$, can be found in the epithelial layer (Char *et al.*, 1990). TCR2 cells proliferate to concanavalin A (ConA), phytohemagglutinin (PHA) and pokeweed mitogen (PWM) and are capable to initiate a GVH reaction, although the responses are found to differ between MHC class II alleles (Char *et al.*, 1991).

Like the TCR2 cells, TCR3 cells home in the periarteriolar sheaths of the spleen, where they tend to form large aggregates. But unlike TCR2 cells, it is often impossible to find TCR3 cells in the intestine (Char *et al.*, 1990). According to Cooper *et al.* (1991), the *in vitro* mitogen response is similar to the response of TCR2 cells, but much higher than of TCR1 cells.

The Humoral Immune System

The humoral immune response is conducted by soluble proteins, called antibodies or immunoglobulins (Ig). An antibody is a tetramer consisting of two identical heavy (H) chains and two identical light (L) chains. Each chain consists of two principal regions: the variable (V) region and the constant (C) region. The V region of a H and a L chain forms the antigen binding site, whereas the C region, especially of the H chain has regulatory functions. The immunoglobulin structure resembles the Y, in which the arms of the Y are identical and each arm has the same antigen-binding site. As in mammals, there are genes coding for the C region and the genes coding for the variable parts. However, antibody diversity and thus the antigen-binding repertoire in poultry is reached differently from mammals (reviewed by Reynaud *et al.*, 1987). Based on differences in the constant chain (Fc), Ig can be divided into classes. In the chicken, three classes are found and analogous to the mammalian system they are called IgG,

IgM, IgA, although only IgM resembles its mammalian counterpart. Moreover, because of differences in structure and characteristics, chicken IgG was initially called IgY, while the IgA-like found in chicken bile was also called IgB. There is evidence for the existence of chicken IgE and IgD (Chen *et al.*, 1982; Faith and Clem, 1973).

Antibody exhibits several functions in the defence of the body. It neutralizes the pathogenity of antigens, for instance by binding to the tissue-receptor of the antigen and thus preventing the antigen from attacking its target tissue. Antibody promotes phagocytosis by opsonisation, because also when antigen has bound to antibody, the constant region of antibodies (Fc) can bind to receptors (Fc-receptors) found on phagocytes. Phagocytosis of the antigen-antibody complex is greatly facilitated in this way. Furthermore, binding of antibodies to heterophils can trigger the release of cytotoxins by these cells, which results in extracellular killing of antigens (Antibody Dependent Cell Cytotoxicity). The classic complement cascade can also be started by binding of antigen to antibody.

Antibodies are made when a special lymphocyte, the B cell, is activated and differentiates into a plasma cell, the actual producer of antibodies. B cells derive their name from the bursa of Fabricius. The function of the bursa in B cell development is comparable to that of the thymus in T cell development. However, the bursa is only found in birds and in mammals no single organ is found to have the same function. This central role in B cell development has been regarded to the bursa since 1956, when Glick and associates described the lack of antibody response in bursectomized chickens (Glick *et al.*, 1956). However, birds bursectomized before the bursa starts to develop, are able to synthesize Ig (Jalkanen *et al.*, 1983; Corbel *et al.*, 1987). Despite the presence of Ig, properly bursectomized birds are not able to mount a specific immune response to many antigens (Ivanyi, 1975; Jalkanen *et al.*, 1983; Corbel *et al.*, 1987). It is now believed that the bursal microenvironment is necessary for the activation of the so-called 'V-gene repertoire', the genes encoding for the V regions of the heavy and light chain of chicken Ig (Jalkanen *et al.*, 1983) and thus determine the antigen binding site of the antibody. Therefore, the antibody repertoire is dependent on the bursa, but Ig gene rearrangement can occur at non-bursal sites. Further details on B cell development in the chicken can be found in the recent reviews of Paramithiotis and Ratcliffe (1994) and Masteller and Thompson (1994).

Each mature B cell expresses surface Ig (sIg), which serves as a receptor for antigen. Because each Ig has an unique binding site, it is restricted to only one antigenic determinant. For activation and maturation of B cells to Ig secreting plasma cells, the sIg has to bind antigen. Luckily, the antigen has usually many antigenic determinants and can therefore trigger a response of several B cells. However, interaction with activated $CD4^+$ T cells and presentation of an antigen fragment by specialized antigen presenting cells (APCs) is also necessary. The products of the MHC on the surface of

these cells have to match with the MHC of the B cells. Furthermore, all cells involved in the interaction can secrete soluble messenger proteins, so called 'cytokines'. To start differentiation and maturation of the B cell into an antibody forming plasma cell, the right profiles of cytokines have to be secreted by the APCs and T cells.

The Avian MHC

To trigger responses of immune cells, an APC has to present antigen(parts) associated with the products of the MHC genes to the immune competent cells. The MHC genes have a high polymorphism and many alleles per gene are known (Goto *et al.*, 1988; Guillemot *et al.*, 1986). The products of each allel differ and have a specific affinity for an antigenic determinant, which might mean that a specific allel might also favour a specific defence reaction. Each individual bird inherits a set of MHC alleles from both parents, resulting in a limited set of MHC-alleles. This set determines to a certain extent the specific immune responses. Below, the structure and function of the avian MHC is only briefly discussed, because recently reviews are published describing the molecular structure and the functions of the chicken Major Histocompatibility Complex (MHC) (Guillemot and Auffray, 1989; Dietert et al. 1991; Pinard *et al.*, 1993[a,b]; Lunney and Grimm, 1994).

In the chicken the MHC was first discovered as the blood group system *B*, hence the name *B*-complex. As in mammals, the avian MHC comprises of Class I and Class II genes, called *B-F* and *B-L*, respectively. The chicken possesses also Class IV or *B-G* genes, of which no counterparts are found in mammals. On the other hand, Class III genes, which encode for complement components in mammals, have as yet, not been detected in the chicken MHC. In the chicken at least 30 *B* alleles have been defined (Briles *et al.*, 1982; Dietert *et al.*, 1991). So far, no recombinants between Class I and Class II genes have been found (Plachy *et al.*, 1992; Lamont, 1993). However, recently a separate locus (*Y*) containing Class I and Class II genes and segregating independent of *B-G* genes has been described (Briles *et al.*, 1993).

The *B*-complex haplotypes are based on serological distinctions (anti-allotype antibodies) and the distinct haplotypes have each an superscript number (e.g. B^{21}). Approximately 30 haplotypes (set alleles at linked loci) are serologically defined (Briles *et al.*, 1982). However, new technics determining differences in DNA might result in the identification of several subtypes of each haplotype (Chaussé *et al.*, 1989).

In the chicken, Class I, or *B-F* proteins are found on nearly all nucleated cells, including erythrocytes. Class II or *B-L* proteins are found on sub-populations of leucocytes including B cells, activated T cells, monocytes and macrophages (Chen and Cooper, 1987). The Class I and II genes are highly polymorphic. The *B*-haplotypes might differ in number of Class I and Class II genes (Kroemer *et al.*, 1990; Chaussé *et al.*, 1989). Within a single haplotype, multiple *B-F* genes can be expressed (Crone *et al.*, 1985;

Guillemot *et al.*, 1988). Class IV, the *B-G* proteins, is found on erythrocytes, but also on many immune system cells (Salomonsen *et al.*, 1991).

The *B*-complex influences the cooperation between cells during an immune response. The $CD8^+$ can only recognize antigen in the presence of *B-F* antigens, while activation of the $CD4^+$ T cells needs presentation of the antigen in association with *B-L* antigens. In the humoral immune response, the *B-L* antigens are necessary for the cooperation between APC and $CD4^+$ (T_{helper}) cells (Vainio *et al.*, 1988) as well as between T-B cell interaction in antibody responses (Vainio *et al.*, 1984). Therefore, it seems not surprising that effects of the *B*-complex have been found on the humoral response to several antigens (Benedict *et al.*, 1975; Pevzner *et al.*, 1975). The *B*-complex also influences the classical cellular immunity, such as skin graft rejections (Schierman and Nordskog, 1961), mixed lymphocyte reaction (Miggiano *et al.*, 1974) as well as macrophage inflammatory responses (Puzzi *et al.*, 1990[a,b]).

Cell Interactions in the Humoral Response

When the sIg at the membrane of a B cell binds an antigen (the possibility depends on the specifity of the sIgs variable part), the B cell can be triggered into an antibody response. However, for the majority of the antigens, the T cell-dependent antigens, help of $CD4^+$ T cells (Vainio *et al.*, 1984; Lassila *et al.*, 1988) and APCs is needed to trigger the B cell. Some antigens, like in the chicken *Brucella abortus* and *Salmonella* H antigen, seem able to activate B cells more or less independently of T cells and APCs. They are therefore called T cell-independent antigens. Presumably they trigger B cells by cross linking several sIg or sIg with other receptors.

For activation of B cells, probably both direct cellular interactions and cytokines are necessary. When the APC has internalized and processed antigen, it presents an antigen fragment at its surface in association with the MHC Class II products. If the MHC matches, $CD4^+$ T cells can bind to the antigen-fragment with their TCR. The production of the cytokine interleukin-1 (IL-1) by the APC and IL-2 by the activated T cell, is necessary to further activate the $CD4^+$ T cells. In mammals, it is known that the $CD4^+$ T cells, can be divided into two distinct subsets, the $T_{helper}1$ (T_H1) and the $T_{helper}2$ (T_H2) cells (Mosmann *et al.*, 1986). The T_H1 cells produce cytokines like interferon-γ and IL-2 and assist cell-mediated immune responses. The T_H2 cells, on the other hand produce IL-4, IL-5 and IL-10, and promote the humoral immune response (Abbas *et al.*, 1991). It seems likely that the APC determines whether T helper cells will differentiate into T_H1 or T_H2 and thus promote a cell mediated or a humoral response, respectively (Scott, 1993). In mammals, naive T cells can be triggered into either T_H1 or T_H2 cells by respectively IL-12 (Hsieh *et al.*, 1993) and IL-4 (Seder *et al.*, 1992). What causes the APC (or other cells) to react with the production of IL-4 or IL-12 to an antigen is still under investigation. Whether the mechanism of T_H1 and T_H2 can also be applied to

the chicken is questionable. Avian homologues of IL-4, IL-10 or IL-12 are, as yet, not described. However, so far most mechanisms found in mammals also apply to chickens. Chicken $CD4^+$ cells are found to play a crucial role in the humoral response (Vainio and Lassila, 1989; Arstila *et al.*, 1994). IL-1 induces high levels of IL-2 receptors and eventually IL-2 secretion on the T cells in chickens (Klasing, 1987). Avian IL-2 can be produced by $CD4^+$ $\alpha\beta$ T cells at significant levels (Vainio *et al.*, 1986), while crude IL-1 administered during an antibody response against SRBC, increased antibody titers in broilers (Klasing, 1987). In contrast to the enhancing effect of the $CD4^+$ T cells on the humoral response, suppressive effects of $CD8^+$ T cells on *in vivo* B cell responses have been reported (Quere *et al.*, 1990[a,b]), which is also in agreement with findings in mammals.

SELECTION FOR IMMUNE RESPONSIVENESS

Bursa weight

Soon after the discovery of the bursa of Fabricius being the organ responsible for B lymphocyte development, experiments were started to study the relationship between the size of the bursa and antibody production. When Single Comb White Leghorn families were selected for a large bursa at hatch, higher antibody levels to *Vibrio fetus* were found than in the small bursae families (Sadler and Glick, 1962). However, selection for increased bursa weight in broilers (Temple and Jaap, 1961) did not affect mortality, growth depression or antibody titer after a challenge with *Salmonella typhimurium*, notwithstanding the increase in bursa weight of 92% compared with non-selected chicks (Jaffe and Jaap, 1966). New Hampshires, several generations divergently selected for bursa size, did not differ in antibody production to Bovine Serum Albumin (BSA) (Glick and Dreesen, 1966), but titers to SRBC were higher in the large bursa line. Surgical bursectomy at hatch or at 3 weeks of age depressed the antibody production to SRBC more in the small than in the large bursa line (Landreth and Glick, 1973). Selection did not affect the total white-blood-cell counts and absolute counts of lymphocytes and of heterophils (Glick and Dreesen, 1967). No consistent differences were found in the weights of body, thymus and spleen (Glick and Dreesen, 1967; Landreth and Glick, 1973), although adrenal weights were larger in 1 and 3 week-old small bursa line chicks (Glick and Dreesen, 1967). Since antibody production is dependent on normal development of the bursa, the embryonal development of the bursae of these lines was investigated. Significant differences in bursa size from day 15 of embryonic life onward and higher bursal cell counts in the large bursa line were found (Kulkarni *et al.*, 1971). The bursa cells from one-week old large bursa line chicks

showed more *in vitro* cell growth than these cells of small bursa line contemporaries (Kulkarni *et al.*, 1971). At hatch, the bursae of the large bursa line had densely populated bursa folds, while in the small bursa line nearly no active follicles were found (Landreth and Glick, 1973). Three weeks after hatch, histologic differences in follicular development had disappeared between the lines (Landreth and Glick, 1973).

Thus, although selection for bursa size is possible and influences the embryonal development, it seems not to influence the antibody response to most antigens.

Antibody production to SRBC

The Biozzi Mice. In mice, Biozzi and associates have done extensive studies on the modifications of the immune system, induced by genetic selection for antibody responsiveness. Several lines were obtained using different antigens (i.e. heterologous erythrocytes, *Salmonella* species) and immunization protocols. Here, only the bidirectional selection for antibody response 5 days after intravenous immunization with SRBC (Selection I) will be discussed. In the high responder line of this selection, maternal antibodies were found to interfere with the response of their offspring after 6 selection generations. Therefore, selection was continued by immunizing alternate generations with either SRBC or pigeon red blood cells. The decrease in the low responder line was faster than the increase in the high responder line, thus the response was asymmetrical. The estimated h^2 were 0.24 to 0.26 in the H line and 0.30 to 0.36 in the L line (Biozzi *et al.*, 1975). After 18 generations the selection plateau was reached and the mice were considered homozygous for all loci determining the trait under selection.

The following modifications of the immune system caused by the selection were found:

a. Number, multiplication and differentiation rates of B lymphocytes were higher in the H than in the L line mice (Biozzi *et al.*, 1972; Unanue *et al.*, 1974).
b. The potentialities of the T cells are similar in the H and L line (Biozzi *et al.*, 1975).
c. Catabolism of antigens occurs faster in L line than in H line mice (Biozzi *et al.*, 1975).
d. Presentation of antigen fragments is prolonged in the H line (Wiener and Bandieri, 1974).

This resulted in a 200 fold interline difference for antibody levels and a lower threshold dose for both T cell-dependent and T cell-independent antigens in the H line (Biozzi *et al.*, 1975).

When disease resistance was considered in the lines, the results were in accordance with what was expected on basis of the immune characteristics of the lines. When antibody is the main defence mechanism, the H line is in advantage and even more

when memory is involved (Mouton *et al.*, 1988). The L line, on the other hand, shows a greater resistance against intracellular parasites (Biozzi *et al.*, 1982). Although, these studies are of great importance, there is no control line, thus it is not possible to determine whether the modifications caused by selection were an increase in one line, or a decrease in the other, or both.

In chickens, similarity was found in the kinetics of naturally occurring antibody responses and the haemagglutinins after immunization with SRBC (Seto and Henderson, 1968). This suggested that levels of haemagglutinins could be an indication for the immune potential of the developing chick. Also heritability of anti-SRBC antibody (anti-SRBC) titers was found to be high in chickens (Van der Zijpp *et al.*, 1983). Thus it was possible to genetically select lines of chickens with high or low antibody response to SRBC, aiming for a chicken with a more general disease resistance.

Chicken Selection lines of Siegel and Gross. Siegel and Gross (1980) selected two lines of White Leghorns for persistence and non-persistence of antibody titer to SRBC after intravenous (IV) immunization with SRBC. The line selected for persistence was relatively more resistant to *Mycoplasma gallisepticum*, *E. coli* and feather mites compared with the non-persistent line (Gross *et al.*, 1980).

Two other lines of White Leghorns were divergently selected for high (HA) and low (LA) antibody titer 5 days after IV immunization with 5% SRBC in phosphate buffered saline (PBS) (Siegel and Gross, 1980). Not only differed the peak titer in height, but the LA line reached the peak titer usually later in response (Ubosi *et al.*, 1985[a]). The IgM titers followed the total titers (IgG + IgM) (Ubosi *et al.*, 1985[a]). Line differences in secondary response depended on the primary immunization dose (Ubosi *et al.*, 1985[a]). The antibody titer to NCD (Gross *et al.*, 1980), horse red blood cells (Siegel *et al.*, 1982) and swine red blood cells (Gross, 1986) were all higher in the HA line. However, when under stressful social conditions, lines did no longer differ in antibody response to SRBC and swine RBC, (Gross, 1986). *Brucella abortus* (BA), a T cell-independent antigen, which activates B cells with little or no help of T cells, but needs accessory cells for antibody response, induced line differences in antibody response similar to SRBC (Dunnington *et al.*, 1992; Scott *et al.*, 1994). The HA line was more resistant to infections with *M. gallisepticum*, *Eimeria necatrix*, feather mites and NCD, but less resistant to *E. coli* and *Staphylococcus aureus* than the LA line (Gross *et al.*, 1980; Gross, 1986). The line difference in antibody response to *E. coli*, however, was dependent on the dose administered. LA line chicks were more susceptible to a natural exposure of MD virus and affected at a younger age than HA line chicks (Dunnington *et al.*, 1986). In both lines males were more susceptible, but this sex difference was more pronounced in the H line (Dunnington et al, 1986). Mortality of adult hens was higher in

the LA line (Siegel *et al.*, 1982).

Body weight was in general lower in the HA line (Siegel *et al.*, 1982). Interestingly, in lines divergently selected for body weight, peak anti-SRBC titers were similar between the lines, but antibodies were more persistent in the low body weight line (Miller *et al.*, 1992). This suggests a functional relation between bodyweight and height of the antibody titer. In addition to the lower body weights in the HA line, higher relative bursa weights and lower relative thymus and spleen weights were found in the HA line (Ubosi *et al.*, 1985[b]). Differences in allelic frequencies of several specific blood groups between the HA and LA line were reported (Dunnington *et al.*, 1984). In the HA line the majority of the chicks was B^{21} and in the LA line B^{13} (Dunnington *et al.*, 1984). The antibody response to SRBC seemed influenced by particular *B* haplotypes, as well as by the genetic background (Dunnington *et al.*, 1989). *In vitro* mitogen responses of peripheral lymphocytes to both ConA and PHA were higher in the HA line, but the responses were not influenced by the different *B* haplotypes (Scott *et al.*, 1991). Thus, this divergent selection for anti-SRBC titer, also divergated the humoral response to several other T cell-dependent antigens, as well as to a T cell-independent antigen. Cellular responses were higher in the HA line. The influence of selection on disease resistance, depended on the disease, comparable with the results in the Biozzi mice. Selection had affected body weight and the lymphoid organs. However, structural differences were not studied.

The Wageningen Selection Lines. A similar bidirectional selection experiment was started with ISA Brown medium heavy layers. Chicks were selected for high (H) or low (L) antibody titer 5 days after immunization with SRBC. However, chicks were intramuscularly (IM) injected with 25% SRBC in PBS (Van der Zijpp and Nieuwland, 1986). Additionally, a random bred control (C) line was maintained. This C line is a great advantage, because whenever comparison between the H and L line reveals an effect of selection, the C line can be used to decide whether the H line was affected or the L line in the opposite direction.

Line differences after IV immunization were similar to the differences found after IM immunization, both in primary and secondary response (Donker, 1989). More anti-SRBC producing cells were found in the H line spleen and spleen weight, relative to body weight was higher, but relative bursa weight was lower in the H line (Donker, 1989). Body weight was generally higher in the L line (Nieuwland, 1990, pers. comm), as was also found in the lines of Siegel and Gross (Siegel *et al.*, 1982). The levels of metabolism-associated hormones, like growth hormone, somatomedin and thyroxine, were similar between the lines (Donker, 1989), only a slightly higher level of triiodothyronine (T_3) was found in the H line (Donker, 1989). Before immunization, the energy utilization of H line was better than of the L line, as concluded from the higher fat deposition in the H line while the metabolic energy intake was comparable between

the lines. After immunization, energy utilization was even less efficient in the L line (Donker, 1989). When the H and L line birds were exposed to acute heat stress, plasma corticosterone levels were increased, but similar in both lines and no effect on titers was found (Donker, 1989). Direct administration of corticosterone did also not affect antibody titers in both lines (Donker and Beuving, 1989).

When prior to SRBC immunization, chicks were injected with carrageenan or carbon, which both block the functions of the reticuloendothelial system (i.e. macrophages), antibody responses to SRBC were depressed in a dose dependent manner. The dose did, however, not affect the line difference (Van der Zijpp *et al.*, 1988; 1989). Higher doses of carrageenan resulted also in depressed BA titers (Van der Zijpp *et al.*, 1989), while carbon had no effect on the BA titers (Van der Zijpp *et al.*, 1989). In contradiction with the results found in the lines of Siegel and Gross (Dunnington *et al.*, 1992; Scott *et al.*, 1994), there was no line difference found in anti-BA titers (Van der Zijpp *et al.*, 1988; 1989).

Selection for antibody responsiveness to SRBC resulted in frequency changes in MHC-haplotypes. In the tenth selection generation, four B types were present in the C line, i.e. B^{14}-like, B^{19}-like, B^{21}-like and B^{24}-like. In the H line, the B^{21} was dominating and in the L line B^{14} (Pinard *et al.*, 1993[a]). The change in frequency of MHC-haplotypes explained only a small part of the line difference in antibody response (Pinard *et al.*, 1993[a]; Pinard and Van der Zijpp, 1993).

Chicks from the L line were more susceptible to challenges with MD virus and died earlier than the chicks from the H line and C line. Total mortality did not differ between the H and C line chicks, but mortality occurred later in the H line than in the C line (Pinard *et al.*, 1993[b]). The MHC genotypes only contributed 3,5% of the total variance of mortality to Marek's disease (Pinard *et al.*, 1993[b]).

Selection had affected the weight of the lymphoid organs, but had no effect on the metabolism associated hormones, although energy utilisation was better in the H line. Blocking the functions of the RES, i.e. phagocytosis and antigen presentation, did not affect the antibody response of the lines differently. Although selection had affected the frequencies of the *B*-haplotypes, this had hardly any effect on the antibody titers. Thus, so far there is no evidence of line differences in the combined effect of phagocytic activity and antigen presentation. The selection effect was not general, at least one T cell-independent antigen did not differ in antibody response between the lines.

Selection of Adult Hens. Another divergent selection experiment with SRBC was conducted by Genzel and Weigend (1989). Adult laying hens were selected for antibody titer after immunization with SRBC in Complete Freunds Adjuvant. The line with the highest increase in titer had also the lowest body weight. However, because no progress in selection could be reached after the first generations, the selection

experiment was ended (S. Weigend, pers. comm. 1994).

As in mice, selection for anti-SRBC response was found to be easily assessable in chickens. However, as the comparable selection experiments with chickens do not always give similar results, it seems likely that the results found in mice can not be applied directly to chickens.

Other humoral responses

White Rock chickens were selected for serum immunoglobulin G (IgG) level by Okada and Yamamoto (1987). This selection did not affect the humoral responses to all antigens, because a higher antibody response to SRBC and to lipopolysaccharides from *E. coli* were found in the high IgG line, but not to BSA (Okada and Yamamoto 1987). The high IgG line had also a higher splenomegaly index in the GVH reaction (Okada and Yamamoto, 1987). Both morbidity and mortality after IM inoculation with MD virus were much higher in the high than in the low IgG-line (Okada and Yamamoto, 1987).

The synthetic amino acid polymer, glutamic acid-alanine-tyrosine (GAT) is also widely used to measure humoral immune responses. Divergent selection for low and high humoral response to GAT was conducted within broilers and layers (Pevzner *et al.* (1989[a,b]). The lines selected for high antibody response to GAT were more resistant to Rous sarcoma (RS), MD, and *S. aureus* than were the low GAT responders (Pevzner *et al.* (1989[a,b]).

White Leghorns, homozygous for either B^1B^1 or $B^{19}B^{19}$ were selected for high or low antibody response to GAT (ir-GAT) (Pevzner *et al.*, 1978[b]). As in the 'at random *B*-haplotype lines', both B^1B^1 and $B^{19}B^{19}$ high GAT responders showed lower mortality after inoculation with MD virus (Pevzner *et al.*, 1981[a]). *In vitro* proliferation of GAT-primed lymphocytes differed between the MHC types, the $B^{19}B^{19}$ having the higher response (Steadham and Lamont, 1993[a]). However, within the $B^{19}B^{19}$, proliferation was higher in the high ir-GAT type than in the low ir-GAT type. Within the B^1B^1, there was no significant difference between ir-GAT types (Steadham and Lamont, 1993[a]). When the cultured cells were separated in $CD4^+$ and $CD8^+$, within the $B^{19}B^{19}$ genotype, it was found that the low Ir-GAT chicks had more $CD8^+$ than the chicks of the high Ir-GAT type. This suggested an increased suppression of antibody response due to high levels of $CD8^+$ cells in the low line. But, the effect was different in the B^1B^1 chicks: the low Ir-GAT had more $CD4^+$ cells (Steadham and Lamont, 1993[a]). Therefore, this can not be considered a general effect of selection for ir-GAT. Moreover, *in vitro* estimation of the ability of APC to process and present GAT indicated a higher ability in the low Ir-GAT chicks of the B^1B^1, in the $B^{19}B^{19}$, the Ir-GAT effect was not significant (Steadham and Lamont, 1993[a]). These results indicate that in the two *B* haplotypes different mechanisms are responsible for the high or low humoral response to GAT. Association studies, using F1 and F2 chicks, produced by backcross matings,

suggested that both MHC-linked and MHC-non-linked genes control the humoral response to GAT (Steadham and Lamont, 1993[b]).

The *B*-GAT lines (Pevzner *et al.*, 1978[a]) were further divided for progressive or regressive Rous sarcoma induced (RS) virus response. In these lines survival seemed to be positively associated with the high ir-GAT response (Kim *et al.*, 1987). The anti-SRBC antibody titers were found not to be associated with MHC type, ir-GAT or RS (Kim *et al.*, 1987). Lamont (1986) first reported effects of selection for RS on the phagocytic activity, but later the RS-selection was found not to affect phagocytic activity (Cheng and Lamont, 1988). Effects of MHC haplotype and ir-GAT type on phagocytic activity were found only in males (Cheng and Lamont, 1988). The antibody responses to simultaneously injected *P. multocida* and *M. gallisepticum* vaccines were higher in the high ir-GAT lines, compared with the low ir-GAT lines, the correlations between the antibody responses to the vaccines were positive, but only significant in females (Cheng and Lamont, 1988). *In vivo* mitogen response to PHA (wingweb) was affected by MHC-type and selection for RS, in males, not in females. Also interactions with ir-GAT type were reported (Cheng and Lamont, 1988). The correlations between wingweb-response and the phagocytic activity were negative in females (Cheng and Lamont, 1988).

Divergent selection for rabbit serum albumin (RSA) was carried out by Okabayashi and Okada (1989). The high RSA-line had higher antibody response to SRBC, *Escherichia coli*, *Salmonella minnesota* lipopolysaccharides and Dog Serum Albumin, but the lines did not differ in response to cattle erythrocytes and BSA (Okabayashi and Okada, 1989). Moreover, the RSA-lines did not differ in morbidity or mortality after a challenge with MD virus (Yamamoto *et al.*, 1991).

T Cell responses

In only a few experiments genetic selection for cell mediated immune responses was conducted. However, selection for GVH reaction competence of chickens has been studied in more detail (Ashikaga *et al.*, 1984; Okada and Yamamoto, 1987). The lines of Okada and Yamamoto (1987) did not differ in antibody response to either SRBC, BSA or lipopolysaccharide (LPS). Slightly higher resistance to MD was found in the low GVH-line compared with the high GVH-line (Ashikaga *et al.*, 1984; Okada and Yamamoto, 1987). The difference in morbidity and mortality after a challenge with MD was influenced by the MHC genotype of the lines (Okada and Yamamoto, 1987). The low GVH $B^{11}B^{11}$ line was significantly more resistance to MD than the high GVH $B^{11}B^{11}$ line. However, the line difference was reverse within the $B^{9}B^{9}$ lines. Tumour incidence and mortality after inoculation with MD virus associated lymphoblastoid cells was much higher in the high GVH lines than in the low GVH lines, independently of MHC-type (Yamamoto *et al.*, 1991). These results (Okada and Yamamoto, 1987; Yamamoto

et al., 1991) indicate that mortality after inoculation with lymphoblastoid cells does not necessarily represent mortality after a challenge with MD in all MHC genotypes.

The GVH-lines were mated with a homozygous B^{12} and the F_2 progeny was further selected for delayed wattle reaction (DWR) to BCG (attenuated *Mycobacterium tuberculosis*) (Afraz *et al.*, 1994). Selection was quite successful, the realized heritability, averaged over 4 generations, was 0.7. There was a frequency shift in B^{12}-haplotype, which increased in the high DWR line, but decreased in the low line. *In vitro* mitogen response to PHA and the capacity to clear carbon from the blood, as well as the antibody responses to a cocktail with SRBC, BSA, BA, dinitrophenyl (DNP) and *Salmonella pullorum* did not differ between the lines. However, disease incidence and mortality after an IM injection with MD virus was significantly higher in the high DWR-line (Afraz *et al.*, 1994). The low DWR-line was heavier than the high line (Afraz *et al.*, 1994).

Multitrait selection

Another approach to improve general disease resistance was the combined selection for 'high' or 'low' responses to several immune traits as conducted by Cheng *et al.* 1991), who selected simultaneously for carbon clearance, antibody responses to *Pasteurella multocida* and *Mycoplasma gallisepticum* and hypersensitivity to phytohemagglutinin (PHA). Although the genetic correlations among the immune traits were generally negative (Cheng *et al.*, 1991), the multitrait selection elevated the level of the diverse immune traits (Kean *et al.*, ,1994[b]). The frequencies of MHC haplotypes differed between the high and low multitrait line. However, also between the replicates of both high and low multitrait selection lines frequency differences were found. Therefore the linkage between MHC-type and multitrait-level could not be a functional one (Kean *et al.*, 1994[a]). The replicates differed also in breeding values calculated from the multitrait-index (Kean *et al.*, ,1994[b]).

SELECTION FOR RESISTANCE TO SPECIFIC PATHOGENS

Newcastle disease (NCD)

Newcastle Disease is caused by a highly contagious paramyxovirus and causes high mortality. Affected birds often show respiratory distress (wheezing and gurgling) or nervous signs (paralysis or twisted necks). There is no treatment for NCD and vaccination is thought to be the only control method, next to eradication procedures.

Gordon *et al.* (1971) described an attempt to create a susceptible and a resistant line to NCD. Although a line difference in the average mortality was found in the first generation, they were unable to increase the line difference further. This was probably

due to the difficulties in standardisation of the NCD challenges. However, selection for antibody response to either inactivated or attenuated NCD virus was possible and correlated positively with resistance to NCD (Peleg *et al.*, 1976). Cahaner *et al.* (1986), selected White Rock broilers for either low or high antibody responses to *E. coli* and NCD vaccines at 18 days of age. It was found that this selection affected the development of the humoral response. In the high line peak antibody titers to NCD were higher and were reached earlier after immunization than in the low immune response line. Moreover, a larger portion of the low line failed to respond to the vaccinations (Pitcovski *et al.*, 1987). Already after 2 selection generations, mortality due to non-specific causes was higher in the low line (Cahaner *et al.*, 1986; Pitcovski *et al.*, 1987). After 3 selection generations, 18-day old chicks of both lines were challenged with live *E. coli* bacteria and 16 % more chicks died in the low immune response line (Cahaner *et al.*, 1986). The high immune response line had higher antibody response to SRBC, more plaque forming spleen cells to *E. coli*, a higher level of total immunoglobulins and IgG, a higher *in vivo* wattle reaction to *Mycobacterium tuberculosis* and higher *in vivo* mitogen responses to PHA and ConA. Also, the high line showed a faster blood clearance of heat killed *E. coli*, both in immunized and non-immunized chicks (Pitcovski *et al.*, 1989). Only the antibody response to BA did not differ between the lines (Pitcovski *et al.*, 1989). Finally, the lines differed in MHC-types (Heller *et al.*, 1991).

Marek's disease

Marek's disease (MD) is a herpes virus-induced lymphoproliferative disease of poultry. Resistant and susceptible lines were developed by selection after exposure to pathogenic MD virus (Morris *et al.*, 1970). Heritability of resistance to MD was estimated to be between 0.07 and 0.18 (Krosigk *et al.*, 1972; Hartmann and Sanz, 1974) and 0.40 in lines selected for antibody response to SRBC (Pinard *et al.*, 1992).

Involvement of the *B*-complex in resistance to MD was first suggested by Hansen *et al.* (1967) and the association between *B*-haplotypes and MD resistance was then intensively studied. Divergent selection for resistance or susceptibility to MD resulted in a 8-fold difference in MD resistance and in differences in the frequency of the B^{21} (Briles *et al.*, 1977). Selection for the *B*-type resulted in line differences in resistance to MD comparable to the differences between lines directly selected for high or low MD resistance (Briles *et al.*, 1976). However, other genes besides the MHC genes, must be associated with resistance to MD (Pazderka *et al.*, 1975; Pinard *et al.*, 1992). Resistance or susceptibility to MD was not associated with mixed lymphocyte reaction or mitogenic responses (Calnek *et al.*, 1989; Gavora *et al.*, 1990).

Selection for reduced incidence of MD seemed possible without a negative effect on economically important traits (Friars *et al.*, 1972; Krosigk *et al.*, 1972; Flock *et al.*, 1975; Gavora *et al.*, 1989).

Coccidiosis

Coccidiosis is caused by intracellular protozoan parasites belonging to several different species of *Eimeria*, which all have the intestinal epithelium as primary target tissue. Selection for resistance and susceptibility to acute caecal coccidiosis by *Eimeria tenella* has been carried out by Johnson and Edgar (1982). Relaxation of selection for resistance resulted in increased mortality, eventually equalizing the unselected control line. Males were more susceptible than females (Johnson and Edgar, 1982). Interestingly, the tissues affected in the resistant and susceptible lines differed, indicating that genes that determine the organ specificity, might also determine the response of the host to the acute coccidiosis (Johnson and Edgar, 1982).

As in many diseases, MHC genes are found to influence the resistance to *Eimeria tenella*, although non-MHC genes are also important (Clare *et al.*, 1985; Lillehoj *et al.*, 1989). Giambrone *et al.*, (1984) found high phenotypic correlation between the ability of the chicken to develop a delayed hypersensitivity skin test and the ability to respond to *E. tenella*.

Escherichia coli

The bacteria *Escherichia coli* (*E. coli*) are often involved in yolk-sac infections and in the secondary bacterial infections after a viral respiratory infection. To study resistance to *E. coli,* two broiler lines were divergently selected for the antibody level 10 days post vaccination with *E. coli*. A special feature of this selection study is the replication of the selection, i.e. two high responder lines and two low responder lines were separately selected for the antibody response to *E. coli* vaccine out of the same base population. Moreover, a non-selected control line was maintained (Leitner *et al.*, 1992). In the first 3 selection generations the response to the selection seemed to be symmetrical, although heritability estimates were higher in the low line (0.32) than in the high line (0.23) (Leitner *et al.*, 1992). Challenge with pathogenic *E. coli* did not show any line difference in mortality or morbidity of non-vaccinated chicks of selection generations 1 and 2. Although the height of the antibody response 10 days post vaccination was positively correlated with resistance to *E. coli* in unselected chicks. However, when chicks were vaccinated prior to challenge, the high line was more resistant (Leitner *et al.*, 1992). The high early antibody response to *E. coli* was, in both replicates, positively associated with a higher antibody response to NCD and SRBC, but not with the response to BSA (Heller *et al.*, 1992). Clearance of carbon from the blood was faster in the high early response line and *in vitro* T cell proliferation response to *E. coli* and ConA, as well as B cell proliferation to pokeweed mitogen (PWM) was increased in the high early response line (Heller *et al.*, 1992). The high and low early response lines differed in 6 of 10 MHC haplotypes, while their replicates differed only for one haplotype (Uni *et al.*, 1993), suggesting no functional association between the

selection and the MHC of the selection lines.

Salmonella pullorum

The bacterium *Salmonella pullorum* causes in chicks typical white diarrhoea, with pasted cloacae and high mortality. Adult breeders show no clinical signs, but have internal lesions in the ovary.

Divergent selection for antibody titers to *Salmonella pullorum* in White Leghorn chickens homozygous for B^1, affected total mortality and susceptibility to MD; both were higher in the high response line (Pevzner *et al.*,1978[a]; 1981[b]).

SCOPE AND AIM OF THIS THESIS

In the previous sections, effects of selection on several immune responses and resistance to specific pathogens are described. Sometimes, genetic selection for enhancement of a certain immune response, simultaneously enhanced a second immune response. However, selection for the second immune response did not always result in the enhancement of the first response (i.e. Okado and Yamamoto, 1987). Moreover, effects found in one selection experiment did not always agree with the results in a comparable selection experiment (Van der Zijpp *et al.*, 1988; 1989; Dunnington *et al.*, 1992; Scott *et al.*, 1994). And effects of selection even differed between replicates of selection lines originating from the same base population (Uni *et al.*, 1993). These results all strongly suggest that there are more genetic pathways to reach the same phenotypic result. The pathway used depends on the alleles of the genes available in the base population and which combination of alleles gave the most favourable response in the selection generations. However, when the selection-plateau is reached, the combination of alleles has become fixed in the population. This means that a given selection line preferably will react on an antigen with its 'genetically fixed pathway'. However, in non of the selection lines has this pathway been revealed.

In mice, Biozzi has shown that approximately 10 genes at independent segregating loci, were responsible for the anti-SRBC response. These genes affected B cell multiplication and differentiation, as well as antigen catabolism and presentation of the antigen. When specific diseases resistance was studied in the Biozzi mice, a large part of the difference in resistance between the high and low line could be attributed to the fixed differences in the immune pathways used by the mice.

As in mice, information of the fixed pathways used by chicken H and L lines, will extend the knowledge of how resistance to a particular disease can be reached. The studies presented in the next chapters of this thesis were conducted to determine which aspects of the immune system have been affected by divergent selection for

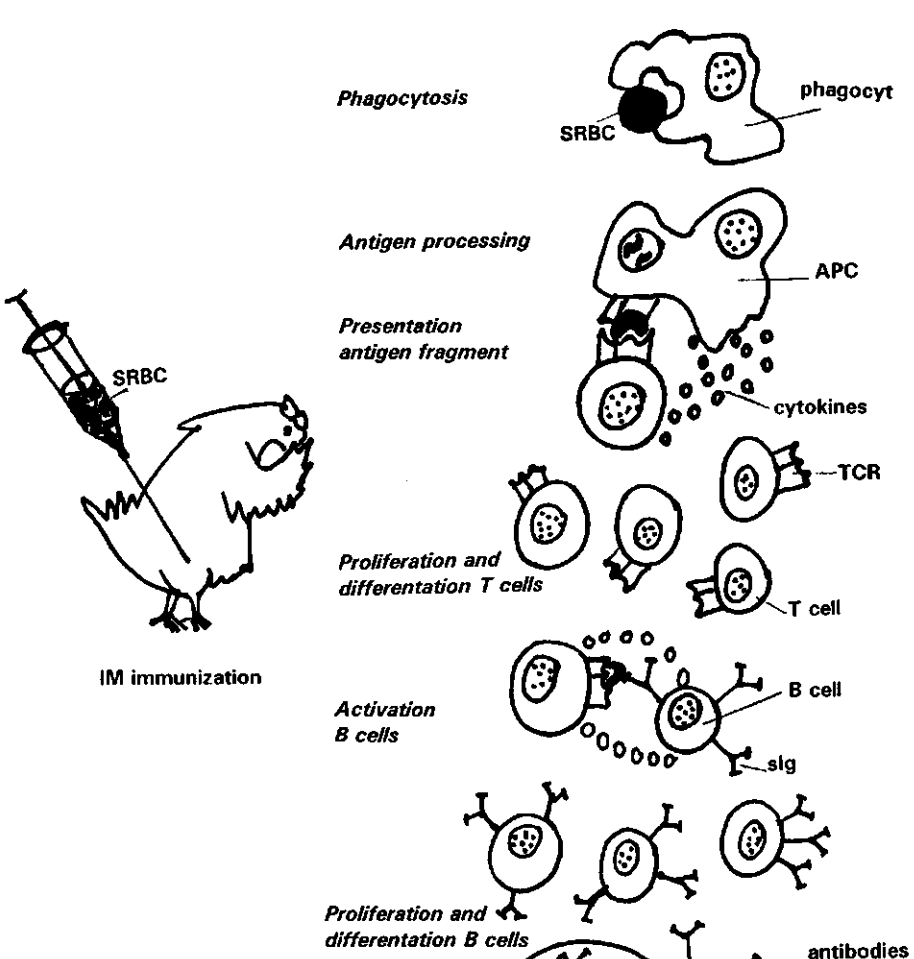

FIGURE 1.1. Cascade of events following IM immunization with SRBC leading to antibody production.
Uptake of the antigen (SRBC) and intracellular destruction (phagocytosis) or processing of the antigen; Presenting part of the antigen on the surface of an Antigen Presenting Cell (APC) in association with a MHC antigen; Binding of the processed antigen to a T cell-receptor (TCR) on a T cell; Release of cytokines; Activation and differentiation of T cell populations; Binding of an antigen fragment to surface Ig (sIg) on a B cell; Release of cytokines; Activation and differentiation of B cells in memory cells and plasma cells; Production of antibodies by the plasma cells.

antibody response to SRBC after IM immunization. A complex antigen like SRBC is probably able to affect many parts of the immune response. The difference in antibody titers between the high and low anti-SRBC lines is the consequence of genetically defined line difference(s) in the cascade of cell interaction events operating to cope with the SRBC. The level of anti-SRBC antibodies measured in the blood is the final result of this cascade, which starts when the SRBC is injected in the muscles of the chick.

In Figure 1.1, a rather simplified summary is given of the cascade of events following IM immunization with SRBC, resulting in antibody production. Each of the single factors comprising this cascade might differ between the lines. These line differences might be quantitative, for instance the number of phagocytes, T cells, B cells, or the amount of antibody produced per plasma cell. But also qualitative differences between the lines might exist, i.e. the MHC-type on the APC, the TCR-type on the T cells, the binding specifity of the TCR and of the sIg on B cells, or the profiles in cytokines. The organization of the immune competent cells in the lymphoid organs might also differ between the lines, resulting in an approach favouring antibody response (the H line) or other ways to cope with the SRBC (the L line).

In the following chapters of this thesis, parts of the cascade of events out lined in Figure 1.1, are investigated to assess differences between the lines selected for high or low levels of circulating antibody 5 days after IM immunization with SRBC.

In the Chapters 2 and 3, differences in the phagocytic and catabolic activity of the two lines are studied. In Chapter 3, the effect of immunization on the phagocytosis of SRBC is discussed. In Chapter 4, the effect of different doses SRBC on the humoral response in both lines is studied in an attempt to determine the threshold dose necessary to stimulate the antibody response in the lines. In the following Chapter (5), the humoral response to SRBC is manipulated by administration of the SRBC by different routes, or in combination with adjuvants. Line differences in the effects of these manipulations might indicate effects of selection at APC, B or T cell level. In this Chapter, also the response to one other T cell-dependent and two T cell-independent antigens is measured to study whether the effect was specific for only SRBC, or more general. In Chapter 6, cellular responses are studied, to gather information about the effect of selection on the T cell responses. In Chapter 7, the percentages of lymphocyte subpopulations are compared between the lines and it is estimated whether selection would affect the numbers of antibody producing cells. In the last chapter, Chapter 8, the results are discussed and, based on the differences and similarities found between the lines, hypotheses on what pathways are followed to destruct SRBC in the H line and in the L line were formulated.

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chapter 2

PHAGOCYTIC ACTIVITY OF TWO LINES OF CHICKENS DIVERGENTLY SELECTED FOR ANTIBODY PRODUCTION

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Phagocytic activity of two lines of chickens divergently selected for antibody production.

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ABSTRACT

Differences in phagocytic capacity of two chicken lines selected for high (H) or low (L) antibody response against sheep red blood cells (SRBC) were studied in eight month old cocks of the seventh selection generation. The H line cocks had significantly higher agglutinin titers after immunization with SRBC than the L line. The total clearance capacity of the phagocytes, measured by the clearance of carbon particles from the blood, did not differ between the lines. The L line cocks had more circulating granulocytes. However, the granulocytes of the H line phagocytized more yeast cells than those of the L line. Neither in immunized nor in non-immunized cocks, were line differences found in the intracellular destruction of antigen by phagocytes, estimated as the superoxide production during phagocytosis and the plasma levels of lysozyme activity and acid phosphatase, before and after immunization. It was concluded that the line difference in antibody response was not due to measurable differences in phagocytic activity.

INTRODUCTION

To gain better understanding of the underlying mechanisms of immune defence in the chicken, selection for immunoperformance can be very important. In mice divergently selected for antibody response to sheep red blood cells, the line difference was partly explained by a higher phagocytic activity i.e. uptake and intracellular destruction of the SRBC in the low (L) antibody production line (Wiener and Bandieri, 1974). Also, better accessory functions, i.e. longer presentation of processed SRBC on antigen presenting cells were found in the high (H) line (Biozzi *et al.*, 1984). In our chicken lines, blocking the phagocytic and accessory functions of the reticuloendothelial system with either carrageenan or carbon (Van der Zijpp *et al.*, 1988; 1989) did not negate the line differences. Immunization with T cell-dependent antigens (BSA, KLH, TNP), but not with T cell-independent antigens (*Brucella abortus*, *Salmonella* H antigen) showed characteristic line differences (Kreukniet *et al.*, 1992; Parmentier *et al.*, 1993). Moreover, line differences diminished when SRBC was given with Freunds Complete Adjuvant. This indicates that the antigen handling preceding T

cell activation may account for the present selection. Therefore we extended our studies on the capacities of phagocytes in both lines. Some easy accessible experimental approaches were used to study the different phases of phagocytosis. The *in vivo* clearance of intravenously injected carbon and the *in vitro* uptake of yeast cells were used to obtain information on the uptake of particles by phagocytes. After internalization of antigens, destruction is accomplished by the lysosomal reservoir of hydrolytic enzymes and by the products of the respiratory burst (i.e. O_2^- and H_2O_2). Hydrolytic enzymes, i.e. lysozyme and acid phosphatase, can be found in the plasma (Butler *et al.*, 1977; Di Luzio, 1979), as a result of the phagocytic activity. Their levels were measured before and at various days after immunization. Also, the release of superoxide during phagocytosis was measured. The results of these assays may provide information on the contribution of the intracellular destruction to the line differences in antibody responses to T cell-dependent antigens in our selection lines.

MATERIALS AND METHODS

Experimental animals and immunization

Cockerels of two lines divergently selected during seven generations for agglutinin response to SRBC (Van der Zijpp and Nieuwland, 1986) were used; in total 35 cocks of the high response (H) line and 39 cocks of the low response (L) line. Lines were housed intermingled, in two floor pens (2x1.5 m^2) covered with wood shavings and with free access to food and water. Vaccinations against Marek's disease, infectious bronchitis, infectious bursal disease, Newcastle disease (twice), infectious laryngotracheitis, egg drop syndrome, avian encephalomyelitis and fowl pox were carried out at the ages of 0, 1, 15, 30 and 56, 42, 56, 70 and 77 days, respectively.

At 192 days of age, cocks were primarily immunized with 1 ml 50% SRBC in phosphate buffered saline (PBS) in the thigh muscle. Heparinized blood was collected at 0, 3, 5, 7, 10 and 13 days post immunization (p.i.) and total and 2-Mercaptoethanol-resistant (2MEr) titers were determined in plasma samples as described by Van der Zijpp and Leenstra (1980).

Assays

Carbon clearance assay. Pelikan Drawing Ink A (Pelikan A.G., Hannover, Germany) was centrifuged (30 min 2000 x g), the supernatant (118 mg/ml) was mixed (1:1) with a 4% gelatin-suspension in PBS. Cocks, 119 days of age, were intravenously (cutaneous ulnaris) injected with 2 ml carbon suspension (30°C) per kg body weight. From this vene, blood samples were taken 5, 10 and 15 min. after the carbon injection. One hundred μl blood was diluted in 3 ml PBS with 5000 IU/ml heparin (10:1), centrifuged (850 x g; 10 min.), and absorption values of the supernatant were

measured at 675 nm using a Perkin-Elmer Lambda 1 spectrophotometer (Oakbrook, IL, USA). One hour after the collection of the last blood sample, cocks were sacrificed and weights of bursa, spleen and liver were determined. Relative organ weights (organ weight/body weight x 100) were calculated.

Yeast cell ingestion assay. Two g of active baker's yeast (*Saccharomyces cerevisiae*) was killed by boiling for 30 min. in 250 ml PBS. After washing 3 times in PBS (850 x g; 10 min), the pellet was resuspended in RPMI 1640 at a concentration of 2.5 x 10^9 yeast cells per ml. Heparinized blood was collected from 126 days old cocks and one ml blood was incubated (41°C) with 100 μl yeast suspension. Blood smears were prepared after 20 min. of incubation and stained with Giemsa (Merck, Darmstadt, Germany). The number of phagocytized yeast cells per 100 granulocytes was counted.

Superoxide production assay. Heparinized blood was collected from 189 days old cocks, diluted 1:1 in RPMI 1640, layered on top of Nycodenz solution (Nyegaard & Co As, Norway; density: 1,086 g/ml) and centrifuged (20 min; 850 x g at 18°C). The buffy coat was collected and washed twice in PBS (10 min; 500 x g at 4°C) and brought at a concentration of 2.5 x10^6 granulocytes/ml RPMI 1640. Samples were tested for superoxide production during phagocytosis in quadruplicate, according to the method of (Pick *et al.*, 1981) with some modifications. One hundred ml of each granulocyte suspension and 50 μl NBT grade III (Sigma Chemical Co., St Louis, MO, USA; 1 mg NBT/ml PBS) were added to 8 wells of 96-well flat-bottom microtiter plates (Omnilabo, Breda, Netherlands). Granulocytes in four wells were stimulated with 10 μl opsonized Zymosan A (prepared from *Saccharomyces cerevisiae*, Sigma Chemical Co.; 1 mg Zymosan A per ml PBS and opsonized with pooled chicken plasma). To the other 4 wells, 10 μl PBS was added. Eight additional wells were used as blanks, containing 160 μl of PBS without granulocytes and eight wells were used as controls, containing 100 μl PBS, 50 μl NBT and 10 μl opsonized Zymosan A. After incubation (40 min.; 41°C) the reaction was stopped by adding 100 μl HCl (1N), washed with PBS (850 x g; 10 min), and the cells were resuspended in 150 μl dimethylsulfoxide (max. 0.03 % water; Merck, Stutchardt, Germany) with 10 μl NaOH (1N) to increase colour intensity. The colour was measured with Multiskan photometer (Flow, Irvine, U.K.) at 690 nm. Mean absorbance of the non-zymosan stimulated samples per animal were subtracted from the absorbance of the stimulated samples, as an indication of the superoxide production during antigen digestion.

Determination of the number of granulocytes. The numbers of granulocytes in heparinized blood, diluted 1:51 in a staining solution (Natt and Herrick, 1952), were counted in a Bürkner chamber.

Lysozyme activity assay. Lysozyme activity in plasma samples was estimated in duplicate according to the method of Lie (1980) with some modifications. Briefly, a standard dilution series was produced by dissolving crystalline lysozyme (grade I, Sigma

Chemical Co.) in a phosphate buffer (11.73 g/l $Na_2HPO_4.H_2O$, pH 6.2) to concentrations of 1.5, 2.0, 3.0, 4.0 en 6.0 µg/ml. Of each concentration, 20 µl was added to two wells of a 96-well microtiterplate, to one well 200 µl phosphate buffer was added to the other 200 µl *Micrococcus lysodeikticus*-solution (600 mg *M. lysodeikticus* (Sigma Chemical Co.) per litre phosphate buffer.) After 15, 30, 45 and 60 min. at 41°C, the absorbance was determined with an eight-channel Titertek photometer at 560 nm. For each concentration, the regression coefficient (*b*) between absorbance (corrected for the absorbance of the controls) and time was calculated as a measure of the activity of the known quantity of lysozyme, causing lysis of *M. lysodeikticus*. A standard curve was produced by plotting *b* of each standard concentration against the quantity of lysozyme. Plasma of the experimental cockerels was treated similarly as the standard solutions. The *b* value for each plasma sample was calculated and fitted into the standard curve, thus estimating the lysozyme-activity of each sample.

Acid phosphatase assay. Plasma was obtained from blood samples collected in glass tubes containing citrate solution (35 g $Na_2C_6H_6O_7.2H_2O$; 2.5 g NaCl in 1000 ml distilled H_2O, diluted 5:1). All plasma samples were tested for acid phosphatase levels in quadruplicate, according to the method of Fishman and Lerner (1953) with modifications. To 8 wells, 20 µl plasma was added and 100 µl substrate buffer (0.41 g $C_6H_8O_7.H_2O$; 0.93 $Na_2C_6H_6O_7.2H_2O$; 0.204 g 4-Nitrophenylphosphate Disodium salt (Merck, Darmstadt, Germany) in 100 ml distilled H_2O at Ph 4.8) was added to four wells, to the remaining four wells (reaction controls) 100 µl NaOH (0.2N) was added. After 30 min at 41°C, 100 µl NaOH was added to the wells containing substrate buffer and 100 µl substrate buffer to the reaction controls. After an additional 30 min., absorbance was measured at 410 nm using a Titertek Multiscan. Means of the four reaction controls per animal were subtracted from each of the four determinations per animal. Substrate buffer (120 µl) was used as a negative control. Pooled plasma was added to eight wells of each microtiterplate and treated as plasma of an individual, for statistical adjustment of differences between microtiterplates.

Experimental Design and Statistical analyses

In Table 2.1, the number of cocks used per assay are given. Line differences were tested by analyses of (co)variance, using the GLM procedure with Line as main effect (SAS, 1989). In the models of the NBT reduction test, acid phosphatase assay and the lysozyme test, 'Titerplate' was also included as a main effect. In the carbon clearance test body weight was used as covariable. When titers of the lines were compared, the number of granulocytes, the decrease in granulocytes, lysozyme activity and increase in lysozyme activity during the antibody response were used as covariable in separate models. Additionally, partial Pearson correlations (correcting for lines) and Pearson correlations within H and within L line between antibody titers and lysozyme activities

TABLE 2.1. Experimental design: number (n) of cockerels of either the H or L line per assay and, when chicks werre immunized, the days post immunization (PI) the assay was conducted.

ASSAY	n COCKERELS		
	H	L	
NON IMMUNIZED			
carbon clearance	10	10	
yeast ingestion	25	29	
superoxide	24	27	
IMMUNIZED			DAYS P.I.
immunization	23	27	
agglutinin titers	23	27	0; 3; 5; 7; 10; 13
n granulocytes	20	26	0; 5; 10
lysozyme activity	23	27	0; 3; 5; 7; 10
acid phosphatase	23	27	-3; 3

at the various days and antibody titers and the in- or decrease in lysozyme activity during immune response were calculated, using the CORR procedure of SAS (SAS, 1989).

RESULTS

Agglutinin titers

On all days after the primary immunization with SRBC, the H line had significantly higher total and 2MEr titers (the 2MEr titers at days 0 and 3 p.i. were not normally distributed) (Figure 2.1). In both lines maximum titers were found at day 10 p.i.

Circulating granulocytes

Before immunization and 5 and 10 days p.i., slightly more granulocytes were found in the L line then in the H line. However, these line differences were not significant. In both lines, the number of granulocytes had significantly decreased five days after immunization. However, although the decrease appeared greater in the H line, there was no significant difference between the lines. Moreover, the number of circulating

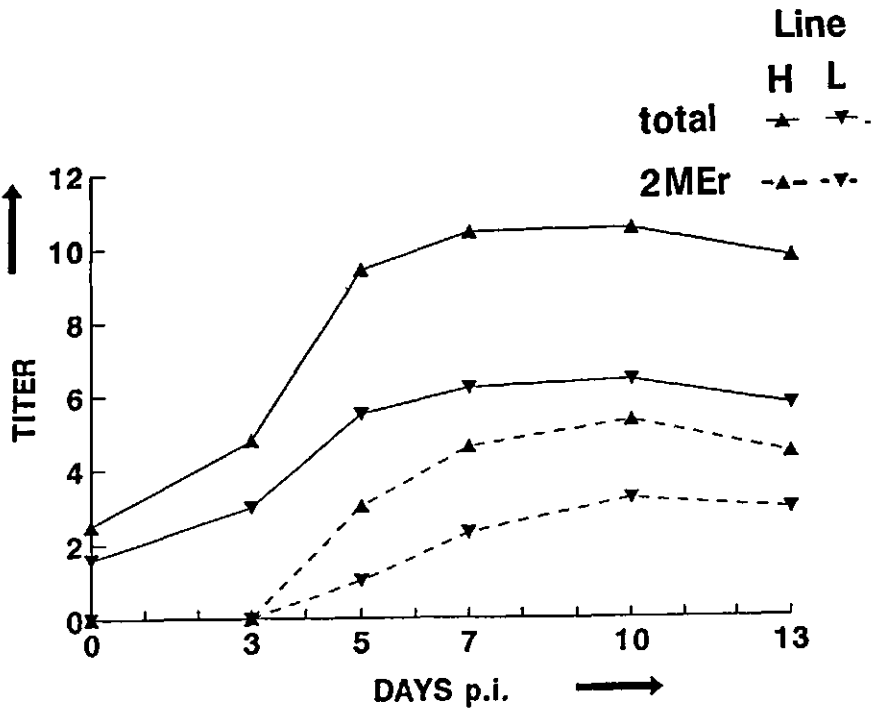

FIGURE 2.1. Mean TOTAL and 2MEr titers per line after primary intramuscular immunization with 1 ml 50% SRBC.

granulocytes, nor the decrease in granulocytes during immune response affected the antibody response, as revealed by covariance analyses.

Uptake and removal of particles by non-immunized cocks

The total phagocytic capacity of the cocks *in vivo* was estimated using the carbon clearance assay. The speed by which the lines removed the injected carbon from the blood did not differ (data not shown). And, although the L line cockerels were slightly heavier (3.1 ± 0.1 kg) than their contemporaries of the H line (2.8 ± 0.2 kg) ($P>0.1$), the carbon clearance was not affected by the body weight. Also, (relative) organ weights did not affect the clearance of carbon, although the absolute liver weight was significantly ($P<0.01$) higher in the L line (42.5 ± 3.2 g) than in the H line (37.7 ± 2.5 g). The relative liver weights did, however, not differ between the lines (2.1 ± 0.1 vs 1.9 ± 0.1). And in addition, no differences were found in relative weights of bursa and spleen between the lines (data not shown).

The *in vitro* uptake of yeast cells by granulocytes differed in number of phagocytized yeast cells per granulocyte (Table 2.2). The H line phagocytized more ($P<0.05$) yeast

cells per granulocyte than the L line (396±31 and 377±25 per 100 granulocytes, respectively). However, similar numbers of granulocytes were phagocytic; in both lines over 98%.

TABLE 2.2. Percentage of H and L granulocytes having *phagocytized various numbers (n)* of yeast cells

n YEAST CELLS	% GRANULOCYTES		
	H	L	
0	0.12 ± 0.33[#]	0.18 ± 0.56	
1	2.96 ± 1.59	4.30 ± 2.55	*
2	9.68 ± 2.94	10.78 ± 3.85	
3	25.04 ± 8.82	26.33 ± 5.65	
4	9.00 ± 5.32	31.70 ± 4.08	*
5	20.96 ± 4.17	18.15 ± 4.15	*
6	8.96 ± 4.57	6.74 ± 3.22	*
7	2.44 ± 2.18	1.44 ± 1.50	o
>8	0.84 ± 1.25	0.37 ± 0.74	o

[#] mean ± standard deviation.
o,*: lines differ (o: $P < 0.1$; *: $P < 0.05$)

Intracellular destruction before and after immunization

No line differences were found in the superoxide production during phagocytosis, and the levels of acid phosphatase and lysozyme activity in plasma of the non-immunized cocks (data not shown).

After immunization with SRBC, the acid phosphatase levels were similar to the levels before immunization and no line differences were found (data not shown). The lysozyme activity in plasma seemed to be slightly enhanced by immunization ($P<0.1$), in the H line from 3.0±0.6 to a maximum 5 days p.i of 3.5±0.9 and in the L line from 2.9±0.9 to maximal 3.5±1.1 at day 10 p.i.. In both lines lysozyme activity at day 7 was the lowest measured. No line differences in lysozyme activity could be detected. Analyses of Covariance revealed that the 2MEr titers were affected by the lysozyme activity. However, the line difference in antibody response could not be explained by the activity of lysozyme, because even after correction for lysozyme, still a significant line difference existed. The results of the correlations between the lysozyme activity at

TABLE 2.3. Partial Pearson correlations (corrected for line effect) calculated between the titers (Total and 2Mercaptoethanol resistant (2MEr)) and the lysozyme activity at various days post immunization (PI) and Pearson correlations within the H line, and within the L line. When not significant ($P>0.05$), only direction (+ or -) of the correlation is given, a 0 indicates a correlation less then 0.1. At day 3 p.i., the values of the 2MEr titers were not normally distributed, this is indicated with NN.

PARTIAL CORRELATIONS OVER LINES

LYSOZYME		TOTAL TITER				2MEr TITER			
	day	3	5	7	10	3	5	7	10
L	3	-.33*	-	-	-	-.49***	-.31*	-.29*	-.39**
Y	5	-	+	+	+.29*	-.60***	0	0	-
S	7	-.34*	-	0	0	-.27*	0	-	-.33*
O	10	-.38**	-	0	0	-.40***	-	-.28*	-.43**

CORRELATIONS WITHIN H LINE

ACTIVITY		TOTAL TITER				2MEr TITER			
	day	3	5	7	10	3	5	7	10
A	3	-.51*	-	0	0	-.83***	-.49*	-.51***	-.80***
C	5	-	-	+	-	-.78***	-	-	-.55**
T	7	-	+	0	-	-.48*	0	-	-.
I	10	-	0	+	+	-.78***	-.42**	-.44**	-.67***
V									
I									
T									
Y									

CORRELATIONS WITHIN L LINE

		TOTAL TITER				2MEr TITER			
	day	3	5	7	10	3	5	7	10
	3	-	-	0	-	NN	-	-	-
	5	0	+	+	+	NN	0	+	+
	7	-	-	-	0	NN	0	-	-
	10	-.42*	-	-	-	NN	0	-	-.17*

*: $P<0.05$; **: $P<.01$; ***: $P<0.001$

the various days p.i, at one hand and the titers (total and 2MEr) at the other, are presented in Table 2.3. The correlations between titers and the change in lysozyme activity during the immune response were very similar and therefore not presented. All correlations calculated between the lysozyme activity at day 0 and the titers at various days were within the range of -0.1 to +0.1. The partial correlations calculated over lines, corrected for the effect of lines were mostly negative, except for the correlation between the lysozyme activity at day 5 and the total titers at days 7 and 10 p.i. (Table 2.3). The high negative correlations between 2MEr titers and lysozyme were even more explicit if calculated only within the H line. However, the results within the L line were not consistent with the other correlations, i.e. correlations were small and often positive.

DISCUSSION

To determine the contribution of phagocytic processes to the line difference in antibody response, we studied diverse aspects of phagocytosis in our two lines of chickens genetically selected for high or low antibody response at an age of 42 days (Van der Zijpp and Nieuwland, 1986). The line differences in antibody titers of the adult cocks used in the present study were of the same magnitude as those of contemporaries at the selection age (Pinard *et al.*, 1992).

Selection for high antibody production in mice had a negative effect on phagocytosis (Wiener and Bandieri, 1974). The present results do not give evidence for the same association in chickens. The *in vivo* clearance of carbon particles, which are filtered out predominantly by phagocytes in the liver, spleen and bone marrow (Chang and Hamilton, 1979) did not differ between the lines, despite differences in liver weight. This is conform the results of Lamont (1986), who found that selection for high or low antibody response to glutamic acidalanine-tyrosine (GAT) in White Leghorn chickens, had no effect on the clearance of carbon. However, in meat-type chickens divergently selected for antibody response to *Escherichia coli* vaccine, a positive association between antibody response and the clearance of carbon was found (Heller *et al.*, 1992). These contradicting findings in different studies, demonstrate that genetic differences in humoral immune response might not always be accomplished by the same immune mechanisms. Moreover, although often chosen to evaluate the effect of treatment or selection on the phagocytic function in chickens (Glick *et al.*, 1964; Heller *et al.*, 1992), the causal connection between antibody production and carbon clearance is questionable. The carbon clearance assay gives only an estimation of the removal of free particles from the blood. For resistance to pathogens, the intracellular destruction of antigens is of major importance. Therefore, superoxide production during *in vitro* phagocytosis and the release of acid phosphatase and lysozyme before and after

immunization were measured to evaluate the intracellular destruction of antigens (Butler *et al.*, 1977; Di Luzio, 1979). However, no line differences were found in any of the tests, indicating that the lines do not differ in the intracellular destruction of antigens. The activity level of lysozyme in plasma was (still) increased 3 days p.i., although this might be to late for an effect of immunization on acid phosphatase level (Butler et al, 1977). Lysozyme is released into the blood when granulocytes die after intracellular destruction of antigen and by the degranulation of macrophages during phagocytosis (Lie, 1980). Consequently, high lysozyme activity in the blood is thus associated with a high destructive activity of phagocytes. The antibody titers, corrected for lines, were negatively correlated with the activity of lysozyme. Yet, within the L line hardly any negative correlation was found, indicating that although intracellular destruction has a negative effect on antibody production, it is not the reason for the line difference in antibody production.

In both lines the number of granulocytes were decreased after immunization. This might be explained by a mild inflammation occurring at the site of intramuscular immunization attracting circulating phagocytes. However, increasing numbers of circulating granulocytes were reported after intravenous immunization with *Brucella abortus* (Trout *et al.*, 1988). The route of antigen adminstration (Donker *et al.*, 1989) and type of antigen might have caused these contradicting results.

We thus found that, although immunization did influence the number of circulating granulocytes and the activity of lysozyme in plasma, the effects were not different for the lines. Also, in non-immunized cocks overall uptake and digestion of antigen by phagocytes did not differ between the lines. We therefore conclude that the line difference in antibody response was not due to measurable differences in phagocytic activity. The lack of difference in phagocytic activity between the lines urges to study differences in other components of the T cell-dependent antibody response, i.e. antigen presentation, T cell subpopulations, and lymphokine profiles.

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chapter 3

EFFECTS OF IMMUNIZATION ON PHAGOCYTOSIS IN CHICKENS

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submitted

Effects of immunization on phagocytosis in chickens.

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ABSTRACT

This study was conducted to investigate whether immunization affects phagocytic activity of peritoneal exudate cells (PECs). To study the possible effect of circulating antibody, two lines of chickens selected for either high or low antibody response to SRBC were used. Chicks were immunized with SRBC. Five days later, PECs were harvested and incubated with SRBC. The percentage of phagocytic PECs and the number of sheep red blood cells (SRBC) phagocytized by these PECs were determined to estimate phagocytic activity.

In general, immunization enhanced the percentage of phagocytic PECs in both lines, while the level of circulating antibodies had no effect on phagocytic activity.

Incubation of the SRBC used for the pagocytosis test with high, low or pooled high and low line sera, did not enhance phagocytosis compared with non-serum treated SRBC. However, less PECs were phagocytic when the SRBC were incubated with L line serum.

It is concluded that immunization enhances pagocytic activity of PECs. This enhancement is probably not due to antibody. It was hypotized that the T cells sensitized by immunization, activate the PECs. Although, the lines do not differ in phagocytic activity, the composition of the sera might differ between the two lines used.

INTRODUCTION

Immunization increases the level of circulating antibodies and therefore may elevate the amount of antigen-antibody complexes. These complexes easily bind to the Fc receptors on phagocytes, which greatly facilitates phagocytosis. Thus, immunization might enhance phagocytosis in an antibody dependent way. However, next to the effects of the circulating antibodies, immunization might cause the release of other factors which affect phagocytosis.

Lymphocytes sensitized by immunization, selectively accumulate in inflammatory reactions (Koster and McGregor, 1970) and are able to activate the microbicidal capacity of macrophages non-specifically (Simon and Sheagren, 1972; Tompkins *et al.*, 1970). Intraperitoneal exudate cells (PECs) are often used to investigate phagocytosis. In chickens, it is necessary to stimulate the peritoneal cavity with an irritant to recover

adequate amounts of PECs (Glick *et al.*, 1964; Trembicki *et al.*, 1984). This irritant causes an acute local inflammatory reaction, which atracts also the sensitized lymphocytes. This implies that immunization might also alter the phagocytic capacity of the peritoneal phagocytes by lymphokines derived from activated lymphocytes, as well as by circulating antibodies.

Present study was conducted to determine whether immunization affects phagocytic activity of PECs in chickens and whether such effect might be mediated by circulating antibodies. Two chicken lines genetically selected for either high (H) or low (L) antibody response to sheep red blood cells (SRBC; Van der Zijpp and Nieuwland, 1986) were used to achieve the necessary differences in antibody levels. These lines differ significantly in antibody response to SRBC (Kreukniet and Van der Zijpp, 1989). However, indirect measurements of the phagocytic and catabolic activity, indicated no difference in phagocytosis between these chicken lines (Kreukniet *et al.*, 1994). Because the levels of anti-SRBC antibodies differ between the lines, the phagocytic activity might be affected differently between the lines. However, also other serum components might differ between the lines. To obtain some information about possible line differences in serum composition, SRBC used for the phagocytic tests were incubated with either H, L or pooled H and L line sera of non-immunized chicks and phagocytosis of these SRBC was compared with non-serum treated SRBC. The objectives of the study were 1) to study the effect of immunization on phagoctytosis; 2) to investigate wether such an effect was mediated by circulating antibodies; 3) to detect whether the selection lines differ in phagocytosis of SRBC and 4) whether there are indications of line differences in serum components facilitating phagocytosis.

MATERIALS AND METHODS

Chicks

Fourty chicks, of the tenth selection generation of lines selected for high (H) and low (L) antibody production (Van der Zijpp and Nieuwland, 1986) were used, equal numbers of both lines and sexes. After hatching, all chicks were vaccinated against Marek's disease, infectious bronchitis, infectious bursal disease and Newcastle disease at 0, 1, 15 and 22 days of age, respectively. Chicks were housed in two-deck commercial battery cages, with a maximum bird density of 10 chicks per cage. Sexes were kept separately. Food and water were available *ad libitum*.

Sheep red blood cells (SRBC)

Blood was collected from Texel sheep in Alsever solution and washed three times in phosphate buffered saline (PBS). The packed cells were diluted to a concentration (v/v) of 25% in PBS for immunization or 5% for phagocytosis in RPMI 1640 medium (Flow,

Irvine, U.K.). Samples of SRBC used for phagocytosis were treated with a 10% (v/v) concentration of serum (30 min.; room temperature). Either H line, L line or pooled serum, containing equal volumes of H and L line serum, was used. The sera were collected from non-immunized chicks, however, low antibody titers against SRBC were found (3.2 in the H line serum and 1.9 in the L line serum).

Immunization

At 38 days of age, twenty chicks, 5 of each line-sex combination, were intramuscularly immunized with 1 ml 25% SRBC in PBS. Blood was collected before (day 0) and 5 days after immunization. Total and 2-mercaptoethanol resistant (2MEr) haemagglutinin titers were determined in plasma (Van der Zijpp and Leenstra, 1980). The total antibody titers five days after immunization were 9.3 ± 5.4 in the H line and 4.5 ± 1.2 in L line, the difference between the lines being significant ($P<0.001$).

Harvesting of Adherent Peritoneal Exudate cells (PECs)

Four days after immunization, a single intraperitoneal injection with sterile Incomplete Freund's adjuvant (IFA) (Difco, Detroit, IL, USA) 1 ml, 10% (v/v) in sterile PBS was given to all 40 chicks. Approximately 24 hours after this injection, the birds were killed by decapitation and the PECs were harvested according to the method of Sijtsma *et al.* (1991). Briefly, 25 ml cold PBS, containing 10 IU heparin/ml was injected into the peritoneal cavity with a blunt needle. After massage of the abdomen, the peritoneal cavity was opened and the PBS was gently recovered, using a 20 ml syringe. The PECs containing fluid was washed twice in PBS (10 min, 400 x *g*, 4° C) and resuspended in RPMI-1640, supplemented with 100 μg streptomycin (Serva, Heidelberg, Germany) per ml, 100 IU penicillin (Serva) per ml and 2mM L-glutamine (Merck, Darmstadt, Germany) and brought at a final concentration of 1.0×10^6 viable cells/ml.

Phagocytosis

Phagocytosis by PECs was measured as described by Qureshi *et al.* (1986) with minor modifications. Briefly, fivehunderd μl of each PECs suspension at a concentration of 1.0×10^6 PECs/ml was layered on glass coverslips (18 x 24 mm) in 8-fold, and incubated in petridishes at 41°C with 5% CO_2 for 60 min. Subsequently the coverslips were washed with PBS (41°C) to remove non-adherent cells.

The coverslips were placed in a 6-chamber petridish, one per chamber. Into each chamber, 1 ml SRBC suspension, either not serum-treated or treated with H, L or pooled H and L line serum, was dispensed; all 4 SRBC-suspensions were tested in duplicate per chick. The cultures were incubated at 41°C in a humidified atmosphere with 5% CO_2.

After 45 min, the coverslips were washed with PBS to remove the not phagocytized SRBC. The cells were then fixed in methanol for 10 min, and stained with Hemacolor (Merck). Per coverslip, 500 PECs were counted and the number of phagocytic PECs and the total number of phagocytized SRBC were scored.

Statistical analyses

After tests for normality, Analyses of Covariance were performed by the GLM procedure (SAS, 1989). The percentage of phagocytic PECs and the mean number of phagocytized SRBC per phagocytic PECs were analyzed within 'serum treatment SRBC', with line, immunization and sex as main effects and either the titer at day 0 or at day 5 after immunization as covariable within immunization. The effects of sex and titers were not significant. Differences between line-immunization groups were tested with Tukey's honestly significant difference (HSD) test.

The parameters were also analyzed with 'serum treatment SRBC' (H, L, H+L, not serum-treated) as repeated factor, contrasts between phagocytosis of serum treated SRBC and not serum-treated SRBC were estimated.

Analyses of Variance were conducted on the total titers at Day 5 after immunization with line, sex, immunization and their interactions as effects. Immunization and line, their interaction as well as the interaction between line and sex affected the titer significantly.

RESULTS

Immunization and line effects when offered not serum-treated SRBC.

A significant line-immunization interaction was found when the offered SRBC were not serum-treated (Table 3.1). No effect of immunization was found in the L line, while in the H line the percentage phagocytic PECs was enhanced by immunization. However, the percentages phagocytic PECs found in the non-immunized L line group were high, compared to the other L and H control groups.

Immunization and line effects when offered serum-treated SRBC.

A significant immunization effect was found within the H, L or pooled H and L line serum used to pre-treat the SRBC. In both lines, the percentages phagocytic PECs were higher after immunization than in the non-immunized control group (Table 3.1). The number of SRBC phagocytized per PECs was not affected by line or immunization within pre-treatment of the SRBC. Consequently, the results of total number of phagocytized SRBC mimicked the results of the percentage of phagocytic PECs.

TABLE 3.1. Phagocytosis (x ± sd) by peritoneal exudate cells (PECs) recovered from two lines selected for high (H) or low (L) antibody response, 5 days after immunization and of their non-immunized controls. The SRBC offered to phagocytize were serum-treated with H, L or pooled H + L line serum, or not serum-treated (non).

	Percentage Phagocytic PECs			
	H line		L line	
serum-treat SRBC	Immunized	Control	Immunized	Control
Non	29.7^{a} ±11.6	15.0^{b} ± 7.4	19.3^{ab} ± 5.3	20.4^{ab} ± 9.5
H	25.6^{a} ±11.7	15.7^{a} ± 7.3	20.0^{ab} ± 9.7	12.7^{b} ± 5.5
L	24.1^{a} ±15.3	10.4^{b} ± 3.6	17.6^{ab} ± 5.4	13.0^{b} ± 5.9
H+L	19.3^{a} ± 5.4	17.9^{a} ± 10.8	27.5^{a} ± 16.6	15.7^{a} ± 9.2
	number of SRBC Phagocytized per Phagocytic PEC			
Non	1.48 ± 0.29	1.52 ± 0.22	1.51 ± 0.20	1.50 ± 0.29
H	1.54 ± 0.45	1.59 ± 0.45	1.37 ± 0.19	1.47 ± 0.32
L	1.47 ± 0.30	1.27 ± 0.15	1.35 ± 0.28	1.38 ± 0.14
H+L	1.40 ± 0.16	1.59 ± 0.54	1.42 ± 0.24	1.58 ± 0.36

^{a,b}: different superscripts within a line differ significantly ($P<0.05$; Tukey's HSD).

Effects of serum-treatment of the SRBC.

In the H line the percentages of phagocytic PECs offered serum-treated SRBC, were of the same magnitude as when the SRBC were not serum-treated. In the non-immunized L line, serum-treatment decreased this percentage. In the statistical analysis using 'serum-treatment of the SRBC' (H, L, pooled H and L serum, or not serum-treated) as a repeated factor, and line, immunization and the interactions as main effects, no significant line effect was found. Immunization and 'serum treatment of the SRBC' affected the percentage of phagocytic PECs and the total number of SRBC phagocytized. No interactions between line and serum-treatment or immunization and serum-treatment were found. Immunization enhanced the percentages of phagocytic PECs and the number of phagocytized SRBC. The effect of serum-treatment of the SRBC was due to a consistent difference between the phagocytized SRBC pre-treated with L line serum and the not pre-treated SRBC. After treatment with L line serum, lower percentages of phagocytic PECs, less SRBC per PEC and lower numbers of

phagocytized SRBC were found. The contrasts between the SRBC treated with H or pooled H and L sera and the not serum-treated SRBC were not significant.

DISCUSSION

The first objective of present study was to investigate wether immunization influences the *in vitro* phagocytic activity of PECs. These PECs were harvested after stimulation with an irritant, thus in an acivated state (Ruco and Smetlzer, 1978; Golemboski *et al.*, 1989). It was found that in general, immunization increased the percentage of phagocytic PECs, in both selection lines. It is tempting to ascribe this increase to the antibodies elicited by the immunization, because passive immunization with antibodies enhanced phagocytosis in turkeys (Arp, 1982). However, no significant effect of circulating endogenous antibody on phagocytosis could be detected and, although lines differed significantly in antibody titers, phagocytic activity did not differ between the lines. No enhancement of phagocytosis was found when the SRBC were incubated with exogenous antibody and other serum factors. Therefore, it seems not likely that antibodies causing the enhancing effect of immunization. Because immunization activates T cells and activated T cells accumulate in inflammatory sites (Koster and McGregor, 1970) and are able to activate macrophages (Simon and Sheagren, 1972), it seems possible that the immunization effect is mediated by these T cells or factors released by the T cells.

In the non-immunized lines, non-serum treated SRBC activated a slightly higher percentage of PECs from the L line compared with the H line. This makes it attractive to speculate that the PECs from the non-immunized L line have a higher phagocytic activity compared to the H line PECs, as in the Biozzi mice (Biozzi *et al.*, 1984). However, this was not statistically proven and sofar, no line differences in phagocytosis were found, both in immunized and non-immunized chicks (Kreukniet *et al.*, 1994). Therefore, line differences in phagocytic activity seem not likely.

In other studies, pre-treatment of SRBC with sera containing subagglutinating levels of antibodies, enhanced the phagocytic activity of PECs (Qureshi *et al.*, 1986; Chu and Dietert, 1988; Puzzi *et al.*, 1990). In the present study, no enhancement was found. However, the sera used were obtained from non-immunized chicks and therefore contained only low amounts of anti-SRBC antibodies, which might explain the lack of enhancement. Moreover, L line serum seemed to contain factors reducing the percentage of phagocytic PECs when compared to SRBC not pre-treated with serum. On the nature of these factors can only be speculated. A difference between the lines in serum factors of the alternative complement pathway has been found (personal communication, F. Demey, Antwerpen, Belgium). However, complement factors are

known to facilitate phagocytosis and in present study non of the sera had a true opsonizing effect.

In general, we can conclude that immunization enhanced phagocytosis of SRBC. No consistenty differences were found between the selection lines and line differences in circulating antibodies did not affect phagocytosis. Therefore, it seems not likely that enhancement of phagocytosis is mediated by circulating antibodies. Thus, although still speculative, the immunization effect might be contributed to activation of PECs by sensitized T cells. Compared with non-serum treated SRBC, the treatment of SRBC with serum had no facilitating effect on phagocytosis. However, this effect differed between serum originating from the H or L line. Therefore the composition of the sera might differ slightly between the lines.

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chapter 4

EFFECTS OF DIFFERENT DOSES OF SHEEP ERYTHROCYTES ON THE HUMORAL IMMUNE RESPONSE OF CHICKEN LINES SELECTED FOR HIGH OR LOW ANTIBODY RESPONSE

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Effects of different doses of sheep erythrocytes on the humoral immune response of chicken lines selected for high or low antibody production.

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ABSTRACT

A study was conducted to determine the influence of dose of sheep red blood cells (SRBC) on humoral response of chicken lines selected for high (H) or low (L) antibody production to SRBC. Chicks were of the fifth selection generation; both sexes were used. The primary doses of SRBC used were $5x10^{-4}$, $5x10^{-3}$, $5x10^{-2}$, $25x10^{-2}$ and $5x10^{-1}$ ml packed cells, resuspended with PBS to 1 ml and injected intramuscularly (IM). All chicks were IM reimmunized with $5x10^{-1}$ ml packed cells in 0.5 ml PBS. Throughout the experiment, H line chicks had higher titers than L line chicks. Level of primary total and 2ME-resistant titers followed dose level. However, in total titers, interactions between line and dose were seen at days 3 and 5 p.i., this was caused by a deviation in the ranking of the doses in the L line. Moreover, the kinetics of the primary response differed between the lines. Generally, in H line peak titers were reached earlier in response. The level sequence of the total titers in secondary response in general, was inverse to the dose level. However, from day 7 of secondary response onwards, dose effects were influenced by line. In L line no effects of dose on responding titers were seen. The 2ME-resistant titers followed dose level in secondary response. Line differences in the reaction to the primary dose level will influence the effectiveness of vaccinations. This should be kept in mind when chickens are bred for disease resistance.

INTRODUCTION

In mice, bidirectional selective breeding studies have been carried out to gain a better understanding of the genetic variation in disease resistance (Biozzi *et al.*, 1979; 1984). Difference in antibody production in lines selected for high (H) or low (L) antibody production to sheep red blood cells (SRBC) could be contributed to a difference between these lines in antigen handling by macrophages (Biozzi *et al.*, 1979; 1984) and in the multiplication rate of B-lymphocytes (Biozzi *et al.*, 1984). A higher catabolic rate in L line mice macrophages caused reduced effectiveness in triggering B cells to antibody response against SRBC. A larger threshold dose SRBC was required to give a detectable antibody response in L line mice, and differences in antibody response

between the lines were more pronounced at low doses (Biozzi *et al.*, 1984).

In chicken lines selected for antibody production after intravenous (IV) immunization with SRBC, the largest differences between H and L lines were found when the lines were immunized with the selection-dosage (Ubosi *et al.*, 1985; Gross, 1986). Thus, selection dose was also the optimal dose in these selection lines. However, when immunized with lower doses, differences between the lines were larger than after immunization with doses above the selection-dosage (Gross, 1986). Differences between the lines in the kinetics of the immune response were not determined (Ubosi *et al.*, 1985; Gross, 1986).

Injections of carrageenan or colloïdal carbon (Van der Zijpp *et al.*, 1988; 1989) in chickens selected for anti-SRBC production after IM immunization, did not produce any difference between the lines in antigen handling by macrophages. However, more antibody produing cells were detected in the H line spleen (Donker, 1989).

The present study is conducted to evaluate the influences of dose of SRBC on humoral responses of chicken lines selected for anti-SRBC production after IM administration of SRBC. In a previous study, using White Leghorn laying hens, a dose of $5x10^{-4}$ml SRBC did not invoke an immune response (Van der Zijpp, 1978). A dosage SRBC ranging from $5x10^{-4}$ ml to $5x10^{-1}$ ml SRBC was used in this study to demonstrate line differences from threshold dose to doses inducing normal immune response.

MATERIALS AND METHODS

Experimental Chicks.

One hundred chicks of the fifth generation selected for high (H) or low (L) antibody production to sheep red blood cells (SRBC) (Van der Zijpp and Nieuwland, 1986) were used. Selection was performed on a basic population of ISA-Warren, medium heavy, brown egg layers. Selection criterion was total agglutination titer 5 days after IM immunization with $25x10^{-2}$ ml packed sheep red blood cells (SRBC), resuspended to 1 ml in phosphate buffered saline (PBS) at 37 days of age.

Lines and sexes were equally divided over the 5 dose-groups without any replicates (n = 5 per dose-line-sex-group). After hatching, all chicks were vaccinated against Mareks disease, infectious bronchitis, infectious bursal disease and Newcastle disease at 0, 1, 15 and 22 days of age, respectively. Chicks were housed in two-deck battery cages, with a maximum bird density of 10 chicks per cage. Sexes were kept separately. Food and water were available *ad libitum*.

SRBC Doses.

Five doses of SRBC were used, $5x10^{-4}$, $5x10^{-3}$, $5x10^{-2}$, $25x10^{-2}$ and $5x10^{-1}$ ml

packed cells, all resuspended to 1 ml with PBS. The SRBC were obtained in a heparin solution from five, unrelated, Texel sheep and washed three times in physiological saline (0.9% NaCl).

Immunization.

At 31 days of age, chicks were bilaterally injected with two 0.5 ml portions of SRBC dose, into the *Musculus pectoralis* (breast muscles). Ten chicks per line, five cockerels and five pullets, were injected with each SRBC dose. At 59 days of age, all 100 chicks were reimmunized IM with $5x10^{-1}$ ml packed cells, ml packed cells, resuspended to 1 ml with PBS.

Preparation of Plasma Samples.

All chicks were repeatedly bled from the wingvein at 0, 3, 5, 7, 10 and 14 days p.i. and at 0, 3, 5, 7, 10 and 13 days post reimmunization (p.r.). After centrifugation (1200 x g) plasma was collected and stored at -20°C until assayed.

Haemagglutination Assay.

Antibody titers, both total and 2mercaptoethanol (2ME)-resistant antibody titers were determined, using a microtiter procedure (Van der Zijpp and Leenstra, 1980). Titers were expressed as the $\log_2$ of the highest plasma dilution giving total agglutination.

Statistical Analysis.

The titers were analysed using the GLM-procedure of SAS (SAS, 1985), with the Model 1. Three final models were chosen to fit the different variables.

$$Y_{ijkl} = \mu + L_i + D_j + S_k + LxD_{ij} + LxS_{ik} + DxS_{jk} + LxDxS_{ijk} + e_{ijkl} \quad (1)$$

$$Y_{ijk} = \mu + L_i + D_j + LxD_{ij} + e_{ijk} \quad (2)$$

$$Y_{ijk} = \mu + L_i + D_j + LxD_{ij} + LxS_{ik} + e_{ijk} \quad (3)$$

$$Y_{ijk} = \mu + L_i + D_j + e_{ijk} \quad (4)$$

Y_{ijkl} = value of the l-th chick: primary or secondary titer
μ = population mean
L_i = effect of the i-th line (i = H, L)
D_j = effect of the j-th dose
(j = $5x10^{-4}$, $5x10^{-3}$, $5x10^{-2}$, $25x10^{-2}$ and $5x10^{-1}$ ml SRBC)
S_k = effect of the k-th sex (k = male, female)
LxD_{ij} = two-way interaction effect of the i-th line and j-th dose
(LxS_{ik}, DxS_{jk}, LxS_{ik}, DxS_{jk} = as LxD_{ij}, but between other main effects)
$LxDxS_{ijk}$ = as above, but three-way interaction
e_{ijkl} = remainder.

Model 2 was the best fit for primary total titers, model 3 for secondary total titers and model 4 for both primary and secondary 2ME-resistant titers.

Variables not conforming to criteria of normality were omitted from further statistical analyses: primary 2ME resistent titers at days 0, 3 and 5 p.i., secondary total titers at day 0 p.i., secondary 2ME-resistant titers at days 0 and 3 p.i. Differences between doses were tested with Tukey's studentized range (HSD) test.

RESULTS

Primary total titers.

Differences between the lines in the kinetics of the responses were apparent (Figure 4.1). Responses were higher in the H line and peak titers were generally reached sooner after immunization than in the L line. The height of the response was also influenced by the SRBC dose administered; a lower dose of SRBC was followed by a smaller anti-SRBC response. However, within days, differences between H and L line tended to be smaller at low doses. At days 3 and 5 p.i., significant (Table 4.1) interactions between line and SRBC dose were found, caused by a deviation in the ranking of the L line. In H line a descending sequence of SRBC dose administered was followed by a similar sequence in antibody level. But in L line chicks the $25x10^{-2}$ ml SRBC dose caused higher total titers than the $5x10^{-1}$ ml SRBC dose (Figures 4.1 and 4.2).

TABLE 4.1. Analysis of variance for the primary total titers (sum of squares) of chicken lines selected for high or low antibody production immunized with different doses sheep red blood cells.

		DAYS p.i.					
Source of variation	df	0	3	5	7	10	14
Line	1	1.0	28.1***	98.0***	136.9***	70.6***	17.6**
Dose SRBC	4	2.1	81.1***	401.1***	310.1***	81.6***	20.4*
Line * Dose	4	2.9	16.1**	22.1*	8.9	1.8	11.0
Remainder	90	55.6	72.1	190.1	249.1	235.8	182.0

* : $P < 0.05$; ** : $P < 0.01$; ***: $P < 0.001$.

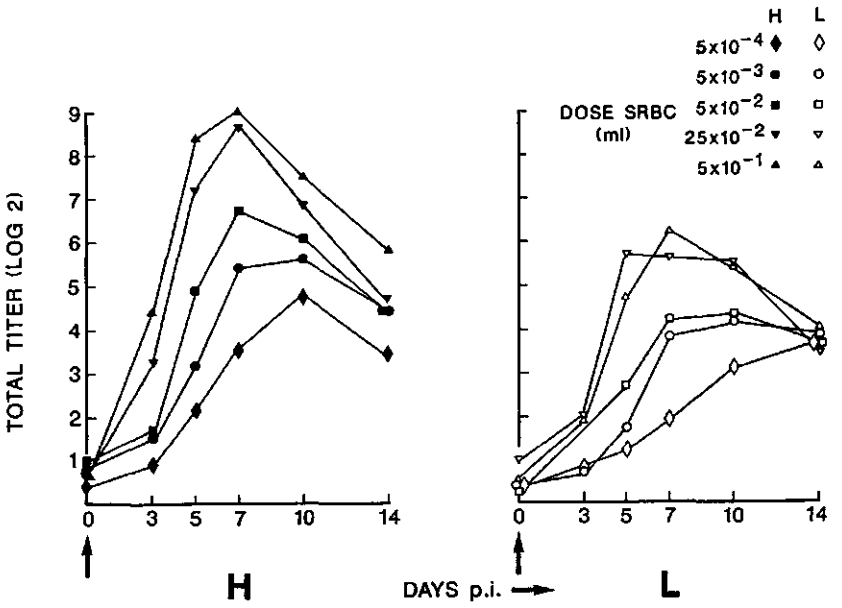

FIGURE 4.1. The kinetics of the primary total antibody response in chicken lines selected for high (H) or low (L) response to SRBC after IM immunization with 5 different doses SRBC.

Primary 2ME-resistant titers.

No IgG-type antibodies were detected at days 0 and 3 p.i. At day 5 p.i. only when $25x10^{-2}$ or $5x10^{-1}$ ml SRBC was injected very small amounts of IgG could be detected in only a few chicks (H < 1.8; L < 0.2). At days 7, 10 and 14 p.i., line and dose affected 2ME-resistant titers. Immunization with low doses of SRBC was followed by a low IgG-type response (Table 4.2). The sequence in response followed the order of SRBC doses. Except for consistently lower titers in L line chicks, no differences between lines were found in the responses to the different doses SRBC.

Secondary total titers.

A complex model was necesarry to fit the results of the total titers after reimmunization. Titers were affected by line, dose and sex at most days, as well as by the interactions line * sex, and line * dose (Table 4.3). In general, H line chicks had higher total titers, but H line pullets had a higher (0.2 - 2 titerpoints) response than the cockerels of this line, while the sexes in L line did not differ.

Besides the differences in the height of the response, also differences in the kinetics between the lines were apparent. In general, H line peak titers were reached on day 5

p.r. and followed by a decrease in titer of 0.5. In L line titers on days 5 and 7 p.r. hardly differed from each other (Table 4.4). Furthermore, the height of the response in H line was affected by the primary SRBC dose administered; the response being more or less inverse to the primary dose level. In L line no relationship between titers and dose could be detected.

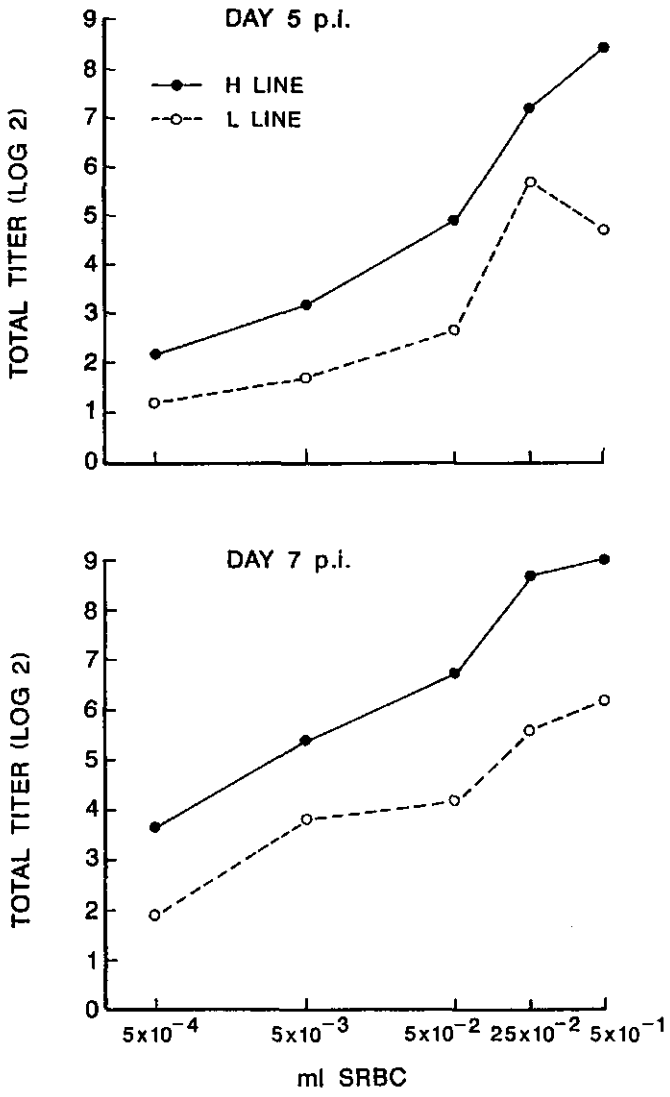

FIGURE 4.2. The primary total titers at days 5 and 7 p.i. of chicken lines selected for high (H) or low (L) response to SRBC after IM immunization with 5 different doses SRBC.

TABLE 4.2. Primary and secondary 2ME-resistant titers within high (H) or low (L) antibody production lines and dose SRBC (ml) (Mean ± standard deviations).

DOSE SRBC	Days post Primary immunization H line		
	7	10	14
$5x10^{-4}$	0.0 ± 0.0^{e}	0.1 ± 0.3^{d}	0.0 ± 0.0^{d}
$5x10^{-3}$	0.2 ± 0.4^{ed}	0.5 ± 0.8^{cd}	0.4 ± 0.7^{cd}
$5x10^{-2}$	0.9 ± 0.7^{cde}	1.6 ± 1.0^{bc}	0.8 ± 0.8^{bcd}
$25x10^{-2}$	2.5 ± 1.8^{ab}	2.7 ± 1.6^{ab}	1.5 ± 1.3^{ab}
$5x10^{-1}$	3.5 ± 1.5^{a}	3.5 ± 1.3^{a}	2.3 ± 1.1^{a}
	Days post Primary immunization L line		
	7	10	14
$5x10^{-4}$	0.0 ± 0.0^{e}	0.0 ± 0.0^{d}	0.1 ± 0.3^{d}
$5x10^{-3}$	0.0 ± 0.0^{e}	0.4 ± 0.5^{cd}	0.1 ± 0.3^{d}
$5x10^{-2}$	0.3 ± 0.5^{de}	0.8 ± 0.9^{cd}	0.2 ± 0.4^{d}
$25x10^{-2}$	1.6 ± 1.0^{bcd}	2.3 ± 0.7^{ab}	1.3 ± 0.7^{abc}
$5x10^{-1}$	2.0 ± 1.4^{bc}	2.6 ± 1.4^{ab}	1.9 ± 0.6^{a}
	Days post Secondary immunization H line			
	5	7	10	13
$5x10^{-4}$	$2.2 \pm 11.3c$	3.6 ± 0.8^{ab}	2.8 ± 1.1^{ab}	2.1 ± 1.2^{b}
$5x10^{-3}$	2.9 ± 1.5^{abc}	4.0 ± 1.2^{ab}	3.3 ± 1.2^{ab}	2.5 ± 1.3^{ab}
$5x10^{-2}$	3.6 ± 1.3^{abc}	4.7 ± 1.5^{ab}	3.7 ± 1.3^{ab}	2.7 ± 1.3^{ab}
$25x10^{-2}$	4.1 ± 1.0^{ab}	4.4 ± 1.2^{ab}	3.8 ± 0.4^{ab}	3.0 ± 10.9^{ab}
$5x10^{-1}$	4.6 ± 0.7^{a}	4.8 ± 0.6^{a}	4.1 ± 0.7^{a}	3.7 ± 0.5^{a}
	Days post Secondary immunzation L line			
	5	7	10	13
$5x10^{-4}$	2.0 ± 1.2^{c}	3.2 ± 1.1^{b}	2.6 ± 1.1^{b}	2.0 ± 1.2^{b}
$5x10^{-3}$	2.5 ± 1.6^{bc}	3.2 ± 1.5^{b}	3.0 ± 1.3^{ab}	2.1 ± 1.0^{b}
$5x10^{-2}$	3.5 ± 0.5^{abc}	4.4 ± 0.5^{ab}	3.5 ± 0.5	3.0 ± 0.7^{ab}
$25x10^{-2}$	3.7 ± 1.1^{abc}	4.1 ± 0.7^{ab}	3.3 ± 0.7^{ab}	3.0 ± 0.5^{ab}
$5x10^{-1}$	3.6 ± 1.0^{abc}	3.9 ± 0.7^{ab}	3.5 ± 1.0^{ab}	2.4 ± 1.1^{ab}

^{abcde} : means within column having the same letter do not signifcantly differ ($P<0.05$)

TABLE 4.3. *Analysis of variance for the secondary total titers (sum of squares) of* chicken lines selected for high or low antibody production, immunized with different dose SRBC.

Source of variation	df	DAYS p.i. 3	5	7	10	13
Line	1	37.2***	110.3***	75.7***	59.3***	13.0***
Dose SRBC	4	4.2	30.4*	39.7***	12.7	4.5
Sex	1	0.1	18.5**	3.6	7.3*	10.2**
Line*Dose SRBC	4	1.8	12.4	18.7*	18.3*	10.5*
Line*Sexe	1	2.3	18.5**	10.9**	7.3*	17.6***
Remainder	88	72.6	194.7	136.2	132.1	89.1

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Secondary 2ME-resistant titers.

Effects of line and dose were found on secondary IgG titers. Again, H line had higher titers. Dose effects were not consistent. In general, the highest primary dose of SRBC also gave the highest secondary IgG response (Table 4.2). No differences between the lines in response to the doses were detected.

TABLE 4.4. Secondary total titers within high (H) or low (L) antibody production line and dose SRBC (Mean ± standard deviation).

LINE	DOSE	DAYS P.I. 0	3	5	7	10	13
H	$5x10^{-4}$	0.5 ±0.5	2.8 ±0.9^{ab}	7.5 ±2.4^{ab}	7.1 ±1.5^{ab}	5.8 ±1.5^{ab}	3.7 ±0.9^{abc}
	$5x10^{-3}$	1.1 ±0.6	3.1 ±0.9^{a}	7.9 ±2.0^{a}	7.9 ±1.1^{a}	6.4 ±2.3^{a}	4.2 ±1.4^{a}
	$5x10^{-2}$	0.9 ±0.7	2.3 ±1.2^{abc}	6.6 ±1.8^{abc}	6.1 ±2.2^{abc}	5.1 ±1.7^{abc}	4.1 ±1.2^{ab}
	$25x10^{-2}$	0.7 ±0.7	2.5 ±1.0^{abc}	6.0 ±1.2^{abcd}	5.5 ±0.8^{ab}	4.6 ±0.7^{bc}	3.0 ±1.3^{abc}
	$5x10^{-1}$	1.2 ±0.8	2.8 ±0.8^{ab}	5.5 ±1.1^{bcd}	5.0 ±0.7^{c}	4.4 ±0.5^{bc}	3.3 ±1.3^{abc}
L	$5x10^{-4}$	0.5 ±0.5	1.8 ±1.2^{abc}	4.8 ±1.7^{cd}	4.8 ±1.3^{c}	3.4 ±1.1^{c}	2.7 ±1.1^{bc}
	$5x10^{-3}$	0.7 ±1.3	1.6 ±0.8^{bc}	4.9 ±1.9^{cd}	4.9 ±1.5^{c}	3.8 ±0.8^{c}	2.5 ±1.3^{c}
	$5x10^{-2}$	0.7 ±0.5	1.3 ±0.7^{c}	4.4 ±1.1^{cd}	4.4 ±0.8^{c}	3.8 ±0.6^{c}	3.2 ±0.8^{abc}
	$25x10^{-2}$	1.3 ±0.7	1.5 ±0.7^{bc}	4.6 ±1.2^{cd}	4.3 ±0.9^{c}	3.5 ±0.8^{c}	3.0 ±0.8^{abc}
	$5x10^{-1}$	0.8 ±0.9	1.2 ±0.8^{c}	4.3 ±1.3^{d}	4.5 ±1.3^{c}	4.1 ±1.4^{bc}	3.3 ±1.2^{abc}

^{abcd} : means within a column having the same letter do not significantly differ ($P<.05$)

DISCUSSION

Objectives of this study were to evaluate line differences in antibody response to SRBC doses ranging from threshold dose to doses inducing normal immune response. Although White Leghorns did not respond to $5x10^{-4}$ ml SRBC, this dose did induce an antibody response in our selection lines. And the dose range of $5x10^{-4}$ to $5x10^{-1}$ ml SRBC in present study did not include the threshold dose in neither line. When the height of the response, which was influenced by the dose level, was not considered, the kinetics of the antibody response to all doses SRBC were consistent with the normal observations in these selection lines (Van der Zijpp *et al.*, 1988, 1989; Donker *et al.*, 1990). The IgG-type antibodies appeared relatively late, compared to the results of other investigators (Martin *et al.*, 1988). However, when our selection lines were IV immunized IgG type antibodies could also be detected earlier in response (Donker *et al.*, 1990), indicating an immunization route effect.

The higher titers of the H line, within dose, agree with the usual observations on our selection lines (Van der Zijpp and Nieuwland, 1986; Donker *et al.*, 1990; Van der Zijpp *et al.*, 1988; 1989). In mice (Biozzi *et al.*, 1984) and chickens (Ubosi *et al.*, 1985; Gross, 1986) differences between selection lines were more pronounced when these lines were immunized with low doses of SRBC. Biozzi and associates (Biozzi *et al.*, 1984) contributed this to the higher catabolic rate of L line macrophages, leaving less SRBC's to trigger B cell response. This hypothesis is not supported by our study; the differences between primary antibody titers of our selection lines even tended to be smaller at low doses (Figure 4.2). Moreover, when macrophages of both lines were killed with carrageenan (Van der Zijpp *et al.*, 1988), line differences were maintained. Also, no line differences in the activity of phagocytes could be determined (Kreukniet *et al.*, 1994), giving strong evidence that other mechanisms than antigen handling by macrophages are responsible for the differences in our chicken lines. Moreover, a higher number of direct plaque forming cells in H line chicks was found (Donker, 1989). At the moment FACS analyses are carried out to study the cell populations in the blood and the lymphoïd organs in greater detail.

In general, the primary immune response followed the level of SRBC dose, while the secondary response in the H line showed an inverse response sequence. In the L line no order in total antibody level could be detected. It is obvious that these within line differences in relation to the primary dose level could have negative consequences on the effectiveness of vaccination. When chickens are bred for disease resistance these effects should be kept in mind.

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chapter 5

EFFECTS OF ROUTE OF IMMUNIZATION, ADJUVANT AND UNRELATED ANTIGENS ON THE HUMORAL IMMUNE RESPONSE IN LINES OF CHICKENS SELECTED FOR ANTIBODY PRODUCTION AGAINST SHEEP ERYTHROCYTES

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Effects of route of immunization, adjuvant and unrelated antigens on the humoral immune response in lines of chickens selected for antibody production against sheep erythrocytes.

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ABSTRACT

Effects of intramuscular (IM), intravenous (IV) and intraperitoneal (IP) primary immunization with the T cell-dependent antigen sheep red blood cells (SRBC) was studied in two chicken lines selected for either high (H) or low (L) antibody response after IM immunization with SRBC. Line differences were affected by the primary route of immunization as was true for the memory induction.

Intravenous immunization with the T cell-dependent antigen Bovine Serum Albumin (BSA) showed line differences similar to those found after IM or IV immunizations with SRBC. Immunizations with the T cell-independent antigens *Brucella abortus* (BA) or Salmonella H-antigen (SHA) both revealed an interaction between selection lines and sex of the chicks, although interaction effects were opposite.

Immunization with SRBC in Incomplete Freund's Adjuvant (IFA) did not affect the line differences, whereas immunization with Complete Freund's Adjuvant (CFA) diminished the line differences.

It is postulated that differences in antibody production between the selection lines might be contributed to differences in T cell activity.

INTRODUCTION

Two lines of chickens were divergently selected for antibody production to sheep red blood cells (SRBC), a T cell-dependent antigen (Van der Zijpp and Nieuwland, 1986). Chicks were selected for either high (H) or low (L) antibody production after an intramuscular (IM) immunization with SRBC.

Since no evidence of differences between the selection lines in macrophage activity was found so far (Van der Zijpp x 1988, 1989). We further studied possible mechanisms responsible for the differences in antibody response between the genetically selected lines. These included:

i) Routes of immunization. The route by which an antigen enters the body determines the lymphoid tissues involved in the immune response. After IV administration of antigen, the spleen will be a major source of antibodies (White *et al.*, 1975). An IM

injection will cause a more local reaction, and the antigen may be transported via draining lymphatic ducts to regional lymphoid tissues. In these functionally lymph node-like structures, antibody responses will be elicited. The spleen is also involved in this response (Donker *et al.*, 1989). After an IP immunization, the lymphoid tissue around the intestine is probably the prime side for antibody production.

ii) Adjuvant. Intramuscular immunization with antigen in either Incomplete or Complete Freund's Adjuvant (IFA and CFA, respectively) enhances and prolongs the antibody response in mammals. IFA and CFA both enhance humoral immunity, however, only CFA elicits cell-mediated immunity (Allison and Byars, 1986). The mycobacterial components in CFA stimulate T cells, among which T helper populations (Allison and Davies, 1971; Waldman and Pope, 1977). Accessory cells are probably involved in this process by releasing factors, such as interleukine-1, stimulating the proliferation of helper T lymphocytes (Allison and Byars, 1986).

iii) Unrelated antigens. Measuring the humoral response of H and L line chickens to 1) a soluble T cell-dependent antigen like Bovine Serum Albumin (BSA), 2) a partially T cell-independent antigen like *Brucella abortus* (BA), or 3) an a-specifically acting T cell-independent antigen, such as *Salmonella* H-antigen (SHA) will extend the knowledge of the effect of selection on T cell involvement in antibody response.

MATERIALS AND METHODS

Experimental Chicks

A total of 160 chicks was used in six experiments. All chicks were of the 4th generation of lines selected for either a high (H) or low (L) hemagglutination titer, measured 5 days after a primary immunization with 1 ml 25% SRBC in phosphate buffered saline (PBS) injected in the *musculus pectoralis* at 37 days of age. Chicks were housed in rearing cages, lines intermingled, but sexes separated. Food and water were applied *ad libitum*

Vaccinations against Marek's disease, infectious bronchitis, infectious bursal disease and Newcastle's disease were done at 1, 2, 16 and 20 days of age, respectively.

Antigens

The SRBC were collected from Texel sheep. Blood was washed three times in PBS and packed cells were diluted to concentrations of either 5% or 25% in PBS (v/v).

Salmonella H Antigen A (SHA) (*Salmonella enteritidis paratyphoid* A) was obtained from Difco laboratories (Detroit, Michigan, USA).

Brucella abortus (BA) (CVI, Lelystad, the Netherlands) containing approximately $5x10^{11}$ bacteria/ml was diluted 1:10 in PBS.

Bovine Serum Albumin (BSA) (Sigma, Chemical Company, St. Louis) was diluted to a

4% dilution in PBS (v/v).

Experimental Design

Route of Immunization. Three experimental groups of 20 chicks each, in which lines and sexes were represented equally, were formed. At 31 days of age groups were either injected intraperitoneally (IP) with 0.5 ml 5% SRBC, intravenously (IV) (*vena cutanea*) with 0.5 ml 5% SRBC, or intramuscularely (IM) with in total 1 ml 25% SRBC, one aliquot at each thigh (*musculus pectoralis*). On days 0, 3, 5, 7, 11 and 14 post immunization (p.i.) blood was collected of all chicks. The IM-group was also bled at days 20, 25, 28 and 33 p.i. Plasma was harvested and stored at -18° C until assayed.

To investigate the effect of primary immunization route on memory induction, all 60 chicks were IM reimmunized at 66 days of age. One ml 50% SRBC was injected in two aliquots, one in each thigh. Blood was collected at 0, 3, 5, 7, 10 and 12 days p.i. Plasma was stored at -18° C. In all plasma samples total and 2-Mercapthoethanol resistant (2MEr) antibodies were determined using the microtiter procedures (Delhanty and Solomon, 1966, McCorkle and Glick, 1980). Antibody titers were expressed as the log2 of the reciprocal of the highest plasma dilution giving complete agglutination.

Adjuvants. Experimental groups consisted of 20 chicks, 5 of each line-sex-combination. Chicks were IM immunized with in total 1 ml containing 0.25 ml packed SRBC, 0.25 ml PBS and either 0.5 ml Incomplete Freund's Adjuvant (IFA) or 0.5 ml Complete Freund's Adjuvant (CFA) (Difco Laboratories, Detroit, Michigan, USA), injected in two aliquots at 31 days of age. The IM-chicks of the route-experiment were used as controls.

Blood was collected at 0, 3, 5, 7, 11, 14, 20, 25, 28 and 33 days p.i. Of the CFA-group, blood samples were also taken at 35, 38, 42, 45 and 47 days p.i.. The IFA group was IM reimmunized (without IFA) at 66 days of age, 35 days p.i., with in total 1 ml 50% SRBC injected in two aliquots. Blood was collected at days 0, 3, 5, 7, 10 and 12 after reimmunization. Total and 2MEr antibody titers were determined.

BSA-immunization. At 66 days of age, 20 chicks, 5 of each line-sex combination, were IV injected with a 4% BSA diluted in PBS (approximately 40 mg BSA per kg body weight; i.e. 1 ml for pullets and 1.1 ml for cockerels). Blood was collected at 0, 3, 5, 7, 10 and 12 days p.i. and titers were determined in an enzyme-linked immunosorbent assay (ELISA) as described by Sijtsma *et al.* (1989).

BA-immunization. At 66 days of age, 5 chicks of each line-sex combination were IV immunized (*vena cutanea*) with 0.1 ml BA in the right wing. Blood was collected at 0, 3, 5, 7, 10 and 12 days p.i. Both total and 2MEr titers were determined using the microtiter procedure. Dilution (1:1) series, were made with 50 ul serum in 50 ul PBS and 50 ul of a 10% BA solution in PBS was added to each cup. After 24 hours storage in a humid box (room temperature), titerplates were held vertical for 30 seconds and

titers were read as the log reciprocal of the highest dilution showing agglutination.

SHA-immunization. At 24 days of age, 5 chicks of each line-sex combination were IV (*vena cutanea*) injected with 0.1 ml SHA. Blood was collected on 0, 3, 5, 7, 10 and 14 days p.i. Agglutination titers were determined using the (Dynatech) microtiter procedure. Dilution series (1:1) were made with 25 ul serum in 25 ul PBS. To each cup, 25 ul 1:5 SHA in PBS was added. After shaking for 15 seconds, titerplates were stored in a humidbox at room temperature for 4 days. Titers were measured as described for the BA-experiment.

Statistical analysis.

All normal distributed data were analyzed using the GLM-procedure of SAS (SAS, 1985), with the appropriate full factorial design. Non significant factors were omitted from the final model. In total 8 models were found which fitted the data best.

(1) $Y_{ijk} = \mu + L_i + R_j + e_{ijk}$

(2) $Y_{ijk} = \mu + L_i + R_j + (L*R)_{ij} + e_{ijk}$

(3) $Y_{ij} = \mu + R_j + e_{ij}$

(4) $Y_{ijk} = \mu + L_i + I_j + e_{ijk}$

(5) $Y_{ijk} = \mu + L_i + I_j + (L*I)_{ij} + e_{ijk}$

(6) $Y_{ijkl} = \mu + L_i + I_j + S_k + e_{ijkl}$

(7) $Y_{ijk} = \mu + L_i + S_k + (L*S)_{ij} + e_{ijk}$

(8) $Y_{ijk} = u + L_i + e_{ijk}$

Symbols represent:

$Y_{ij(kl)}$	= depending variable
μ	= population mean
L_i	= line effect (i = H or L)
R_j	= route effect (j = IM, IV or IP)
I_j	= immunization method effect (j = IM or IFA or j = IM or CFA)
S_k	= sex effect (k = male of female)
$(L*R)_{ij}$	= effect of line-route interaction
$(L*I)_{ij}$	= effect of line-immunization method interaction
$(L*S)_{ik}$	= effect of line-sex interaction
$e_{ij(kl)}$	= remainder.

Model 1 fiited best for primary total titers in the route experiment; Model 2 for secondary total titers in the route experiment; Model 3 for both primary and secondary 2MEr titers in the route experiment; Model 4 for primary total and 2MEr titers and secondary total titers in the IFA-experiment; the 2MEr titers in the CFA-experiment; Model 5 for the total titers in the CFA-experiment; Model 6 for the data of the secondary 2MEr titers in the IFA-experiment; Model 7 for the data of both the BA- and

SHA-experiments and Model 8 for the data of the BSA-experiment.

Differences between the three immunization routes were tested with Bonferoni's test and the line differences within a route group with the F-test. The two adjuvant-experiments were separately analyzed, with a full factorial design, including line, sex, immunization method (IM and IFA or CFA) and interactions. In both adjuvant experiments the line effect within immunization method was tested with a F-test.

RESULTS

Route of Immunization.

The kinetics of the immune response of the IM, IV, and IP primed groups are presented in Figures 5.1, 5.2, and 5.3, respectively. After primary immunization, the IV group produced higher titers than both other groups. No Line*Route effects were found. However, in the primary response, line differences in total titers could be detected within the IM and the IV group, but not in the IP group. Independently of route of immunization, or line, titers peaked at 5 days p.i. The primary 2MEr titers generally followed the kinetics of the total titers.

All chicks were IM reimmunized, and in all groups, total titers peaked at 5 days after reimmunization. Although the secondary 2MEr titers were generally higher than the primary, the secondary total titers were only higher than the primary in the IP group. The height of the secondary total antibody response was influenced by a Line*Route interaction. In the IM and the IP group lines differed (Figures 5.1 and 5.3). However, in the IV group no line differences were found. In the L line even a higher response was observed (Figure 5.2).

IFA.

A negative effect of IFA on the height of both total and 2MEr titers was found, when compared to the control IM group (Figures 5.1 and 5.4). The kinetics of the total antibody responses were comparable, but in the IFA group antibody titer peaked later than in the IM group (day 7 versus day 5 p.i.) The response in the IFA group consisted nearly completely of 2MEr antibodies from day 25 p.i. onwards (Figure 5.4).

Also after reimmunization, antibodies in the IFA group were mainly of the 2MEr-type, which was in the IM group true to a lesser extend. Both total and 2MEr secondary responses were low, compared to the primary response and to the secondary response of the IM group.

Within the IFA group, line differences were apparent in the primary response: the H line had the higher titers. The L line followed the response of the H line on a lower level.

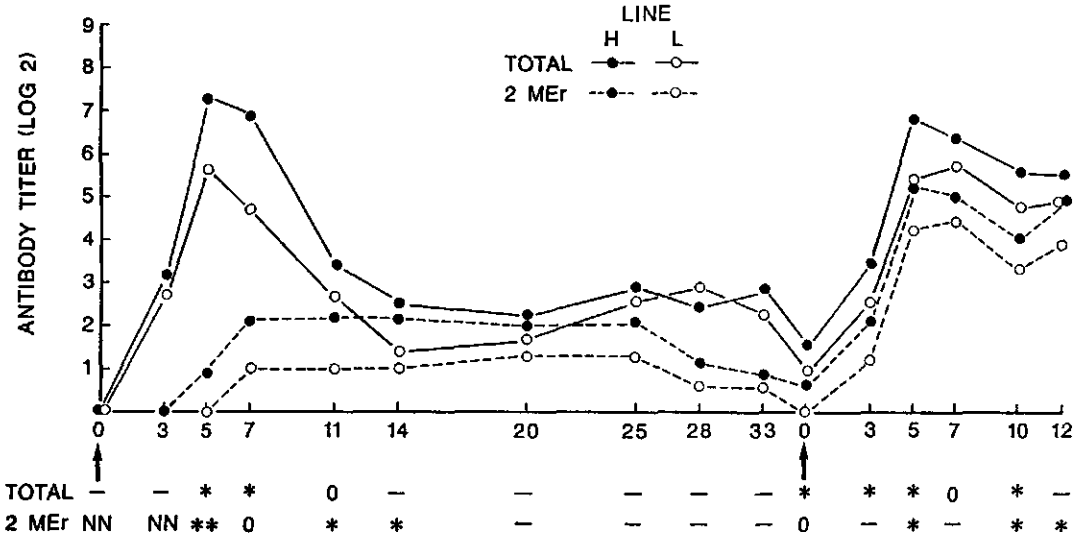

FIGURE 5.1. The kinetics of the total and 2MEr antibody response in lines selected for high (H) or low (L) antibody response after IM immunization and IM reimmunization with SRBC. Line differences are indicated (F-test; NN: non normal distribution of values; -: $p>0.1$; 0: $P<0.1$; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

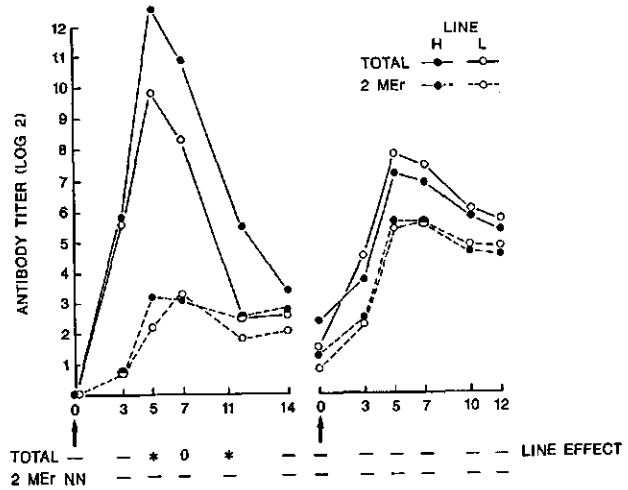

FIGURE 5.2. The kinetics of the total and 2MEr antibody response in lines selected for high (H) or low (L) antibody response after IV immunization and IM reimmunization with SRBC. Line differences are indicated (F-test; NN: non normal distribution of values; -: $p>0.1$; 0: $P<0.1$; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

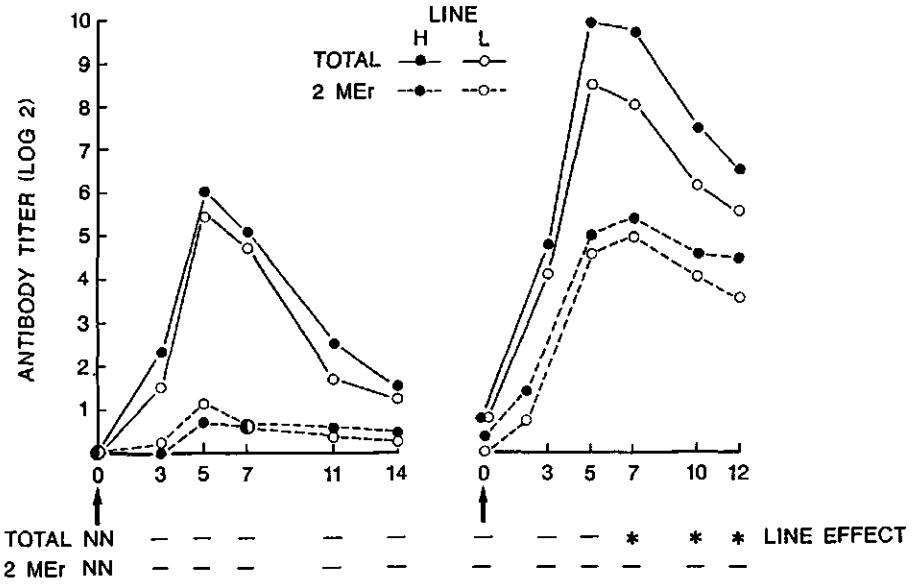

FIGURE 5.3. The kinetics of the total and 2MEr antibody response in lines selected for high (H) or low (L) antibody response after IP immunization and IM reimmunization with SRBC. Line differences are indicated (F-test; NN: non normal distribution of values; -: $p>0.1$; 0: $P<0.1$; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

CFA.

Striking differences between the CFA and the IM group were found (Figures 5.1 and 5.5). In both groups titers peaked at day 5 p.i. followed by a decline, however, the level of antibodies rose in the CFA group without reimmunization, to the level of the first peak. It remained on this level from day 25 untill the end of the experiment (day 47 p.i.). In contrast to the IM group the antibodies elicited after CFA administration completely consisted of 2MEr antibodies (Figure 5.5). Also, CFA affected the line differences in antibody level significantly at several days. Within the CFA group line differences could only be detected at day 11 p.i. (Figure 5.5), where the H line had higher titers. This tended to be also the case at day 14 p.i. Throughout the rest of the response, no line differences could be detected, often the L line was even higher ($p>0.1$).

BSA-immunization.

Antibodies against BSA could already be detected in both lines at day 0 p.i. At day 3 p.i., however, titers were lower than at day 0 p.i. (Figure 5.6). Line differences were apparent; the H line having the higher titers.

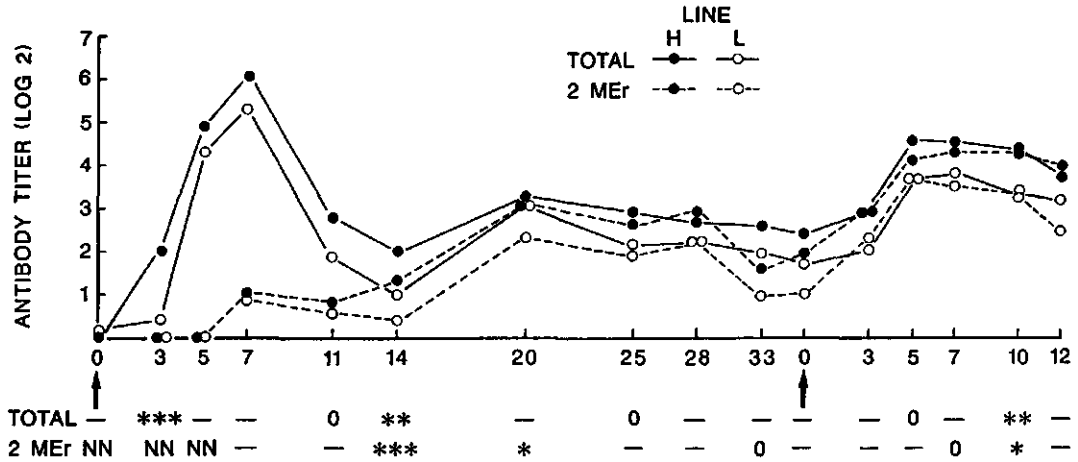

FIGURE 5.4. The kinetics of the total and 2MEr antibody response in lines selected for high (H) or low (L) antibody response after IM immunization with SRBC in Incomplete Freund's Adjuvant and IM reimmunization with SRBC. Line differences are indicated (F-test; NN: non normal distribution of values; -: $p>0.1$; 0: $P<0.1$; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

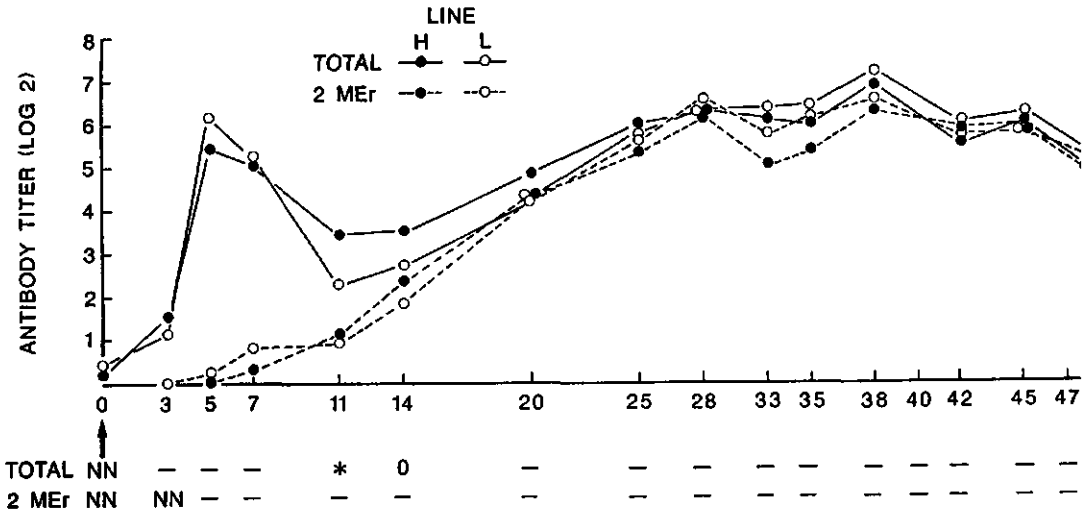

FIGURE 5.5. The kinetics of the total and 2MEr antibody response in lines selected for high (H) or low (L) antibody response after intramuscular immunization with SRBC in Complete Freund's Adjuvant. Line differences are indicated (F-test; NN: non normal distribution of values; -: $p>0.1$; 0: $P<0.1$; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

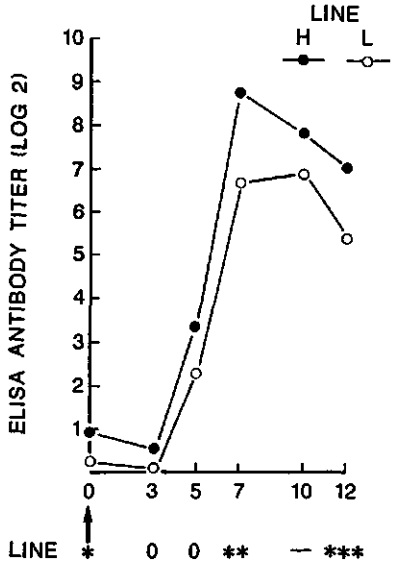

FIGURE 5.6. The kinetics of the antibody response to an IV imunization with Bovine Serum Albumin (BSA), of two lines selected for high (H) or low (L) antibody response. Line differences are indicated (F-test; NN: non normal distribution of values; -: p>0.1; 0: P<0.1; *: P<0.05; **: P<0.01; ***: P<0.001).

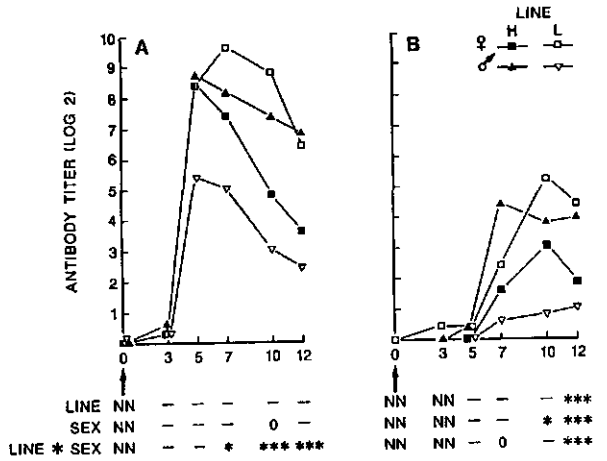

FIGURE 5.7. The kinetics of the antibody response to an IV immunization with *Brucella abortus*, A: total titers and B: 2MEr titers of two lines selected for high (H) or low (L) antibody response. Results of an ANOVA with line, sex and their interaction are presented (NN: non normal distribution of values; -: P>.1; 0: P<.1; *: P<.05; **: P<.01; ***: P<.001).

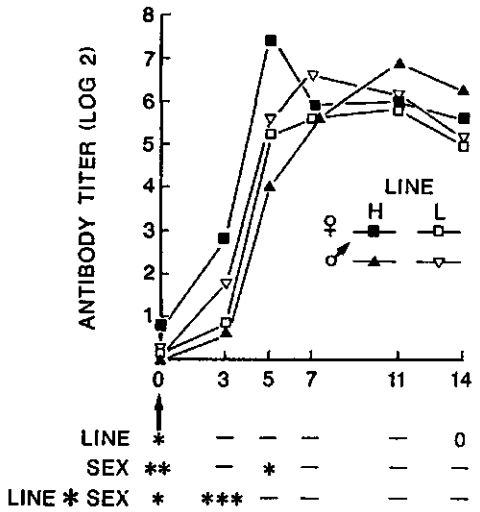

FIGURE 5.8. The kinetics of the antibody response to an IV immunization with *Salmonella* H-antigen (SHA) of two lines selected for high (H) or low (L) antibody response. Results of an ANOVA with line, sex and their interaction (NN: non normal distribution of values; -: $p>0.1$; 0: $P<0.1$; *: $P<0.05$; **: $P<0.01$; ***:$P<0.001$).

BA-immunization.

When chicks were immunized with BA; sexes interfered with line differences in both total and 2MEr titers (Figures 5.7A and B). In general, L line pullets had the highest total titers, followed by H line cockerels, H line pullets, and L line cockerels in descending order. The 2MEr titers showed more or less the same sequence (Figure 5.7B).

SHA-immunization.

Low titers to SHA were already detected at day 0 p.i. (Figure 5.8). Until day 7 p.i., line and sex effects showed interactions. The H line pullets had the highest titers, followed in descending order by L line cockerels, L line pullets and H line cockerels. From day 7 p.i. onwards no significant effects of line or sex could be detected. On the other hand, the H line tended to have higher titers than the L line at day 14 p.i.

DISCUSSION

The antibody responses to SRBC elicited after IM and IP immunizations were lower than after IV immunization, confirming earlier findings of Seto and Henderson (1968)

and Van der Zijpp *et al.* (1986). In present study, antibody levels peaked at day 5 p.i., independently of route of administration. Van der Zijpp *et al.* (1986) found differences in the timing of the peak titer between the routes (IV: day 5; IP and IM: day 7).

The line differences were affected by the route of immunization, which indicates that in the different tissues the mode of action used to cope with SRBC was not altered to the same extend by the selection. In addition, memory induction differes between the primary route groups, illustrated by the absence of the line differences in the secondary response only after IV immunization.

In the H line, processes stimulating antibody response may be more efficient when SRBC enters the body. On the other hand, the H line may possess higher numbers of SRBC-specific B cells. Both may explain the higher number of plaque forming cells (Donker, 1989) and the higher antibody production in the H line than in the L line. However, the differences in antibody producing capacity can not be solely contributed to a larger number of B cells that can react to SRBC. This is illustrated by the responses followed after immunization with either IFA or CFA. In both experiments representative samples from both lines were taken, differences in B cell numbers between experiments within lines will therefore not be expected. Line differences disappeared after immunization with CFA, but were still found with IFA. CFA stimulates, besides humoral immunity also cell-mediated immunity (Allison and Byars, 1986). Additional T cell signals may result in an enhanced antibody production from day 14 p.i. onwards in both CFA immunized selection lines. This indicates that due to T cell help, enhanced by CFA, lines can produce antibodies equally well, independently of numbers of SRBC-specific B cells. Moreover, similar line differences were found after immunization with SRBC and the protein BSA, another T cell-dependent antigen. In contrast, no line differences in antibody response were detected when the (partially) T cell-independent antigens BA or SHA were injected. This suggests that the differences in antibody producing capacity might be caused by differences in the T cell activity as a result of selection.

When the adjuvant activity of IFA is based on an enhanced phagocytic activity, (Allison and Byars, 1986), the difference in antibody production between the lines can not be contributed to line differences in activity of phagocytes. However, after administration of IFA and CFA an enhancement of antibody response is expected. In the present study, IFA appeared to have a depressing effect on the peak titer in both lines, and moreover, the response was not sustained. The lack of stimulation may not be unusual in chickens, as Steinberg *et al.*, (1970) found that in chickens IFA did not always enhance the antibody response. Antibody levels against human serum albumin (HSA) maintained on a higher level after the peak was reached (Steinberg *et al.*, 1970). This sustained response to HSA was probably a result of the persisting stimulation caused by the depot of antigen formed by the water in oil emulsion, from which

continuously low doses of antigens were released. In the present study, this persisting stimulation of the immune system by a low dose of SRBC did not alter the line differences, as was expected, while line differences did not disappear even after low doses of free SRBC (Kreukniet and Van der Zijpp, 1990). The slow release of SRBC, resulting in lower effective doses SRBC to stimulate B cells, might also explain the lower and later peak, as was found when low doses of free SRBC were administered (Kreukniet and Van der Zijpp, 1990). Kinetics of the response after CFA and IFA were similar to the results of Steinberg *et al.* (1970) and French *et al.*, (1970).

We concluded that results of the CFA, BA and SHA experiments give rise to the hypothesis that divergent selection for antibody production is based on indirect selection for T cell responsiveness. Studies are in progress to elucidate the differences between the selected chicken lines in the involvement of T cells in antigen processing.

ACKNOWLEDGEMENTS

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chapter 6

IN VITRO T CELL ACTIVITY IN TWO CHICKEN LINES DIVERGENTLY SELECTED FOR ANTIBODY RESPONSE TO SHEEP ERYTHROCYTES.

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In vitro T cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes.

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ABSTRACT

Four experiments were conducted to determine possible differences in the *in vitro* concanavalin A (ConA) response between two lines selected either for high (H) or low (L) antibody response 5 days after intramuscular immunization with SRBC. In all four experiments, the cell proliferation after stimulation with ConA was higher, although not always significantly so, in the L line than in the H line, independently of dose of ConA and source of lymphocytes. It can be concluded that selection for anti-SRBC antibody response affected the cellular response in chickens. Previously reported results in other, chicken lines selected for humoral response to SRBC after intravenous immunization with SRBC, showed an opposite line difference in mitogen response. These opposite results point to the fact that comparable selection protocols for immunological parameters do not necessarily have a comparable influence on the diverse components of the immune response.

INTRODUCTION

Knowledge of how the avian immune system copes with entering antigens is important to understand the mechanisms of resistance and susceptibility. To clarify the immunological pathways used by chickens to cope with entering antigen, the various lines genetically altered for a specific immune response (Jeffers *et al.*, 1969; Siegel and Gross, 1980; Pevzner *et al.*, 1981a,b; Van der Zijpp and Nieuwland, 1986; Yamamoto *et al.*, 1988; Tamaki *et al.*, 1988) can be a great advantage. One of the immune responses most intensively studied is the selection for high or low antibody production against SRBC (Siegel and Gross, 1980; Van der Zijpp and Nieuwland, 1986). The latter two selection experiments differed with respect to the chicken strain used in the base population, the immunization route, and dose of SRBC administered. However, both selections resulted in large line differences in the humoral response to SRBC (Siegel and Gross, 1980; Pinard *et al.*, 1992) and in a change of *Ea-B* haplotypes (Dunnington *et al.*, 1984; Pinard *et al.*, 1993). The lines of Siegel and Gross differed also in mitogen responses; their high antibody producing line showed a higher concanavalin A (ConA) and phytohemagglutinin-M (PHA) response than their low line (Scott *et al.*, 1991).

Previous studies conducted with the selection lines used in present study (Van der Zijpp and Nieuwland, 1986) also indicated differences between the high (H) and low (L) antibody production lines in T cell help during antibody response (Kreukniet *et al.*, 1992). The present study was conducted to search for differences in *in vitro* ConA responses in these lines.

MATERIALS AND METHODS

Experimental Chicks.

Four experiments (1 to 4) were conducted with chickens of the 11th generation of two lines selected for high (H) or low (L) antibody response to SRBC (Van der Zijpp and Nieuwland, 1986). In Experiment 1, the mitogen response of 9-mo-old hens was determined. Out of the mothers of the 12th selection generation, 10 H line hens with the highest antibody titers (mean titer of 12.9) and of 10 L line hens, all with titer of 0, were used. The hens were intramuscularly immunized with 1 ml of 25% SRBC at 37 days of age, and 5 days later, antibody titers to SRBC were determined (Van der Zijpp and Leenstra, 1980). In Experiments 2 to 4, four to nine week-old pullets, genetically identical to the 11th selection generation were used. In Experiment 1, hens were a sample of the MHC class IV haplotypes in the lines, whereas in Experiment 2 to 4, all chicks were homozygous B^{24} (Pinard *et al.*, 1993). All chickens were housed in battery cages with a maximum bird density of 13 chicks per cage and free access to feed and water. Adult hens were individually housed. Vaccinations were only carried out in the adult hens, to conform to the selection protocol (Pinard *et al.*, 1992). The chicks in experiments 2 to 4 were not vaccinated.

Immunizations.

In Experiment 1, hens were immunized with SRBC according to the selection protocol, as mentioned above, at 37 days of age. The 9 H and 9 L line chicks used in Experiment 4 were not immunized. In both Experiments 2 and 3, 8 H and 8 L line pullets were immunized with BSA (Sigma Chemical Co., St Louis, MO, 63178-9916) conjugated with trinitrophenyl sulphonic acid (TNP; Sigma Chemical Co.) to a ratio of 13 to 25 TNP molecules per molecule of BSA (molecular weight of BSA was set on 67,000). All immunizations were done intramuscularly (breast muscle) in 2 aliquots of 0.5 ml BSA-TNP (2 mg/ml). Immunizations with BSA-TNP were carried out, respectively 30 (Experiment 3) and 37 days (Experiment 2) prior to the mitogen stimulation test.

Cell Isolation.

In Experiment 2, spleen lymphocytes were used. Pullets were killed by decapitation

and suspensions of spleens were made by teasing the organs through a stainless steel mesh. Peripheral blood lymphocytes (PBL) were used in all the other experiments and were obtained through venipuncture. Lymphocytes were prepared by passing the spleen suspensions and heparinized blood over Ficollpaque (Density 1.072; Pharmacia Nederland B.V., Woerden, The Netherlands). Spleen lymphocytes were brought to a concentration of 5×10^5 (Experiment 2) and PBL to a concentration of 1×10^6 (other experiments) cells per millilitre in RPMI 1640 containing 10% Nu-serum (Flow, Irvine, United Kingdom).

Lymphocyte Stimulation Test.

Aliquots of 200 μl containing either 5×10^5 or 1×10^6 lymphocytes and either 0, 2 or 10 μg/ml ConA (Type IV; Sigma Chemical Co.) were cultured, in duplicate, in 96-well tissue culture plates for 48 to 84 h to evaluate a possible effect of culture time, in 5% CO_2-air humified atmosphere at 40°C. The last 12 hours before harvesting, cultures were pulsed with 20 μl 20 μCi ^{3}H-thymidine (Amersham International plc, Amersham, United Kingdom). After incubation, the cells were harvested and counts per minute were determined with a beta-scintillator counter (Beckman Liquid Scintillation System LS 1701, Beckman instruments, Fullerton, CA, 92634).

Statistical Analyses.

In all experiments, the Stimulation Index (SI) was calculated as (counts per minute of mitogen treated cells) / (counts per minute of untreated cells) per observation. Means and standard deviations of the SI were calculated per line within experiment and *t*-tests were performed to determine significant differences using SAS® software (SAS Institute, 1989). Also a two-way variance analyses with line and experiment as main effects on the combined data of all experiments was performed.

RESULTS AND DISCUSSION

In various studies with chickens, mitogen responses were associated with the MHC-genes (Morrow and Abplanalp, 1981; Scott *et al.*, 1988; Knudtson *et al.*, 1990). However, also non-MHC loci are involved in the responses to ConA (Pink and Miggiano, 1977; Morrow and Abplanalp, 1981; Fredericksen and Gilmour, 1983; Knudtson *et al.*, 1990). The lines used in the present study were found to differ in MHC-haplotypes. The B^{21} haplotype was predominant in the H line in the form of $B^{21}B^{21}$ genotype, whereas in the L line a B^{14} haplotype was most frequent in the form of $B^{14}B^{14}$ and $B^{14}B^{24}$ genotype (Pinard *et al.*, 1993). The adult hens of the H and L line used in Experiment 1, were a mixture of the genotypes available, and differed significantly in anti-SRBC response. Although not significantly so, the line difference in *in vitro*

response to ConA in this experiment was reverse to the antibody response: high in the L line, low in the H line (Table 6.1). To avoid possible influences of the MHC on the mitogen response, chicks used in Experiments 2 to 4 were all homozygous B^{24}. Nevertheless, the same trend in line differences in ConA response was found: the L line having always higher SI than the H line. This means that the ConA response in the present lines is not linked with the *Ea-B* loci of the MHC complex. The line difference was seen in all experiments, although not significant in each experiment, indicating that the line effect was not dependent on stimulation time, low or high doses ConA and source of lymphocytes. Moreover, Analyses of Variance over all data, showed a significant ($P<0.05$) effect of the lines on the SI, as well as an experiment-effect ($P<0.01$).

TABLE 6.1. In vitro mitogen response of chicken lymphocytes to concanavalin A (ConA) in four different experiments.

Exp.[1]	LINE	ConA µg/ml	Stim.[2] hour	ConA[3] CPMx10[3]	BG[4] CPMx10[2]	SI[5]
1	H	2	84	10.6[6] ± 5.1	2.5 ± 0.6	46.1 ±30.3
	L			16.7 ± 14.3	2.8 ± 1.3	78.6 ±83.1
2	H	2	84	1.4 ± 2.0	1.7 ± 0.9	14.6 ±26.5
	L			3.1 ± 2.6	1.5 ± 1.1	34.5 ±48.3
3	H	10	48	35.7 ± 17.3	2.8 ± 0.6	127.5 ±60.4*
	L			38.5 ± 12.8	1.9 ± 0.5**	215.6 ±89.8
	H		72	21.5 ± 10.8	6.8 ± 5.4	46.8 ±35.4
	L			21.3 ± 6.0	4.1 ± 3.0	90.2 ±85.4
4	H	10	60	15.3 ± 0.8	1.6 ± 0.8	11.1 ± 7.4*
	L			21.4 ± 12.8	1.2 ± 0.7	21.2 ±11.4

[1] Experiment; [2] hours of culturing the cells, including 12 Hours pulsing with 0.5 uCi ^{3}H-thymidine; [3] Counts per minute x 10^3 in the presence of ConA; [4] counts per minute x 10^2 in the abscence of ConA; [5] Stimulation Index : CPM in the presence of ConA / CPM in the absence of ConA;[6] mean ± standard deviation.

* Lines differ $P<0.05$; ** lines differ $P<0.01$.

Chicken lines genetically selected for antibody response to SRBC differed also significantly in *in vitro* mitogen response (see also Scott *et al.*, 1991). Therefore, genetic selection in chickens for antibody response against SRBC, can result in differences in the cellular response. However, in the selection lines of Siegel and Gross, the mitogen responses of their high line to both ConA and PHA were higher than those of their low line (Scott *et al.*, 1991). In the lines studied here the response to ConA

was higher in the L line. After stimulation with PHA, no line differences were found in these lines (preliminary data, not shown). In other studies with chickens, the mitogen responses to ConA and PHA were also not positively associated (Miggiano *et al.* 1976; Morrow and Abplanalp, 1981; Pink and Miggiano, 1977). Moreover, the line difference in *in vitro* mitogen responses between the present lines were opposite to *in vivo* hypersensitivity responses to PHA and BSA in these lines (Parmentier et al., 1994). This might be caused by differences in the responding T lymphocyte subsets in *in vitro* and *in vivo* test, or by the mechanisms *in vivo* facilitating local lymphocyte proliferation.

The reverse line differences for *in vitro* ConA response in the two selection experiments (Scott *et al.*, 1991; present study) indicate that the different T cell responses are not necessarily a functional consequence of the selection for antibody response, but might be merely a side effect of this selection. Similarly Steadham and Lamont (1993), reported that several *in vitro* T lymphocyte responses to Glutamic Acid-Alanine-Tyrosine (GAT) did not mimic the difference in humoral response in lines selected for antibody response to GAT. However, antibody responses to several antigens, and *in vitro* mitogen responses were higher in chicken lines selected for early antibody production to *Escherichia coli* vaccine (Heller *et al.*, 1992). In the present selection lines, the high T cell activity in the L line could just be a compensation mechanism for survival, because the antibody response to several T cell-dependent antigens has been severely reduced (Kreukniet *et al.*, 1992; 1995).

It is noteworthy that divergent selection for total antibody response to SRBC, either after an intramuscular immunization with 25% SRBC (Van der Zijpp and Nieuwland, 1986) or after intravenous immunization with 0.5% SRBC (Scott et al, 1991), gives rise to diverse line differences in other types of immune responses. This might be caused by differences in immunization protocol or the different breeds used in the base population. Because genetic improvement of disease resistance is an important issue in the poultry industry, effects of selections should be taken in consideration with respect to vaccination protocols. Vaccination scheme used, might not always give rise to the desired immunity to a disease in all modern poultry populations.

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chapter 7

THE B CELL COMPARTMENT OF TWO CHICKEN LINES DIVERGENTLY SELECTED FOR ANTIBODY PRODUCTION: DIFFERENCES IN STRUCTURE AND FUNCTION

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The B cell compartment of two chicken lines divergently selected for antibody production: differences in structure and function

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ABSTRACT

In the present study differences in the B cell compartment of two chicken lines selected for either high (H) or low (L) antibody response to sheep red blood cells (SRBC) are investigated. In non-immunized chicks, flow cytometry revealed generally more circulating Ig^+ leukocytes in the H line, while in the L line slightly more $CD4^+$ and in week 5, more $CD8^+$ cells were found. In the L line spleen more $CD8^+$ were found and in the H line spleen more $CD4^+$ cells. In week 6, half of the chicks were immunized. Both lines were similarly affected by immunization. Immunization reduced the percentages of the circulating T cell subpopulations, while Ig^+ cells were enhanced, compared to non-immunized chicks.

Histological determinations with specific mAb's on spleens of young, non-immunized chicks, showed large dense T cell areas in the L line, while in the H line more and larger germinal centres were found. In the H line, also more B cells were found in the peri-ellipsoid lymphoid sheaths (PELS). No line differences in mononuclear phagocytes were found other than associated with line differences in numbers of PELS and germinal centres.

After immunization with TNP-BSA, both higher numbers of TNP-specific antibody producing cells and higher levels of circulating antibody were found in the H line. Moreover, more TNP-specific plasma cells were found in non TNP-immunized H line chicks, than in the L line chicks. The H line had also higher ELISA-titers to KLH five days after immunization with KLH.

Therefore it was concluded that selection for antibody response has affected the B cell compartment. The H line has relatively more B cells. Moreover, the splenic structure of the H line differs from the L line, in the H line probably resulting in a more optimal organization for antibody response to T cell dependent antigens.

INTRODUCTION

Several lines of chicken have been divergently selected for specific immune parameters to explore the possibilities of genetic improvement of disease resistance and immunoperformance (Jeffers *et al.*, 1969; Siegel and Gross, 1980; Pevzner *et al.*, 1981[a,b]; Van der Zijpp and Nieuwland, 1986; Okada and Yamamoto, 1987; Pitcovski *et al.*, 1987; Yamamoto *et al.*, 1991; Heller *et al.*, 1992; Steadham and Lamont, 1993).

These selections must have affected the cascade of events following an invasion of antigen, resulting in the line differences in the immune response selected for. When the effects of selection are antigen non-specific, it might have consequences for disease resistance in these lines. Understanding the cellular events that cause the immune differences in the selected lines, will give more general clues on how resistance or susceptibility to a pathogen is achieved in chickens. In none of the selection lines, however, are these immune mechanisms completely disentangled.

In the present study, the B cell compartment of two chicken lines genetically selected for either high (H) or low (L) antibody response to the T cell-dependent antigen sheep red blood cells (SRBC) (Van der Zijpp and Nieuwland, 1986), is studied in more detail. Previous investigations indicated that there were no detectable differences between the lines in the accessory and phagocytic functions (Van der Zijpp *et al.*, 1988; 1989; Kreukniet *et al.*, 1994[a]). However, results of Donker (1989) demonstrated a higher number of antibody forming cells in the H line after immunization with SRBC. Whereas Siegel *et al.* (1992) and Parmentier *et al.* (1995) reported higher percentages B cells in blood and spleens of the H line. This indicated that differences in the number of B cells might correspond to the selection difference. In the present study, the postnatal development of circulating lymphocyte populations was followed in both lines using flow cytometry. At the selection age, i.e. six weeks of age, percentages of lymphocyte populations were also determined in lymphoid organs and blood of both immunized and non-immunized chicks. In addition, possible differences between the lines in the *in situ* distribution of lymphoid and non-lymphoid cells in the spleen were investigated. To determine whether effects of selection were limited to SRBC and BSA, as previously described (Kreukniet *et al.*, 1992; Parmentier *et al.*, 1994), the T cell dependent antibody responses to keyhole limpet haemocyanin (KLH) and trinitrophenyl (TNP) were determined, as well as the number of splenic cells producing TNP-specific antibody in both lines.

MATERIALS AND METHODS.

Experimental chicks.

Experiments were carried out with chickens of two lines, genetically selected for either high (H) or low (L) antibody titers 5 days after intramuscular (i.m.) immunization with 1 ml of 25% SRBC at 37 days of age (Van der Zijpp and Nieuwland, 1986). All chicks were housed in wire cages with a maximal bird density of 13 birds/cage. Birds had free access to food and water.

Flow cytometry analyses were carried out on leukocytes of in total 65 H and 65 L line chicks, chosen at random of the 10th selection generation. Sexes were determined at 6 weeks of age.

Histological observations were conducted on spleens of 15 H and 15 L line pullets of 41 days of age. All pullets were of the 11th selection generation and offspring of *B-G24* (Pinard *et al.*, 1993) homozygous parents. These pullets were not immunized.

Specific antibody responses were measured in 24 H and 24 L line *B-G24* homozygous pullets, all of the 11th selection generation. Of each line 16 pullets were i.m. immunized with trinitrophenyl conjungated bovine serum albumin (TNP-BSA). The remaining 8 of each line were immunized with KLH.

Immunizations.

All antigens were intramuscularly injected as 2 aliquots of 0.5 ml, one aliquot in each tight muscle.

SRBC: 25% SRBC in PBS (Van der Zijpp and Nieuwland, 1986); reimmunization with 50% SRBC.

TNP-BSA: per ml PBS 2 mg Bovine serum albumin (BSA; Sigma Chemicals Co, St Louis, MO, USA) conjungated with 13-25 molecules trinitrophenyl (TNP; Pierce, Rockford, IL, USA) per molecule BSA.

KLH: 2 mg Keyhole limpet hemocyanin (KLH; Pierce) per ml PBS.

Histological observations.

After decapitation of the pullets, the spleens were removed, frozen in liquid nitrogen and stored at -20°C. Cryostat sections of 8 μm thickness were picked up on slides and stored over silica gel. Slides were fixed in pure acetone for 10 minutes, air-dried, and incubated for 1 hour with mAb's (Table 7.1) diluted in PBS containing 0.6% BSA at the appropriate concentration. Slides were rinsed in 0.01M PBS and covered for 30 minutes with peroxidase-conjugated rabbit anti-mouse Immunoglobulin (Dakopatts, Glostrup, Denmark) diluted in PBS with 0.6% BSA. Peroxidase activity was developed with a solution of 0.5 mg 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma Chemical Co.) and 0.01 % H_2O_2 per ml Tris-HCl buffer (0.05 M; pH 7.6). The slides were counter stained with haematoxylin, dehydrated, and mounted in DePex (BDH, Poole, UK). Control slides were incubated as described above, except that the mAb's were omitted.

Flow cytometry

In weeks 2, 3, 4, 5, 10, and 12, blood samples were taken of 25 H and 25 L line chicks to determine the frequencies of the lymphocyte subpopulations in the blood. Heparinized blood was passed over Ficoll Paque (d 1.072; Pharmacia, Upsala, Sweden) and brought on a concentration of $2x10^6$ per ml with RPMI 1640 containing 2% FCS and 0.1% NaN_3. The percentages of cells expressing the different markers (Table 7.1), were determined as described by Joling *et al.* (1994). At 44 days of age, 13 H and 12

L line chicks were immunized. And at 49, 72, 96 days of age (respectively week 6, 10 and 12), blood samples were taken of these chicks and of the 12 non-immunized H line and 13 non-immunized L line chicks. The immunized chicks were i.m. reimmunized with SRBC at 96 days of age and blood samples were taken at 101 days of age (week 13) to determine the lymphocyte percentages and hemagglutinin titers (Van der Zijpp and Leenstra, 1980).

In week 6, an additional 40 H and 40 L line chicks were used to determine frequencies of lymphocyte subpopulations in bursa, thymus, spleen and blood. These chicks were randomly divided in 4 groups (Immunization Groups) of 10 H and 10 L line chicks. Of each group 5 chicks per line were immunized with SRBC; one Immunization Group at 37, at 38, at 39 and one at 41 days of age. Exactly 5 days after immunization the 10 immunized and 10 non-immunized chicks of each Immunization Group were sacrificed and the frequencies of lymphocyte populations determined in blood and cell suspensions of the organs.

TABLE 7.1. Monoclonals used in the flow cytometry and histological experiments with specifity and references.

ASSAY	ANTIBODY	SPECIFITY
Flow cytometry	2-6[1,2]	CD4
	11-38[1,3]	CD8β
	RaChIgG(H+L)[4]	Ig
Histology	HIS -C1[5]	B cells
	CVI-ChT-74.1[6]	CD8
	CVI-ChNL-68.1[7]	mononuclear phagocytes
	CVI-ChNL-68.2[8]	ellipsoid associated reticulum cells
	CVI-ChNL-74.2[9]	some macrophages
	CVI-ChNL-74.3[9]	follicular dendritic cells

[1] Kindly donated by dr O. Vainio, Turku, Finland; [2]Vainio *et al.*, 1989; [3] Ratcliffe *et al.*, 1993; [4] Nordic, Tilburg, The Netherlands; [5] Jeurissen *et al.*, 1988[a]; [6] Noteborn *et al.*, 1991; F. Davison, Compton, UK, pers comm.; [7] Jeurissen *et al.*, 1988[b]; [8] Jeurissen *et al.*, 1989; [9] Jeurissen *et al.*, 1992.

Specific antibody responses.

Sixteen H and 16 L line pullets were immunized with TNP-BSA at 25 days of age. An additional 8 H and 8 L line pullets were immunized with KLH; 4 of each line at an age of 25 days, the others at 39 days of age. Plasma samples were collected at days 0, 3, 5 and 7 post immunization (p.i.) and of the chicks immunized at 25 days, also at 10, 14 and 21 p.i. and were stored at -20° C till assayed for antibodies against KLH and TNP.

At 46 days of age, eight of the TNP-BSA immunized chicks per line and four of each KLH-immunized 'age' group per line were killed by decapitation. At 53 days of age the remaining 16 TNP-BSA immunized chicks were killed. Single spleen cell suspensions were made and passed through Ficoll Paque (d 1.072; Pharmacia) and brought on a concentration of $1x10^7$ cells/ml in RPMI-1640$^+$ (RPMI-1640 supplemented with 10% NU-serum (ICN Biomedicals, Costa Mesa, CA, USA), penicillin (100 IU/ml; Serva, Heidelberg, Germany), streptomycin (100 μg/ml; Serva), 2-mercapthoethanol ($5x10^{-5}$ M), L-glutamine (2mM; Merck, Darmstadt, Germany) and 0.03 M sodium hydrogen carbonate). The spleen cell suspensions of the chicks immunized with TNP-BSA were directly used in the ELISpot assay, while those of the KLH immunized pullets were incubated for 2.5 hour with TNP-KLH (150 μg/ml) prior to the ELISpot assay (500 μl suspension with 500 μl KLH-TNP; 41°C; 5% CO_2-humified conditions).

ELISpot-assay: The number of antibody forming cells in the spleens was estimated as described by Bianchi *et al.* (1990) with some slight modifications. Briefly, after precoating, microtiterplates were coated with 100 μg/ml of TNP-OVA (Egg white albumin (Sigma Chemicals Co) conjugated with TNP to a ratio of 20 TNP molecules per molecule of OVA) in 0.04 M PBS-13 at 4°C overnight. The non-specific binding-sites were blocked and 100 μl of either 5 x 10^7, 1 x 10^7, or 5 x 10^6 leukocytes was added and incubated for 3-4 hours in a humified atmosphere at 37°C. After washing, 100 μl of 1:250 dilution of goat anti chicken IgG(H+L) alkalic phosphatase (Nordic) was added to each well and incubated overnight at 4°C. Spots were coloured and counted.

ELISA's for TNP and KLH: Microtiterplates were coated with 100 μl 2.5 μg/ml TNP-OVA in carbonate buffer (1 L distilled water containing 28.6 g Na_2CO_3-$10H_2O$; pH 9.6) per well for TNP-binding antibodies, or 100 μl 1.25 μg/ml KLH in the carbonate buffer for KLH-binding antibodies (17 h; 4°C). Remaining binding sites were blocked with 100 μl PBS with 1% BSA and 0.05% Tween-20 per well (30 min.; 37°C). Per well, 100 μl plasma was added and two-fold dilution series were made with PBS (pH 7.2), containing 0.05 % Tween-20 and either 0.1% OVA (for TNP) or 2% FCS (for KLH). After incubation (1 h; 37°C), microtiterplates were washed and 100 μl of an optimal dilution of RaChIgG(H+L)PO (Nordic, Tilburg, The Netherlands) was added. After 30-60 min. at 37°C, 100 μl substrate (100 ml orthophenylene diamine + 133 μl H_2O_2 in buffer made of 14.71 g $C_6H_5Na_3O_7$-$2H_2O$; 17.8 g $Na_2HPO_4.2H_2O$ diluted in 800 ml distilled water, brought at pH 5 by adding HCl, and then brought to a volume of 1 L with distilled water) was added. After 30 min. at room temperature in the dark, the reaction was stopped by adding 50 μl 2.5 NH_2SO_4 per well. Extinctions were measured at 450 nm with a Titertek Multiscan MCC (ICN Biomedicals).

Statistical analysis.

The data of the flow cytometry was, following tests of normality, analyzed with

analysis of variance (SAS, 1989). A full factorial design was used, containing line, sex, and group (in which markers were determined during the test weeks). During the postnatal development in weeks 2 to 5, the data of the lymphocyte markers fitted best in Model 1.

In week 6, immunization (I) and immunization group (IG) and their interactions were included as main effects. The ratio's of Ig^+:$CD4^+$, Ig^+:$CD8^+$ and $CD4^+$:$CD8^+$ cells were calculated for spleen and blood at week 6 and tested with the same model. The non-significant effects were omitted from the final model, resulting in Model 2 for the data of organs and blood, except the ratio's of blood lymphocytes, for which Model 3 fitted best. The data of weeks 10, 12 and 13, were best described by Model 4.

$$y_{ijkl} = \mu + Line_i + Sex_j + Group_k + e_{ijkl} \quad \text{Model 1.}$$
$$y_{ijkl} = \mu + Line_i + Sex_j + I_k + IG_l + e_{ijklm} \quad \text{Model 2.}$$
$$y_{iklm} = \mu + Line_i + I_k + IG_l + e_{iklm} \quad \text{Model 3.}$$
$$y_{ilm} = \mu + Line_i + e_{il} \quad \text{Model 4.}$$

Symbols represent:

$y_{i(jk)l(m)}$ = depending variable
μ = population mean
$Line_i$ = Line effect
Sex_j = Sex effect
$Group_k$ = Group effect
I_k = Immunization effect
IG_l = Immunization Group effect
$e_{i(jk)l(m)}$ = remainder

(Partial) Pearson's correlations over lines and within lines, were calculated between the haemagglutinin titers five days after reimmunization and the percentages of $CD4^+$-, $CD8^+$-, and Ig^+- leukocytes.

The data of the ELISpots were analyzed with ANOVA, with 'Line' and 'Repeat' (TNP-BSA-immunized group) or 'Line' and 'Age at Immunization' (KLH-immunized group) and the interaction. The antibody response to KLH was analyzed with the same ANOVA as used for the ELISpot. The line differences in antibody response to TNP were tested with a *F*-test.

RESULTS

LYMPHOCYTE POPULATIONS

Postnatal development in blood.

In general, the H line had higher percentages Ig^+ cells in the blood (Figure 7.1). On the other hand, after week 2 percentages of the T cell marker CD4 were higher in the L line. In week 5, $CD8^+$ cells were also determined and percentages were higher ($P<0.05$) in the L line (11.5%), than in the H line (8.2%).

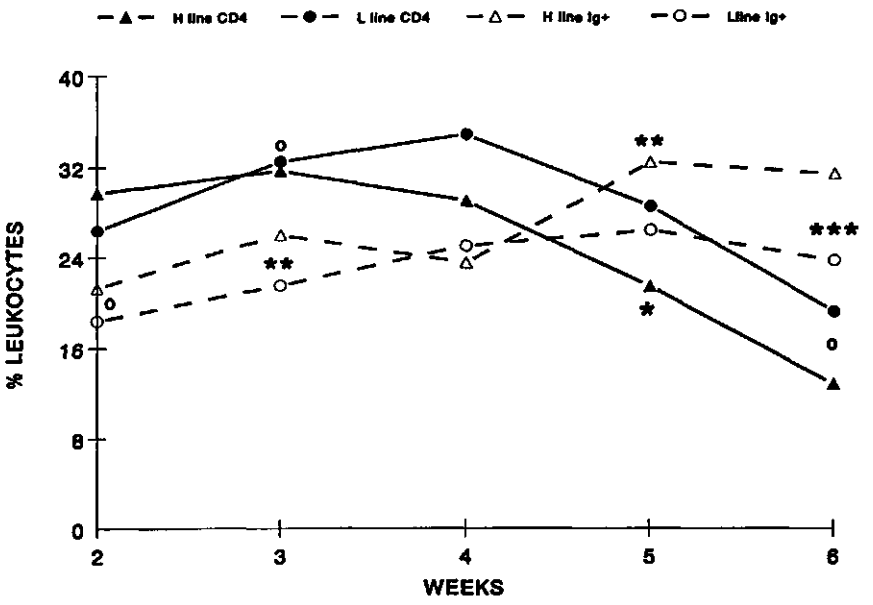

FIGURE 7.1. LSmeans per line (n = 25) of the percentages $CD4^+$ and Ig^+ peripheral leukocytes *in the weeks post hatching, estimated with Model 1. Significant line effects in* percentages of $CD4^+$ and Ig^+ cells are indicated (O:$P<0.1$; *:$P<0.05$; **:$P<0.01$; ***:$P<0.001$).

After the selection age, at weeks 10 and 12, similar line differences in T and B cell profiles existed (data not shown). In week 13, 5 days after re-immunization, no line differences could be detected in percentages of T cell markers. The percentage Ig^+ leukocytes differed significant ($P<0.01$) between the lines: 75.8 % in the H line and 54.0 % in the L line. Also haemagglutinin titers differed ($P<0.001$) between the lines (4.9 in H and 2.3 in L line).

In both lines, higher percentages of Ig^+ (weeks 2, 3, 4; $P<0.05$) and $CD4^+$ (weeks

3, 4, 5; $P<0.05$) were found in cockerels than in pullets. In week 5, the percentage of $CD8^+$ cells differed ($P<0.05$) also between the sexes (males: 11.8%; females: 7.9%).

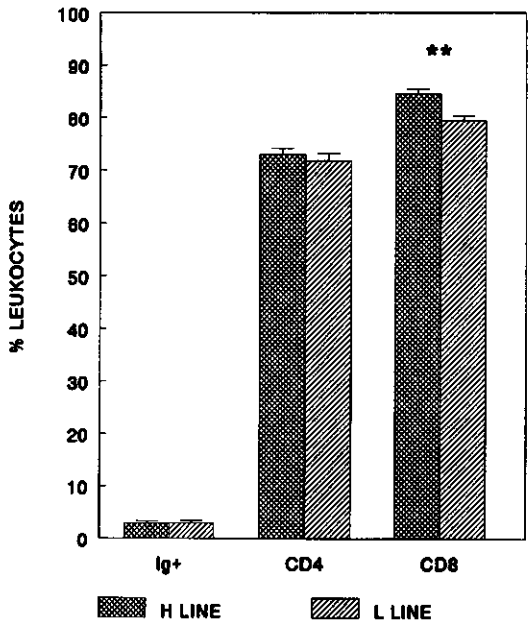

FIGURE 7.2. LSmeans (Model 2) per line ($n=40$) of percentages of $CD4^+$, $CD8^+$ and Ig^+ leukocytes in the thymus. Standard errors of the mean and significant line differences are indicated (**:$P<0.01$).

Lymphoid organs

Thymus. Only the percentage $CD8^+$ differed between the lines, the H line having more $CD8^+$ (Figure 7.2).

Bursa. In the bursa, over 95 % of the cells was Ig^+, consequently percentages of $CD4^+$, and $CD8^+$ were low. No line differences were detected.

Spleen. In the spleen, more $CD4^+$ cells were found in the H line, while in the L line more $CD8^+$ cells were found (Figure 7.3). Moreover, the ratio $CD4^+$:$CD8^+$ was significantly higher ($P<0.05$) in the H line. When the ratio's between Ig^+ cells and T cells were considered, Ig^+:$CD4^+$ was significantly higher ($P<0.01$) in the L line, while Ig^+:$CD8^+$ was, although not significantly higher ($P<0.1$) in the H line (Figure 7.3).

Histological observations on the spleen corresponded well with the flow cytometry results. When spleens of H and L line chicks stained with mAb CVI-ChT-74.1 were compared, a striking difference was found. This mAb, specific for the CD8 α chain, was used to demarcate the T cell areas, as $CD4^+$ cells are always located intermingled with $CD8^+$ T cells. The T cell areas of the H line were small, whereas in the L line large, dense T cell areas were found (Figure 7.4A, C).

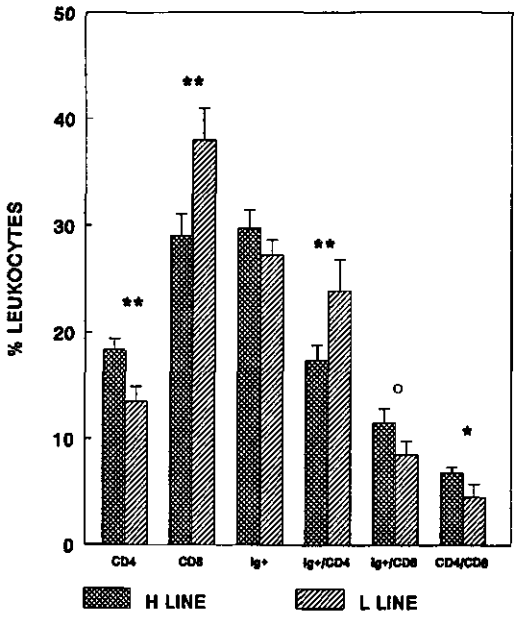

FIGURE 7.3. LSmeans (Model 2) per line (n=40) of the percentages splenic leukocyte populations and the ratio's (x 10) at 6 weeks of age. Standard errors of the mean and significant line effects are indicated (O:P<0.1; *:P<0.05; **:P<0.01).

Clear differences between the lines were also found when mAb HIS-C1, specific for B cells, was used. The H line had many peri-ellipsoid lymphoid sheaths (PELS) of B cells, whereas this number was lower in the L line. In addition, both number and size of germinal centres was larger in the H line. Consistent with the results on B cells, H line spleens contained much more ellipsoids than the L line spleens (Figure 7.4B, D) as indicated by mAb CVI-ChNL-68.2, specific for ellipsoid-associated reticulum cells. As expected, after staining with mAb CVI-ChNL-74.2, which recognizes, besides red pulp macrophages, rings of macrophages around the PELS, more rings were found in the H line. The numbers of red pulp macrophages were, however, comparable between the lines.

The mononuclear phagocytes (monocytes, interdigitating cells, and red pulp macrophages), specifically stained by mAb CVI-ChNL-68.1 showed no remarkable

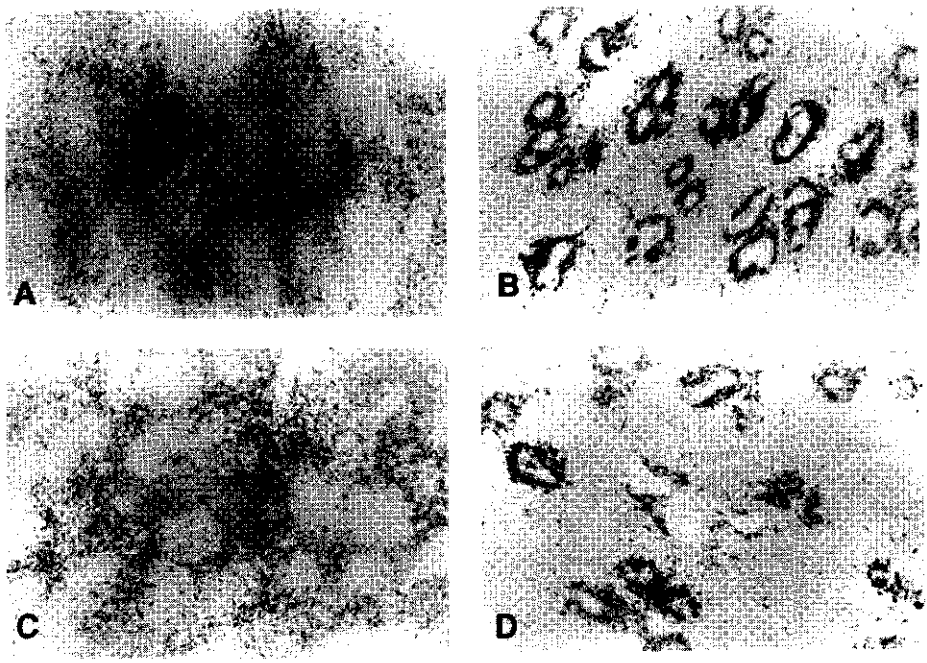

FIGURE 7.4. Immunohistochemical staining of spleens of high (A,B) and low (C,D) line chickens with monoclonal antibodies CVI-ChT-74.1, specific for T lymphocytes (A,C) and CVI-ChNL-68.2, specific for ellipsoid-associated reticulum cells (B,D).

differences between the lines. Also after staining with the mAb CVI-ChNL-74.3, which is specific for follicular dendritic cells and their precursors, no remarkable differences were found between the H and L line other than those due to the higher number of germinal centres in the H line, consequently, the H line had also more follicular dendritic cells.

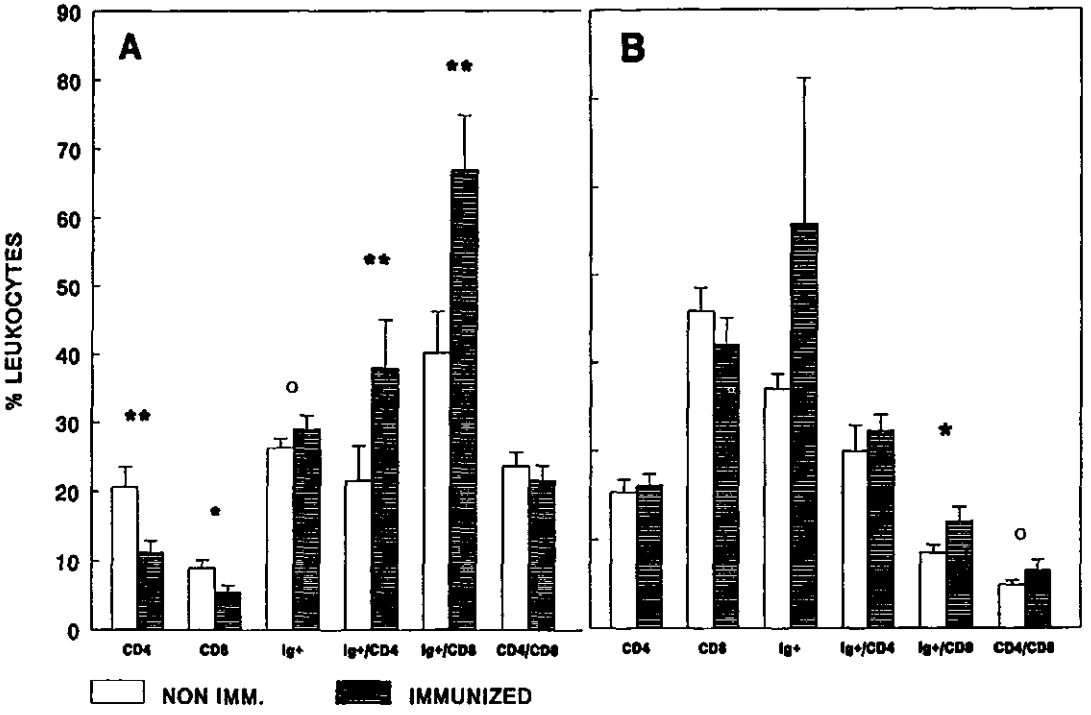

FIGURE 7.5. LSmeans of the percentages peripheral (A) and splenic (B) leukocyte populations (x 10^1) (Model 2) and the ratio's (x 10^{-1}) (Model 3) of non-immunized and immunized chicks at 6 weeks of age. Standard errors of the mean and significant immunization effects are indicated (O:$P<0.1$; *:$P<0.05$; **:$P<0.01$).

IMMUNIZATION EFFECT

Blood. Immunization did not affect the percentages of lymphocyte populations differently between the lines. However, immunization significantly reduced the percentages of circulating T cells (Figure 7.5A), while the percentage of Ig^+ cells was slightly enhanced ($P<0.1$) (Figure 7.5A). Therefore, the B cell:T cell ratio's were enhanced after immunization, but similar in the lines.

In weeks 10 and 12, no effect of immunization on the percentages of circulating

lymphocytes were found (data not shown).

In week 13, both the percentages of lymphocyte populations and the antibody titers were determined. Lines differed in percentages of Ig^+ cells and in the antibody titer, however, no significant correlations between agglutinin titer and the percentages of $CD4^+$ and $CD8^+$ cells were found. Moreover, partial correlations, (corrected for line effect) were not significant. The correlation between the haemagglutinin titer and percentage Ig^+ leukocytes was positive, +.36 ($P<0.10$). However, when corrected for line effect, the partial correlation was -.20, but not significant. Within the H line, also a negative (-.58) correlation was found ($P<0.05$), while this correlation was positive (+.11) but not significant ($P>0.10$) in the L line.

Thymus and bursa. In both lines slightly enhanced ($P<0.10$) percentages of $CD4^+$ cells were found in the thymus after immunization (immunized: LSmeans = 74.0, non-immunized: LSmeans = 70.8). In the bursa no effects of immunization were found.

Spleen. Immunization did not significantly affect the percentages lymphocytes, however, the splenic ratio's Ig^+:$CD8^+$ and $CD4^+$:$CD8^+$ were both enhanced by immunization (Figure 7.5B).

SPECIFIC HUMORAL RESPONSES.

Antibody producing cells. In the H line pullets immunized with TNP-BSA, always more spots were counted in the ELISpot-assay in both repeats and at all cell concentrations used (Table 7.2).

TABLE 7.2. Number of ELISpots to TNP, counted in cell suspensions made of spleens of pullets 21 days (test 1) or 28 days (test 2) previously immunized with BSA-TNP (mean ± standard deviation).

TEST	CONCENTRATION of cells	H	L	P<#
1	$5x10^7$	33.3 ± 10.9	19.6 ±13.4	.04
	$1x10^7$	19.8 ± 7.6	11.0 ± 5.7	.02
	$1x10^6$	9.8 ± 4.2	4.1 ± 2.1	.004
2	$5x10^7$	31.1 ± 20.5	9.7 ±13.0	.03
	$1x10^7$	32.7 ± 14.9	8.8 ±12.3	.004
	$1x10^6$	24.3 ± 10.6	6.8 ± 8.2	.002

Probability of line difference within cell concentration and test.

Also when pullets were only immunized with KLH and pulsed *in vitro* with KLH-TNP, significantly more ($P<0.05$) TNP-specific B cells were found in the H line spleen than in the L line (data not shown). Age at immunization had no effect on the number of spots.

TABLE 7.3. ELISA titers of H and L line chickens immunized with KLH at 25 or 39 days of age (mean ± standard deviation).

	COMBINED AGES		25 DAYS OLD		39 DAYS OLD	
DAY	H	L	H	L	H	L
0	0.1 ±0.3	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.1 ±0.4	0.0 ±0.0
3	0.7 ±0.8	0.4 ±0.6	0.2 ±0.3	0.1 ±0.1	1.1 ±1.0	0.7 ±0.8
5	3.0 ±1.8	1.9 ±1.0*	2.0 ±0.8	1.2 ±0.7*	4.0 ±2.0	2.6 ±1.0
7	4.3 ±2.2	4.9 ±1.9	3.2 ±1.3	3.5 ±1.4	5.4 ±2.3	6.3 ±1.1
10	3.2 ±1.4	3.9 ±1.7	3.2 ±1.4	3.9 ±1.7		
14	2.7 ±1.2	4.1 ±1.0	2.7 ±1.2	4.1 ±1.0		
21	2.6 ±1.0	3.3 ±0.6	2.6 ±1.0	3.3 ±0.6		

* lines differ $P<0.05$.

Antibody response to KLH. The early KLH-immunized chicks had significantly lower ($P<0.001$) ELISA-titers than the late immunized chicks (Table 7.3). The KLH titers were higher in the H line, however only significant on day 5 p.i. From day 7 p.i. onwards, titers of the L line were - not significantly - higher.

TABLE 7.4. Elisa titers for TNP of 16 H line and 16 L line chicks immunized with TNP-BSA (mean ± standard deviation).

DAY	H	L	
0	1.9 ± 1.2	0.3 ±0.5	***
3	2.4 ± 1.1	0.6 ±0.5	***
5	3.5 ± 0.9	1.3 ±0.5	***
7	3.8 ± 0.8	1.6 ±0.6	***
10	3.5 ± 0.7	2.0 ±0.8	***
14	3.4 ± 0.9	1.9 ±1.0	***
21	2.5 ± 0.9	1.5 ±1.5	***

***: lines differ $P<0.001$

The antibody response to TNP. At all days after immunization, the H line had higher TNP titers than the L line ($P<0.001$; Table 7.4).

DISCUSSION

In the present study, several approaches were used to investigate differences in the B cell compartment between two chicken lines selected for high or low antibody response to SRBC. The results obtained with IgG(H+L) to identify Ig^+ cells in present study were similar to those found with monoclonal AV20, specific for B cells (Parmentier *et al.*, 1995). The line difference in percentage of circulating Ig^+ cells, previously found at 6 weeks of age (Siegel *et al.* 1992), the age on which selection for humoral response is conducted in these lines, was now found to exist already at 2 weeks of age. Although in the present study lines did not significantly differ in percentage of splenic B cells, the ratio Ig^+:$CD8^+$ cells was higher in the H line and Ig^+:$CD4^+$ was higher in the L line. This indicates that the cellular organization of the spleen differs between the lines. Which agrees with the higher percentages of PELS and germinal centres found in the spleens of non-immunized H line chicks. Since the (relative) spleen weight is higher in the H line (Donker, 1989; Parmentier *et al.*, 1995), it is reasonable to assume that the H line has also absolutely more antibody producing cells, PELS and germinal centres in the spleen. The higher number of PELS, the chicken equivalent of the marginal zone in mammalian spleen (Jeurissen *et al.*, 1992) in the H line, indicates a constitutional difference between the lines, because the branching of arterioles is thought to be intrinsic and not influenced by antigenic challenges. The higher number of germinal centres in the H line should be considered as the result of previous contact with antigen. Nevertheless, because chickens of both lines were housed intermingled, it can be assumed that both lines were under the same antigenic pressure. Still the H line reacted with a higher number of germinal centres, demonstrating a functional difference between the immune systems of the lines. This difference is not SRBC-specific, because the chicks were not immunized. Moreover, early levels of antibodies to KLH, as well as TNP were higher in the H line, while also higher numbers of anti-TNP-producing cells were found in the spleen of non TNP immunized H line pullets. This emphasizes again the antigen non-specific effect of selection. The secondary antibody titers did not correlate with the percentage of circulating B cells in the H line. However, this is not surprising, because circulating antibodies are released by plasma cells as the result of a immune response in the peripheral lymphoid tissue (i.e. spleen).

As in mammals, the antibody responses to SRBC, TNP and KLH, in chickens will also depend on the support of helper T cells, characterized by the CD4 antigen on their surface. In general, the percentages of circulating T cell subsets were higher in the L line. However, soon after immunization many T cells are trapped in the peripheral lymphoid tissues, to assist with the elimination of antigen. Siegel *et al.* (1992) described a larger increase of $CD4^+$ cells in the spleens of the H line at 12 hours after

immunization, while in the L line the percentage of splenic $CD8^+$ cells was increased. In the present study, no line difference in immunization effect was found. However, in the 5 days following immunization the different reaction of the lines to immunization might have passed unseen. The line differences in T cell subsets were, however, similar to the results of Siegel *et al.* (1992) and Parmentier *et al.* (1995). The results found so far, implicate that the H line with a higher percentage of splenic $CD4^+$ cells and B cells is better equipped for antibody response to a T cell dependent antigen than is the L line. Because T cells with CD8 antigen on their surface are probably suppressor or cytotoxic effector cells, the L line has presumably other mechanisms selected to deal with the entering antigen. This was also suggested by the higher mitogenic response to Con A in the L line (Kreukniet *et al.*, 1994[b]).

From present studies it seems valid to conclude that divergent selection for antibody response has resulted in a relatively higher number of B cells in the H line. Moreover, in the H line the organization of the spleen, and probably also other peripheral lymphoid organs, favours antibody response to T dependent antigens. However, selection has also affected the T cell subsets. To what extend the T cell populations contribute to the line differences found in the B cell compartment, is presently being studied.

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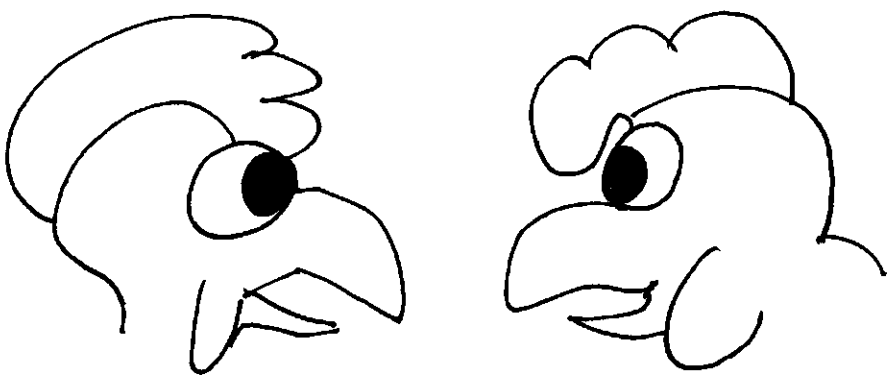

chapter 8

GENERAL DISCUSSION

GENERAL DISCUSSION

Introduction

In the last few decennia, the housing systems of livestock have dramatically changed. Intensification, thus more animals per m^2, was found economically favourable. However, not only the profit increased, but also the risk on infectious diseases, diminishing the profits. Medication, vaccination and improved hygienic measures helped to prevent outbreak and spread of diseases, but are costly. These costs and the incomplete protection of these measures, initiated the search for other ways to protect livestock and this reactivated the research on genetic improvement of disease resistance.

In 1980 a selection experiment was started to evaluate the possibilities of genetically improving general disease resistance in chickens by selection for either high (H) or low (L) antibody response to sheep red blood cells (SRBC; Kreukniet *et al.*, 1994). In addition, a control (C) line was maintained. This C line originates from the same base population and was maintained under identical conditions as the selection lines, but mated at randomly. The differences in antibody level, measured as haemagglutinin titers, increased nearly each generation, both between the H and L line and between the selection lines and the control (C) line (Figure 8.1).

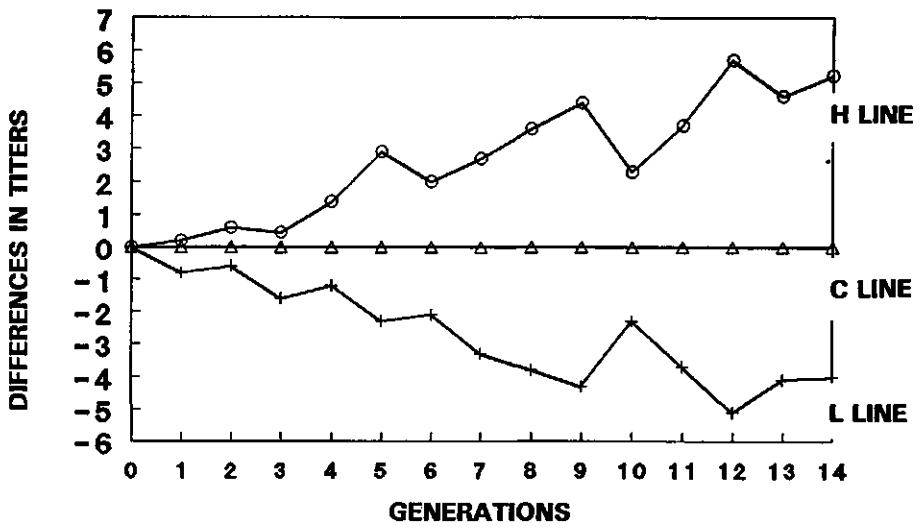

FIGURE 8.1. Mean titers in each selection generation of the H and L line relative to the mean of the C line.

Although selection for the phenotypic trait 'antibody production' was relatively easy, the knowledge of the cellular pathways used by each line to reach the selection goal is still limited. To make meaningful use of selection lines, it is important to characterize the immunological pathways used by the lines to realize the trait under selection as much as possible.

The genes responsible for the trait under selection become more fixed each selection generation. Therefore, the selected lines can be used as an *in vivo* model to study protective immune responses to pathogens in chickens, but also to study the genes responsible for the antibody response. And, when fully characterized at cellular level, it is also possible to pinpoint the genes for all known steps of the pathways leading to this response. In addition, in immunologically characterized selection lines the genes indirectly affected by selection can be studied. For instance, body weight is found to be negatively associated with antibody response (Chapter 1). Thus the selection lines can be used to study the genetics of the underlying mechanisms like release of growth hormones. On the other hand, because continuous selection has fixed several genes in both lines, particular immune responses will be impossible in the selection line, while others are obligate. To know how an individual from a selection line is bound to react (or is not able to react) upon an invading antigen, can give useful information on how resistance to pathogens can be reached in chickens.

TABLE 1. Cascade of events following IM immunization with SRBC.

1. Phagocytosis of the SRBC by phagocytes
2. Processing of the antigen
3. Presentation of antigen fragments by APC* to T and B cells
4. Release of cytokines
5. Proliferation and differentiation of T cell subpopulations
6. Proliferation and differentiation of B cells
7. Production of antibodies by plasma cells

* Antigen Presenting Cells

The main aim of the series of studies described in this thesis, was to investigate which pathways, at cell level, are used by the H line to produce high levels of antibody and which are used by the low (L) line for clearance of the SRBC. In Chapter 1, a review of other 'immune trait' selection lines was presented. It was found that non of the described selection lines were fully characterized. In Figure 1.1, (Chapter 1) a simplified scheme of the cascade of events activated after the intramuscular injection with SRBC, resulting in antibody production, is outlined. This figure is summarised in

Table 8.1. The studies described in the Chapters 2 to 7 were conducted to define the differences in immune mediated clearance of SRBC between the two selected lines of chickens. In the present Chapter, the results will be discussed in relation with other studies to identify these parts of the cascade that differ between the lines and to speculate on how the high antibody response is reached in the H line and on how the L line copes with the SRBC. The cascade as described in Figure 1.1, pictures the events resulting in antibody production. This approach will probably not be the most favourable to identify the mechanisms used by the L line to clear the SRBC, because it is likely that low antibody production is not reached by the inverse mechanisms or reactions which enhanced antibody response in the H line. However, this approach is chosen because much more is known of activation than of suppression of antibody response.

When the selection lines are found to differ for an immune parameter, it is not known whether the selection for high response has altered the immune parameter or that it has been altered in the opposite direction by selection for low response (or both). Fortunately, when differences were found, in several cases an additional study was conducted including the C line, which makes it possible to determine the direction of the selection effect.

Phagocytosis

The antigen non-specific or innate immune system is the body's first line of defence against invading antigens. In the case of an intramuscular (IM) injection, a large part of this defence is circumvented. Still, several a-specific responses i.e. phagocytes and several enzymes in the blood, will protect the body before the specific system is triggered. The cell damage caused by the IM injection induces the release of chemotactic factors which attract phagocytes. If the phagocytes are able to clear all the SRBC, there is no need for a specific response like antibody production. In mice divergently selected for antibody production against SRBC, the high phagocytic activity of the low line could explain a large part of the interline difference in antibody response (Wiener and Bandieri, 1974).

In our chicken selection lines, high plasma levels of lysozyme released from phagocytes during destruction of the SRBC after immunization were negatively correlated with antibody level (Chapter 2). These results suggest a comparable mechanism as found in the Biozzi mice (Wiener and Bandieri, 1974). However, the lines did not differ in lysozyme activity. Moreover, no line differences could be detected in the other assays estimating uptake or destruction of antigen (Chapter 2). Also, the uptake of SRBC by peritoneal cells did not differ between the lines (Chapter 3). Because the assays described in Chapters 2 and 3 were mostly *in vitro* assays, results might not mimic the *in vivo* reaction. However, the lack of effect on the interline

difference of the low SRBC-doses (Chapter 4), as well as the results of immunization with IFA (Chapter 5) further support the hypothesis that line differences in phagocytic activity do not cause the difference in antibody response to SRBC. When phagocytic activity is high, a very small dose of injected SRBC is likely to be destroyed before it is able to stimulate antibody response. Thus, a small dose of 0.5×10^{-3} ml packed SRBC, as well as the continuous release of small quantities of SRBC from the depot in IFA, are both likely to be cleared before the humoral response can be activated in the situation of high phagocytic activity. In both studies the interline differences were more or less of the same magnitude as after an injection with the normal dose (25×10^{-2} ml) or higher doses of SRBC. Thus, line differences in phagocytic activity (uptake and destruction) were not eminent, and therefore line differences in antibody response can not be attributed to phagocytic activity

Presentation of SRBC

To trigger an antibody response to a T cell-dependent antigen, the antigen has to be internalized and processed within an Antigen Presenting Cell (APC). Presentation of a part of the antigen at the surface of the APC to $CD4^+$ T cells, can activate these $CD4^+$ cells. These cells then respond with proliferation and the release of the cytokines. necessary to activate B cells. Activation of $CD4^+$ cells is in chickens, as in mammals, Class II (*B-L* in chickens) restricted (Vainio *et al.*, 1988). T cells will only respond to APC with corresponding *B-L* molecules at their surface. In chickens, associations with particular *B-G* haplotypes and Marek's disease are reported (see review Chapter 1). Because *B-G* genes and *B-L* and *B-F* genes are found to be rather tightly linked, it was hypothesized that particular *B*-products are better in stimulating protective immune responses (Briles *et al.*, 1977). Similarly, it can be hypothesized that particular *B-L* products might be better than others in stimulating $CD4^+$ lymphocytes to certain antigens. That is, a particular antigen/*B-L* combination might be recognized more effectively by a given T cell receptor (TCR) or alternatively, particular *B*-complex products might associate better with this specific antigen and therefore are more effective in stimulating antibody responses. As the frequencies of *B*-haplotypes differ between the lines (Pinard *et al.*, 1993[b]), it is tempting to speculate on differences in presentation efficiency between the lines. The H line might be better equipped to stimulate T helper cells and B cells to respond to SRBC than the L line. However, the *B*-genotypes explained only 3.5% of the total variation in the antibody response estimated in the F2 population (produced by crossing the products of a cross from H and L line chickens; Pinard and Van der Zijpp, 1993). Therefore it is not likely that the line differences in *B*-haplotypes are responsible for the difference in antibody production. On the other hand, the *B*-haplotypes measured are products of *B-G* genes,

which are not involved in the presentation of antigens and in generations of selection associations with *B-G* and *B-F* or *B-L* might have altered between the lines.

To activate T cells, binding to the antigen fragment on the APC is essential, but also other co-stimulatory signals are necessary. One of these is the stabilizing surface molecule CD28, expressed on all $CD4^+$ $\alpha\beta$ T cells in chickens (Young *et al.*, 1994). Binding to this CD28 molecule and to the antigen receptor, causes enhancement of cytokine production (Arstila *et al.*, 1994). Recently the peripheral and splenic percentages of CD28 molecules were determined in the H and L line. However, differences were not consistent in time (Parmentier *et al.*, 1995). And percentages splenic $CD28^+$ cells were similar between the lines both in immunized and non-immunized chicks (Kreukniet *et al.*, unpublished results).

To summarize the information concerning presentation of antigen as yet known, the two aspects studied, i.e. the *B*-complex and the CD28 molecule, do not indicate any differences in antigen presentation between the selection lines. However, in chickens, the counter part of the CD28 molecule is not been determined and it is possible that this molecule, or other co-stimulating factors (i.e. cytokines) differ between the lines, resulting in $CD4^+$ T cell activation favouring antibody production in the H line.

Cytokines

As stated before, not only the association of TCR and other accessory molecules on the surface of APC with their ligands is necessary, but also certain cytokines, i.e interleukines and interferon, are essential for the activation of B cells. Interleukine-1 (IL-1), produced by activated APCs, induces IL-2 receptors on T cells. After prolonged exposure to IL-1, chicken T cells will secrete IL-2 (Klasing, 1987). IL-1 and IL-2 are necessary to activate $CD4^+$ cells. Differences between the lines in production of IL-1 or other interleukines will lead to differences in immune responses. The release of the certain cytokine profiles, is found to direct the differentiation of $CD4^+$ into either T_H1 or T_H2 cells in mammals. T_H1 cells function as helper cells in the cellular response and T_H2 cells in the humoral response (Mosmann *et al.*, 1986; Abbas *et al.*, 1991). However, as yet, no avian homologues of the interleukines inducing this differentiation (IL-4, IL-5, IL-10 and IL-12) have been found. The existence of the T_H1 and T_h2 cells is therefore hypothetic in poultry.

To study our selection lines in more detail, attempts have been made to measure IL-2 and interferon γ. Unfortunately, the assays used seemed poorly reproducible with our chickens. Therefore, no direct information about these cytokines is available. But, also in poultry seems T cell growth during *in vitro* mitogen response to depend on IL-2 (Arstila *et al.*, 1993). Therefore, the higher *in vitro* responses to the mitogen ConA in the L line (Chapter 6) might mean higher IL-2 production in the L line (Pink and Vainio, 1983), produced either by T cells or APC. On the other hand, it is also possible that

ConA is better in stimulating growth of $CD8^+$ cells. This would explain the higher response in the L line, because relatively more $CD8^+$ cells are found in the L line (Chapter 7; Siegel *et al.*, 1992; Parmentier *et al.*, 1995).

T cells

In Chapter 5, it was found that interline differences diminished in the second (spontaneously appearing) peak after an IM injection of SRBC in Complete Freund's Adjuvant (CFA). This effect was attributed to the mycobacteria in CFA, because Freund's Adjuvant without the mycobacteria (IFA) did not enhance the L line response to the H line level. Mycobacteria are believed to stimulate phagocytes and T cells. It is not known whether T cells are activated directly by the mycobacteria, or by cytokines released by phagocytes. However, the mycobacteria might have resulted in a profile of cytokines favouring differentiation of $CD4^+$ T cells into T_H2 cells stimulating antibody response. Hypothetically, the immune system of the H line might normally react with T_H2 cells, while the L line usually reacts with differentiation into T_H1 cells. The mycobacteria might shift the differentiation in the L line towards T_H2 cells. This genetic predisposition to specific antigens has been described before in mice. Certain strains (Balb/C) are very susceptible to Leishmania and were found unable to respond with T_H1 cells, while the strains like C57 BL/6 responding with T_H1 cells to this parasite survive (Bancherreau, 1991). In our chicken lines, both lines might react with T_H2 cells, due to the mycobacteria, resulting in a high antibody response in both lines. However, this is rather hypothetic, because CFA is also found to stimulate cellular responses (Allison and Davies, 1971; Allison and Byars, 1986). Possibly, the total number of activated T_H1 and T_H2 cells are elevated, enhancing both cellular and humoral response simultaneously. As yet, there is in chickens no prove that T_H1 and T_H2 cells exist, thus it is not possible to differentiate between these T cell populations in.

In order to estimate the T cell activity of the lines, traditionally *in vitro* and *in vivo* T cell stimulation tests are conducted. In Chapter 6, *in vitro* T cell responses to ConA were described. *In vivo* T cell responses (wingweb) to PHA or the antigen BSA were estimated in the lines by Parmentier *et al.* (1993). However, the *in vitro* results were contradicting with the *in vivo* results. *In vivo*, higher T cell activity was found in the H line, while *in vitro* the responses were higher in the L line. These differences might be due to differences in T cell subpopulations stimulated. As reported, the lines differ in percentages of $CD4^+$ and $CD8^+$ cells (Chapter 7, Siegel *et al.*, 1992; Parmentier *et al.*, 1995) and of TCR1 and TCR2 cells (Parmentier *et al.*, 1995). Moreover, it is also not known how the different assays used to evaluate T cell activity correspond with the function of T cells *in vivo*. Therefore, it is very speculative to suggest relatively higher or better T cell activity in one of the lines based on these assays. The reverse order in

the lines comparing the mitogen responses *in vitro* and *in vivo*, might just reflect the line differences in T cell populations. Also, because antibodies mediate the reaction measured in the *in vivo* T cell response (Parmentier *et al.*, 1993), line differences found might be the effect of differences in antibody production and not of T cell responses.

Although the different assays conducted to estimate T cell activity in the lines do not show consistent results, line differences in the T cell compartment are indisputable. The percentages of the $CD8^+$ and $CD4^+$ cells (Chapter 7, Siegel *et al.*, 1992; Parmentier *et al.*, 1995) and of TCR1 and TCR2 (Parmentier *et al.*, 1995) differ between the lines, while also structural differences in the T cell areas of the spleens are found (Chapter 7). The $CD8^+$ cells are, at least *in vitro*, capable of suppressing the antibody response to SRBC (Quere *et al.*, 1990[a,b]). It can be hypothesized that selection for low antibody response has favoured the $CD8^+$ T cell populations. These cells might be able to suppress the antibody response. Although they might also reflect a more active cytotoxic defence in the L line, because it is not possible to differentiate between these functional types of $CD8^+$ cells. In the H line selection might be based on high numbers of the $CD4^+$ T_{helper} cell phenotype. However, the difference in antibody response might also be the result of differences in cytokine profiles, causing higher numbers of H line $CD4^+$ to differentiate into putative T_H2 cells and L line $CD4^+$ to differentiate into putative T_H1 cells. These combined effects of selection might have resulted in the high and low anti-SRBC response lines.

Proliferation and differentiation B cells

Although, it is clear that differences in the T cell compartment between the lines exist (see above), there are also differences in the B cell compartment. It is noteworthy that both lines have an antibody peak at day 5 or 7 p.i. This shows that the L line is a true low responder to SRBC and not a delayed anti-SRBC responder, because the height of the response is the only line difference in the kinetics of the antibody response. The line differences in circulating antibodies reflect the line differences found in both total and IgG antibody forming spleen cells (AFC) after immunization with SRBC (Donker, 1989). This might be the result of the higher number of B cells found in the H line compared with the L line and the C line (Chapter 7; Siegel *et al.*, 1992; Parmentier *et al.*, 1995), which thus allows more B cells to be activated. On the other hand, a higher proliferation rate of each activated B cell will also result in more AFC.

For activation, binding of the antigen to the sIg at the surface of the B cell is necessary. This means that only specific B cells can be activated. Higher antibody responses against BSA, KLH and TNP are found in the H line (Chapters 5 and 7; Parmentier *et al.*, 1994), suggesting that the higher number of B cells in the H line is a general phenomenon. This general effect is emphasised by the higher number of TNP specific antibody producing cells in non TNP-immunized H line chicks (Chapter 7).

However, although the percentage of B cells and the anti-SRBC antibody levels differ between the H and C line, anti-BSA levels are comparable between these lines, but lower in the L line. Therefore the relatively higher number of B cells does not necessarily result in higher antibody production. Moreover, after SRBC immunization it was possible to elevate the L line to the H line level, by administering CFA. In addition, the antibody responses to SRBC and BSA do not correlate (Parmentier *et al.*, 1994). Thus although there are in total more B cells which can be activated in the H line, also other mechanisms supporting antibody response must differ between the H and L line. The lack of difference in antibody responses against T cell-independent antigens (Chapter 5) indicates that T cells must also be involved and, as discussed above, the T_H cell populations and thus the cytokine profiles are good options.

Antibody repertoire

If, among other signals, there is antigen bound to their sIg, B cells can be activated, proliferate and differentiate into plasma cells. Each B cell has sIg of only one specific antigenic determinant and can produce only this one specific Ig. However, the total B cell repertoire of an individual will cover most antigens. It might be possible that the low antibody response in the L line reflects a limited recognition of SRBC by B cells, due to a small B cell repertoire, resulting in only a few plasmacells. During differentiation of the B cells, slight modifications in the antibody specifity occur. As the chick is continuously under antigenic pressure, the antibody repertoire will change during life. However, despite the equal housing conditions, relatively more B cells, as well as a higher number of splenic germinal centres at 6 weeks of age, were found in the H line. Therefore, the H line might have created a larger antibody repertoire during embryonic life and the first weeks after hatch. The response to many antigens is higher in the H line and it is tempting to attribute this to the higher number of B cells in the H line, compared with the L and the C line (Parmentier *et al.*, 1995). However, when the antibody response to BSA of H and L line chicks is compared with the C line, it was found that, although the response in the H line is higher than in the L line, the C and the H line do not significantly differ (Parmentier *et al.*, 1994). Therefore it might be better to speak of genetic suppression of antibody responses in the L line instead of enhancement in the H line. However, as BSA and SRBC are different types of antigen, the intracellular processing of the soluble protein BSA by the APC, will probably differ from that of the particle SRBC. The differences between the L, C and H line in processing SRBC, might not exist for BSA. Processing of BSA (and possibly other soluble antigens) might differ between the C and L line, but not between the C and H line.

Hypothesis

Although only a few facets the cascade of events following immunization with SRBC have been studied in both lines, a hypothesis can be based on these results. Firstly, both antigen clearance by phagocytes and antigen presentation by APC seem not to attribute to the differences in antibody response to SRBC between the lines. Thus, there is in this no parallel between present chicken lines and the Biozzi mice. However, selection seems to have altered the B cell compartment, because relatively more B cells are found in the H line than in the C and L line. The differences in percentage B cells might explain the difference in anti-SRBC antibodies, but can not be extended to all other T cell-dependent antigens, because the H line does not always differ from the C line (Parmentier *et al.*, 1994). Higher percentages of $CD4^+$ cells were also found in the H line. The $CD4^+$ cells have helper functions in both cellular and humoral responses, therefore one can not contribute differences in antibody response directly to the relative line differences in helper cell populations. Which immune response is supported by the $CD4^+$ cells - cellular or humoral - depends probably on the APC and the cytokines produced. This differentiation process seems to depend on the kind of antigen processed by the APC. The supposed genetic fixation of differentiation into putative T_H2 cells in the H line only covers the response to SRBC. Although the suppression of antibody response is less dependent on the kind of T cell-dependent antigen (Chapters 5 and 7; Parmentier *et al.*, 1993; 1994).

Line differences were found to depend on the route of immunization (Chapter 5). Selection has altered the splenic organization between the lines (Chapter 7). But, because the place of entrance of the antigen determines to a large extent in which lymphoid organ the immune response takes place, not all the other peripheral lymphoid organs are similarly affected.

In addition to the differences in $CD4^+$ cells, in the L line higher percentages $CD8^+$ T cells are found. $CD8^+$ cells are able to suppress antibody responses, at least *in vitro*. Thus selection for low antibody production might be reached by a relative high number of $CD8^+$ cells. In contrast to the enhancement of antibody response in the H line, the suppression of antibody response in the L line seems also true for BSA. Thus genetic selection for suppressed antibody response might have caused general suppression against T cell-dependent antigens, while enhancement was antigen specific in our selection lines.

Practical Implications

Poultry breeders would have welcomed it hearty when selection for (high) antibody production would have increased general disease resistance. At the beginning of the selection experiment, this was one of the aims for the lines. The first challenge with Marek's disease was promising, indeed less mortality was found in the H line than in

the L line (Van der Zijpp and Nieuwland, 1986). However, comparison with the C line revealed that in fact the L line was more susceptible, while resistance in the H line was not enhanced (Pinard *et al.*, 1993[a]). As discussed, selection for high antibody response was found not to enhance the total scope of antibody responses, while the opposite selection for low antibody responses seemed to be more a-specific. As susceptibility is obviously not an economically favourable trait, selection for antibody response to SRBC seems not to have a direct practical implication. However, these effects should not be generalized, as the defence against invading pathogens does not necessarily involve antibodies. Moreover, selection has enhanced the percentages of $CD8^+$ cells in the L line. These $CD8^+$ cells are believed to play a key role in the resistance to avian coccidiosis. Therefore present L line might be resistant to infectious oocysts.

Although in mice, phagocytic activity could explain part of the difference in antibody response (Wiener and Bandieri, 1974), no such effect was found in our chicken lines. This warns us against direct projection of the results found in one species on other species. Furthermore, when studies with present selection lines are compared with other chicken lines selected for antibody response against SRBC, the differences in results are striking (Scott *et al.*, 1991, Scott *et al.*, 1994, Van der Zijpp *et al.*, 1988, 1989; Chapter 6). And it has also been reported that even replicated selection lines, selected from the same base population, differ in effects on the immune traits and *B*-complex (Kean *et al.*, 1994[a,b]). Therefore, it should not be expected that results found in one selection line, will be repeated when the selection is repeated with other animals of the same strain.

On the other hand, it also indicates that selection for a certain immune response to increase general (or specific) disease resistance, which might be highly correlated in parts of the breeding population, might not have the desired effect in the commercial - hybrids - populations.

Scientific Implications

The immune system is very complex and, although working following the same rules within a species, the outcome differs between individuals as the immune pathways followed will differ. After selection for several generations, the genes responsible for the phenotypic trait (level of the anti-SRBC titer) will finally become fixed, resulting in a limited repertoire of immune pathways in a selection line. The two lines divergently selected for antibody response will thus finally result in two lines with fixed genes. The lines thus have to follow predictable immune pathways to reach the immune responses. If the pathways are antigen non-specific or specific for a defined group of antigens, the lines can be very useful as an *in vivo* model to study the immune responses in chickens and their effectiveness in resistance to different pathogens.

On the other hand, in the future, when the lines are at the selection plateau and the pathways are fully identified in the lines, they can be used to determine the genes responsible for each step of the pathway. This makes direct selection for the most favourable genotypes, instead of phenotype, possible. However, to be able to do this, first should be solved which genotype is the most favourable.

A direct advantage of our selection lines is the existence of a C line. Although at present, the selection lines are not fully immunologically characterised, the C line can be used to determine whether the found selection effects are to be attributed to the H line or the L line. This makes it possible to directly use the information available. For example, of most pathogens the route by which pathogenicity is achieved is still under investigation. The known interline differences in, for instance, the percentages of lymphocyte subpopulations might be very useful in determining how susceptibility or resistance to a given pathogen is reached. So far, our selection lines have only been challenged with Marek's disease virus (MDV). Therefore we can use MDV as an illustration of the possibilities selection lines have to speed up progress in understanding the interactions between the Marek virus and host cells. In the challenge with MDV, the control line was included and it was found that the L line was more susceptible, while the H line and C line hardly differed in resistance to MDV (Pinard *et al.*, 1993[a]). MDV is a DNA herpes virus and it is believed that B cells are the primary lymphocytic targets of the early phase of the infection (Schat, 1991). T cells become activated, express *B-L* antigen and can be infected with MDV (Calnek, 1986). These latently infected T cells are thought to be responsible for the spread of MDV to other organs. Reactivation of the virus in these cells can lead to cytolitic infection in lymphoid organs. (see Calnek, 1986). The final stage of infection is often a neoplastic transformation of T lymphocytes (Nazerian et al., 1973). The target cells for MDV induced tumorgenesis appear to be mature T cells and cell activation may be a key element in the transformation process (Schat, 1991). Schat *et al.* (1991) reported that TCR1 cells are rarely the targets for MDV infection and transformation, although this might be attributed to other *in vivo* growth requirements of TCR1 cells.

If TCR1 cells are less susceptible, it could be hypothesized that selection for antibody response has resulted in less TCR1 type T cells in the L line, which is contradicting with the findings in our lines (Parmentier *et al.*, 1995). Although B cells might be the first cells infected by MD, this infection does not necessarily cause the pathogenic effect, as it would incline that the H line, having more B cells, should be more susceptible, which is not found. Therefore the key to resistance or susceptibility to Marek's disease might be the activation of T cells or the cytokine profile.

Concluding remarks

In this thesis a start is made with the characterisation of the immune pathways used

by the two selection lines. However, much research has to be done to complete this task. The C line was not included in the studies presented in this thesis, however, the use of this line was inevitable to be able to comment on the direction of the selection effects found.

The lines are found to differ in both the B cell and T cell compartment of the immune system. Unfortunately, progress in characterisation of the lines has been hampered by the incomplete knowledge of the structure and function of the avian immune system. Therefore, similarities with the mammalian system have been assumed. Differences in cytokine profiles causing activation of $CD4^+$ cells, either stimulating antibody response or cellular responses remain hypothetic in our chicken lines.

Extension of both the knowledge of the immune repertoire of the selection lines and the resistance and susceptibility to other diseases is a necessity to make full use of the lines as *in vivo* models with fixed immune responses.

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SUMMARY

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Introduction

The housing of large quantities of pigs or poultry on small areas has increased the risk on infectious diseases. One of the solutions to prevent diseases is keeping genetically resistant animals. Therefore, different research groups have started to study the possibilities of breeding animals with so called 'general disease resistance'. Several lines of chickens have been divergently selected for immunological traits or for resistance and susceptibility to a certain disease to study genetic disease resistance. These lines are reviewed in Chapter 1, after a brief overview of the immune system of chickens. In the following chapters 2 to 7, two chicken lines selected for either high (H) or low (L) antibody response to sheep red blood cells (SRBC) are studied. Both these lines were selected from the same base population of medium heavy brown egg layers (ISA Brown), in addition a random mated control (C) line was maintained from the same base population.

In Chapter 1, it was concluded that of non of the selection lines the mechanisms underlying the immune response are known. However, when these selection lines are to be used to study the genetic aspects of those immune responses which protect against pathogens, this knowledge is necessary. After an antigen invades the body, a cascade of reactions is triggered and all the steps of this cascade together will eventually result in the immune response selected for. In each selection generation all the genes responsible for each step in the cascade become more fixed in the lines. Finally this will result in lines which genes can only produce a limited repertoire of reactions to SRBC and possibly also to other antigens. If the limitations of the lines and the antigen specifity are known, they can be of great use in studying the pathogenity of diseases and the possible useful defensive reactions of chickens. On the other hand, when all the steps of the cascade of reactions following the invasion of the antigen are known, the lines can be used to study the genes responsible for each step.

Therefore studies were conducted to start the immunological characterisation of the H and L selection lines. In the Chapters 2 to 7, several steps of the cascade were studied to find differences between the H and L line. In Chapter 8, the results of these studies and additional studies, including the C line, are discussed to set up a hypothesis how high antibody response is established in the H line and how the SRBC is cleared in the L line.

Phagocytic activity

In Chapters 2 and 3, the approach of Biozzi was followed, because in the Biozzi mice selection for antibody response to SRBC affected the phagocytic activity and accessory

functions of macrophages. The mice L line phagocytized more antigen and the antigen was degraded faster. In the H line mice the retention time of antigen on the surface of macrophages was much higher. This lead to the postulate that differences in handling of the entering antigen was the cause of line difference in antibody production. Because when in the L line all entered antigen was trapped and destructed, other protective mechanisms, like antibody response are not necessary. Therefore first the phagocytic capacity of present chicken lines was investigated.

Generally, no line differences were observed. However, more circulating granulocytes were found in the L line. On the other hand, *in vitro* phagocytosis of yeast cells was higher in the H line. Blood clearance of carbon was similar in the lines and no line differences were found in the destruction phase of phagocytosis (Chapter 2). In Chapter 3, intraperitoneal cells (PEC) were harvested from non-immunized chicks and of chicks intramuscularly immunized with SRBC five days previously. The lines did not differ in percentage of phagocytic PEC, nor in number of SRBC phagocytized.

From these results it was concluded that the line difference in antibody production after immunization with SRBC was not caused by differences in phagocytic activity between the selection lines. This was confirmed by the studies described in Chapters 4 and 5. When different doses SRBC were intramuscularly injected in young chicks, both lines still responded when only $5x10^{-4}$ packed SRBC were injected (Chapter 4). Moreover, line differences remained on the same level, independently of the dose given (Chapter 4). Small doses of free SRBC can also be established by immunizing with a depot-forming-adjuvant. Experiments were carried out with SRBC in Incomplete Freund's Adjuvant (IFA) (Chapter 5). The oil in this adjuvant causes a continuous release of very small amounts of SRBC to the surroundings, yet line differences still existed.

T cell activity

When Complete Freund's Adjuvant (CFA), which contains oil, but also mycobacteria, was used, the titers decrease only a little after the first peak was reached and then, without reimmunization, increased to remain, in both lines, at the peak level of the H line for several weeks (Chapter 5). Because this was not found after immunization with sole SRBC or IFA, it is believed that the mycobacteria, which are thought to stimulated T cells, caused the high antibody response in both lines. Therefore differences in T cell activity might be responsible for the line differences in antibody response to SRBC.

Immunization with the T cell-dependent antigens BSA (Chapter 5), Keyhole limpet haemocyanin (KLH) and trinitrophenyl sulphonic acid (TNP; Chapter 7) resulted in divergent antibody responses similar to the responses to SRBC. Additional studies, including the C line (discussed in Chapter 8), showed that antibody response to BSA did not differ between the H and C lines. On the other hand, the antibody responses to

Brucella abortus (BA) and *Salmonella* H antigen, both considered T cell-independent, did not show the characteristic line differences. However, responses differed between sexes, pullets having a higher response in one line, the cockerels in the other (Chapter 5). These differences in antibody response between immunization with a T cell-dependent and a T cell-independent antigen indicate also line differences in T cell activity.

Because of the suggested differences in T cell activity between the selection lines, *in vitro* T cell activity was studied in Chapter 6. The mitogen response to ConA were higher in the L line than in the H line, independently of dose ConA, or lymphocyte source (peripheral or splenic). However, *in vivo* hypersensitivity reactions, also a measure of T cell activity, showed higher response in the H line than in the L line. This might be attributed to the role of antibodies eliciting hypersensitivity *in vivo*. On the other hand, also differences in T cell populations stimulated in the assays applied or differences in activity state of T cells after immunization might account for the opposite results found. This is supported by the line differences in T cell populations. In the H line higher percentages $CD4^+$ cells are found, while in the L line the percentages $CD8^+$ cells are slightly higher (Chapter 7).

B cell compartment

Histological determinations with specific monoclonal antibodies on spleens of non-immunized chicks, showed that the selection had affected the cellular organization of the spleen (Chapter 7). Large dense T cell areas were found in the L line, while in the H line more and larger germinal centres were found. In the H line, also more B cells were found in the peri-ellipsoid lymphoid sheaths (PELS). The higher number of PELS in the H line, indicates a constitutional difference between the lines, because the branching of arterioles is thought to be intrinsic and not influenced by antigenic challenges. The number of germinal centres should be considered as the result of previous contact with antigen. Nevertheless, because chickens of both lines were housed intermingled, it can be assumed that both lines were under the same antigenic pressure. Still the H line reacted with a higher number of germinal centres, demonstrating a functional difference between the immune systems of the lines. This difference seems not SRBC-specific, because the chicks were not immunized.

In Chapter 7, also the percentages of lymphocyte subpopulations are studied in blood and lymphoid organs using flow cytometry. In spleens of non-immunized chicks, more $CD8^+$ cells were found in the L line and in the H line spleen more $CD4^+$ cells. Immunization had a similar effect in both lines (Chapter 7). In general more circulating Ig^+ leukocytes in the H line. Moreover, in non-TNP-immunized chicks, higher numbers of splenic anti-TNP antibody forming cells were found in the H line.

From the studies described in Chapters 2 to 7 it seems valid to conclude that

divergent selection for antibody response has resulted in a relatively higher number of B cells in the H line. While in the H line also the organization of the spleen favours antibody response to T cell-dependent antigens. However, as the line difference depends on the route of immunization (Chapter 5) not all peripheral lymphoid organs might be affected to the same extend.

Hypothesis

Selection affected the B cell compartment differently in the two lines. A relative higher number of B cells was found in the H line, which might attribute to a higher antibody response in this line. The lines also differed in cellular organization of the spleen, which might favour antibody response in the H line. On the other hand, higher percentages $CD8^+$ cells were found in the L line and these cells can suppress antibody response. Also the percentages of $CD4^+$ cells differed between the lines. The $CD4^+$ cells are able to stimulate immune responses. Which response is stimulated - cellular or humoral - depends on the cytokines released by the immune cells. It might be hypothized that in the H line more $CD4^+$ cells differentiate in T helper cells favouring the antibody response. However, the cytokines responsible for the differentiation of $CD4^+$ cells in mammals, have, as yet, not been discovered in chickens.

The high antibody response is largely SRBC specific (when compared with the C line). This might be due to the processing of the antigen by Antigen Presenting Cells and the following release of cytokines. The low antibody response in the L line seems to be less antigen specific.

Concluding remarks

In this thesis a start is made with the characterisation of the immune pathways used by the selection lines, however much work has to be done to complete this task.

Extension of both the knowledge of the immune repertoires, including the antigen specifity, of the selection lines and of the resistance and susceptibility to other diseases in the lines are a necessity to make full use of the lines as *in vivo* models with fixed immune responses.

Comparison of present lines and other lines selected for antibody response to SRBC, and also other unrelated replicated selection experiments, show that replication of an selection experiment will not necessarily respond to the selection with the same immune pathways, although the same phenotypic result is reached. Thus, each line, even replications from the same base population should be immunologically characterized if to be used as an *in vitro* model. On the other hand, even when in parts of the breeding populations a certain immune trait is highly correlated with 'general' (or specific) disease resistance, selection for this immune trait might not give the desired result in the hybrids used in commercial practice.

SAMENVATTING

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Introductie

Het houden van grote aantallen varkens en kippen op kleine oppervlakten heeft de kans op ziekten verhoogd. Een van de oplossingen om ziekten te voorkomen is het houden van genetisch resistente dieren. Daarom is men op verschillende plaatsen begonnen met onderzoek naar de mogelijkheden om dieren te fokken met 'algemene ziekte resistentie'. Er zijn onder andere verschillende divergente kippenlijnen geselecteerd op een hoge danwel lage immuunrespons, ofwel op resistentie danwel vatbaarheid voor een bepaalde ziekte. Een overzicht van deze divergente selectielijnen is gegeven in Hoofdstuk 1, hetgeen voorafgegaan wordt door een kort overzicht van het immuun-systeem van de kip. De hoofdstukken 2 tot en met 7 beschrijven twee kippenlijnen, die divergent geselecteerd zijn op antilichaamproduktie tegen schapen rode bloed cellen (SRBC). Beide lijnen werden geselecteerd uit dezelfde basispopulatie middelzware ISA Brown legkippen. Daarnaast werd ook een niet-geselecteerde lijn als controle (C) lijn aangehouden.

Uit Hoofdstuk 1 bleek dat van geen van de selectielijnen bekend is welke mechanismen op cellulair niveau verantwoordelijk zijn voor de immuunrespons waarop geselecteerd is. Echter, indien men gebruik wil maken van deze selectielijnen om bijvoorbeeld die genetisch aspecten van immuunresponsen te onderzoeken die beschermen tegen pathogenen, dan is deze kennis noodzakelijk. Het immuunsysteem reageert immers met een cascade van reacties, die uiteindelijk leiden tot de immuunrespons waarop geselecteerd is. In iedere geselecteerde generatie komen er meer dieren waarvan de genen die verantwoordelijk zijn voor de deelstapjes van deze cascade aan elkaar gelijk zijn. Uiteindelijk zullen alle dieren van een selectielijn een vastliggend repertoire deelstapjes kunnen uitvoeren. Indien het vastliggende immuunrepertoire van een lijn bekend is en tevens bekend is voor welke antigenen dit geldt (de antigeenspecificiteit), dan kunnen deze lijnen een zeer zinvolle bijdrage leveren aan het onderzoek naar hoe ziekte tot stand komt en welke immuunreacties beschermend zijn bij de kip. Daarnaast kunnen, indien alle stappen van de cascade volgend op het binnendringen van het antigeen bekend zijn, ook de genen verantwoordelijk voor iedere stap bestudeerd worden.

Daarom zijn in de volgende hoofdstukken experimenten beschreven die tot doel hadden de hoge (H) en lage (L) selectielijnen immunologisch te karakteriseren. In Hoofdstuk 2 tot en met 7 zijn verschillende stappen van de cascade bestudeerd om verschillen tussen de lijnen te vinden. In Hoofdstuk 8 zijn de resultaten van deze en aanvullende onderzoekingen (waarin ook de C-lijn) bediscussieerd, zodat hypotheses opgesteld konden worden over hoe de H-lijn een hoge antilichaamrespons bereikt en hoe de L-lijn tegen SRBC optreedt.

Fagocytose-activiteit

In de Hoofdstukken 2 en 3, is de aanpak van Biozzi gevolgd. In Biozzi's muizen bleek de selectie op antilichaamrespons tegen SRBC de fagocytose-activiteit en de antigeen-presenterende functies van macrofagen te hebben veranderd. De L-lijn muizen fagocyteerden meer antigeen en braken het antigeen ook sneller intracellulair af. Bij de H-lijn muizen daarentegen konden gedurende langere tijd stukjes antigeen op het celoppervlak van macrofagen gevonden worden. Hieruit werd afgeleid dat door verschillen in behandeling van het antigeen, verschillen in antilichaamproduktie werden veroorzaakt. Immers, indien in de L-lijn door fagocytose al het antigeen is vernietigd, worden andere beschermende mechanismen, bijvoorbeeld antilichaamproductie, niet meer geactiveerd. Daarom is eerst de fagocytose-activiteit in onze beide kippenlijnen bestudeerd.

Over het algemeen zijn er geen verschillen tussen de lijnen gevonden. In het bloed van de L-lijn werden wel meer granulocyten gevonden, maar in de H-lijn was de *in vitro* fagocytose van gistcellen hoger. Koolstof werd in beide lijnen even snel uit het bloed verwijderd, terwijl ook geen verschillen tussen de lijnen werden gevonden in de vernietiging van antigeen (Hoofdstuk 2). In Hoofdstuk 3 werd de fagocytose van SRBC door intraperitoneale (buikholte) cellen (PEC) bestudeerd. De PEC waren afkomstig uit niet-geïmmuniseerde kippen en uit kippen die 5 dagen eerder waren geïmmuniseerd met SRBC. De lijnen verschilden niet, noch in het percentage fagocyterende PEC, noch in het aantal SRBC opgenomen per actieve PEC.

Uit de resultaten van Hoofdstuk 2 en 3 kan worden geconcludeerd dat het lijnverschil in antilichaamproduktie na immunisatie met SRBC niet wordt veroorzaakt door verschillen in fagocytose-activiteit tussen de lijnen. Ook de resultaten van Hoofdstuk 4 en 5 ondersteunen deze conclusie. De dosis SRBC geïnjecteerd in de spier had geen invloed op het lijnverschil in antilichaamtiter (Hoofdstuk 4). Beide lijnen reageerden zelfs nog met een antilichaamrespons na een dosis van slechts $5x10^4$ SRBC (Hoofdstuk 4). Kleine hoeveelheden SRBC kunnen ook vrijkomen na immunisatie met SRBC in een depot-vormend-adjuvant. Experimenten werden uitgevoerd met SRBC in Incompleet Freund's Adjuvant (IFA) (Hoofdstuk 5). De olie in dit adjuvant zorgt ervoor dat continu een kleine hoeveelheid SRBC vrijkomt. Echter de lijnverschillen werden er niet door beïnvloed.

T-cel-activiteit

Indien werd geïmmuniseerd met Compleet Freund's Adjuvant, hetgeen naast olie ook mycobacteriën bevat, namen de antilichaamtiters slechts een beetje af na de eerste piek, daarna nam de titer toe, zonder reïmmunisatie, om weken lang, in beide lijnen op het niveau van de H-lijn te blijven (Hoofdstuk 5). Omdat dit niet gevonden werd na immunisatie met enkel SRBC, of SRBC in IFA, zijn waarschijnlijk de mycobacteriën

verantwoordelijk voor de hoge antilichaamrespons in beide lijnen. Aangenomen wordt dat mycobacteriën T-cellen stimuleren, daarom zijn mogelijk verschillen in T-cel-activiteit de oorzaak van de verschillen in antilichaamrespons.

Immunisatie met de T-cel-afhankelijke antigenen Bovine Serum Albumine (BSA) (Hoofdstuk 5), Keyhole Limpet Haemocyanine en Trinitrofenyl sulfer zuur (TNP) (Hoofdstuk 7) gaf lijnverschillen in antilichaamtiter vergelijkbaar met de verschillen na immunisatie met SRBC. Uit aanvullende experimenten (bediscussieerd in Hoofdstuk 8) bleek echter dat de antilichaamrespons tegen BSA niet verschilde tussen de H en de C-lijn, maar wel tussen de C- en de L-lijn. De antilichaamrespons tegen *Brucella abortus* (BA) en *Salmonella* H antigeen, beide T-cel-onafhankelijk, vertoonde niet de karakteristieke lijnverschillen. Echter, de responsen verschilden tussen de sexen, waarbij in de ene lijn de hennen de hoogste antilichaamtiter hadden, en in de andere lijn de hanen (Hoofdstuk 5). De verschillen in antilichaamrespons na immunisatie met T-cel-onafhankelijke antigenen en T-cel-afhankelijke antigenen zijn ook een indicatie voor verschillen in T-cel-activiteit.

De verschillen in T-cel-activiteit werden in Hoofdstuk 6 verder bestudeerd met behulp van *in vitro* mitogeenresponsen tegen ConA. De T-cel-groei was groter in de L-lijn dan in de H-lijn, onafhankelijk van de dosis mitogeen die gebruikt was en of de T-cellen afkomstig waren uit bloed of milt. Echter, de *in vivo* overgevoeligheids-reacties, ook een maat voor T-cel-activiteit, bleken tegenovergestelde lijnverschillen te geven. De respons was hoger in de H-lijn dan in de L-lijn. Mogelijk kan men dit toeschrijven aan de antilichamen die een rol spelen in de overgevoeligheids-reacties. Maar ook verschillen in de T-cel-populaties die gestimuleerd worden in de toegepaste testen of verschillen in de activiteit van de T-cellen op het testmoment en na immunisatie zouden de verschillen in de volgorde van de lijnen tussen de *in vivo* en *in vitro* testen kunnen verklaren. Dit wordt ondersteund door de verschillen in T-cel-populaties tussen de lijnen. In de H-lijn zijn hogere percentages $CD4^+$ T-cellen gevonden, terwijl in de L-lijn de percentages $CD8^+$ T-cellen hoger zijn (Hoofdstuk 7).

Het B-cel-compartiment

Histologisch werk met monoclonale antilichamen specifiek voor bepaalde cellen, toonde aan dat in niet-geïmmuniseerde hennen, selectie de cellulaire organisatie van de milt had veranderd (Hoofdstuk 7). In de L-lijn werden grote, dicht bevolkte T-celgebieden gevonden, terwijl in de H-lijn meer en grotere kiemcentra werden gevonden. Deze kiemcentra zijn een gevolg van een antilichaamrespons. In de H-lijn werden ook meer B-cellen gevonden in de 'peri-ellipsoid lymphoid sheaths'(PELS). Het grotere aantal PELS geeft aan dat er een wezenlijk verschil is tussen de lijnen, omdat de mate van vertakking van de haarvaatjes aangeboren is en niet afhankelijk is van de antigenen waarmee het dier in aanraking komt. Het aantal kiemcentra moet echter wel

gezien worden als het gevolg van contact met antigeen. Echter, omdat de beide lijnen onder identieke omstandigheden zijn gehuisvest, kan aangenomen worden dat zij beide aan eenzelfde hoeveelheid antigenen hebben blootgestaan. De H-lijn reageerde echter met meer kiemcentra, hetgeen aangeeft dat er een functioneel verschil is tussen de immuunsystemen van beide lijnen. Dit verschil lijkt niet SRBC afhankelijk, immers de hennen waren niet geïmmuniseerd.

In Hoofdstuk 7 zijn met behulp van 'flow cytometry' de percentages van de lymfocyten subpopulaties gemeten in het bloed en lymfoïde organen. In niet-geïmmuniseerde kippen werden in de milt van de L-lijn meer $CD8^+$ T-cellen en in de H-lijn milt meer $CD4^+$ T-cellen gevonden. Immunisatie gaf in beide lijnen dezelfde veranderingen in lymfocyt-percentages. In het bloed van de H-lijn werden meer Ig^+ cellen gevonden. Daarnaast werden in de milt van niet TNP-geïmmuniseerde H-lijn dieren ook meer Antilichaam-Vormende-Cellen tegen TNP gevonden dan in de L-lijn.

Uit de experimenten beschreven in Hoofdstuk 7 kan geconcludeerd worden dat de selectie voor antilichaamrespons heeft geresulteerd in een relatief groter aantal B-cellen in de H-lijn. Daarnaast is de organisatie van de milt in de H-lijn zodanig dat op T-cel-afhankelijke antigenen eerder met een antilichaamrespons wordt gereageerd. Echter, omdat het lijnverschil afhangt van de immunisatie-route (Hoofdstuk 5), is het niet waarschijnlijk dat de selectie alle secundaire lymfoïde organen in dezelfde mate heeft veranderd.

Hypothese

Het effect van de selectie op antilichaamrespons verschilde tussen de twee lijnen. Een relatief hoger aantal B-cellen werd gevonden in de H-lijn, wat mogelijk de hoge antilichaamrespons heeft veroorzaakt in deze lijn. Er werden ook verschillen tussen de lijnen gevonden in de cellulaire organisatie van de milt. Mogelijk is deze in de H-lijn zodanig veranderd dat een antilichaamrespons makkelijker plaatsvindt dan in de L-lijn. In de L-lijn werden echter hogere percentages $CD8^+$ cellen gevonden, deze cellen kunnen een verlagend effect op de antilichaamrespons hebben. Ook de percentages $CD4^+$ cellen verschillen tussen de lijnen. De $CD4^+$ cellen zijn in staat een immuunrespons te stimuleren. Echter welke immuunrespons wordt geactiveerd - cellulaire of antilichaamrespons - hangt af van de cytokinen in de omgeving van deze T-cel. Mogelijk differentiëren er in de H-lijn meer $CD4^+$ cellen in de richting van T-helper-cellen specifiek voor de antilichaamrespons. Echter, de cytokinen die in zoogdieren verantwoordelijk zijn voor de differentiatie van $CD4^+$ T-cellen, zijn in de kip nog niet aangetoond.

De hoge antilichaamrespons blijkt in de H-lijn (vergeleken met de C-lijn) SRBC-specifiek te zijn. Dit kan mogelijk worden toegeschreven aan de verwerking van antigeen door de zogenaamde Antigeen-Presenterende-Cellen en de daarop volgende

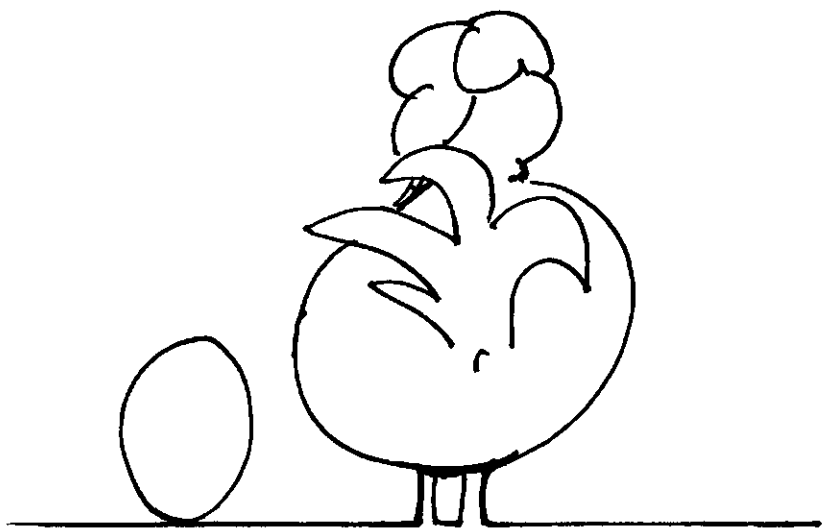
cytokinenproduktie. De lage antilichaamrespons in de L-lijn blijkt daarentegen veel minder antigeen-specifiek.

Afsluitende opmerkingen

In deze dissertatie is een begin gemaakt met de immunologisch karakterisering van twee op antilichaamrespons geselecteerde kippenlijnen. Echter, er moet nog veel werk gedaan worden voordat deze karakterisering compleet is.

Een volledige karakterisering van het repertoire aan immuunresponsen, inclusief antigeen-specifiteit, van beide selectie lijnen en, in mindere mate, van de resistentie of gevoeligheid voor pathogenen is noodzakelijk om goed gebruik te kunnen maken van deze lijnen als *in vivo* modellen met genetisch vaststaande immuunresponsen.

Vergelijking van deze lijnen met andere kippenlijnen geselecteerd op antilichaamrespons tegen SRBC en andere onafhankelijke herhaalde selectie-experimenten, tonen aan dat het herhalen van een selectie experiment niet noodzakelijkerwijs hoeft te leiden tot selectielijnen met hetzelfde repertoire aan immuunresponsen. Daarom moet iedere selectielijn die men wil gebruiken als *'in vivo'* model opnieuw immunologisch gekarakteriseerd worden. Ook betekent dit dat selectie op een bepaalde immuunrespons in commerciële populaties (hybriden) niet tot verbeterde genetische resistentie hoeft te leiden, ook al is in delen van deze populaties aangetoond dat er een hoge correlatie is tussen deze imuunrespons en resistentie.



Curriculum Vitae

Maaike Bernardina Kreukniet werd op 10 april 1961 te Gorinchem geboren. Het diploma ongedeeld VWO werd in 1979 behaald aan het Koningin Wilhelmina College te Culemborg. In datzelfde jaar werd de studie Zoötechniek aan de toenmalige Landbouw Hogeschool te Wageningen gestart. In 1986 studeerde zij af met als hoofdvakken Gezondheids- en Ziekteleer (verzwaard) en Ethologie en het bijvak Pedagogiek en Didactiek. In de periode april 1986 tot januari 1992 was gedurende in het totaal 4 jaar en 6 maanden, steeds voor 20 uur in de week, afwisselend als toegevoegd universitair docent en toegevoegd onderzoeker werkzaam bij de vakgroep Veehouderij van de Landbouw Universiteit te Wageningen. Tijdens deze perioden werd onderzoek verricht aan het immuunsysteem van twee op antilichaamproductie geselecteerde kippenlijnen. Van juli 1994 tot januari 1995 was zij aangesteld als Onbezoldigd Promovenda bij de Landbouw Universiteit, hetgeen haar in staat stelde, naast deelname aan congressen en cursussen, dit proefschrift af te ronden.