Avian T Helper One/Two Immune Response Balance Can Be Shifted Toward Inflammation by Antigen Delivery to Scavenger Receptors

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ABSTRACT Whether immune responses are dominated by inflammation or antibody production is often key to surviving infections. Therefore, differential control of these immune pathways determined by CD4 T cells is of fundamental interest for vaccine design. Little is known about how inflammatory [T helper cell (Th) type 1 (Th1)] versus antibody-inducing (Th2) choices are controlled in domestic fowl. To address this, MHC-matched chickens were immunized to test whether antibody-dominated Th2 or inflammatory Th1 responses could be preferentially activated, and our findings subsequently extended to outbred broiler breeders. Strategies used were known to shift the response in mice from Th2 to Th1 by delivering the injected antigen preferentially to macrophages. The model antigen, BSA, was maleylated to allow binding to scavenger receptors (SR) present on mammalian macrophages. Maleyl-BSA bound well in receptor-specific fashion to a chicken macrophage cell line. Compared with native BSA, immunization with SR-binding, maleyl-BSA modulated the immune response toward the Th1 pathway, as evident by increases in the magnitude of in vivo inflammatory reactions and declines in antibody-making responses. Initiation of a maleyl-BSA Th1 pathway is further supported by the enhanced ability of splenocytes to express mRNA for interferon-γ in response to antigens. Together, these data establish the presence and functional relevance of SR in domestic fowl as well as provide a system for investigating the mechanisms controlling Th1/Th2 pathways in chickens. Moreover, the ability to direct immune responses toward either pathway by antigen maleylation will contribute significantly to the development of better vaccines for poultry diseases.

(*Key words*: lymphocytes, macrophages, T helper subsets, cytokines, scavenger receptors)

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INTRODUCTION

Adaptive immunity to pathogens is critically dependent on T cell responses, particularly CD4 T helper (Th) cell responses, in mammalian and avian species (Arstila et al., 1994). Upon activation, mammalian CD4 T cells make several cytokines that fall into two groups, referred to as Th1 and Th2. A given mammalian T cell is likely to make either Th1 or Th2 cytokines (Mosmann et al., 1986; Cherwinski et al., 1987; Pfeiffer et al., 1991). The Th1 cytokines include tumor necrosis factor-β and interferon-γ (IFNγ). The IFNγ induces inflammation and activates macrophages to kill resident intracellular pathogens, such as mycobacteria or salmonellae (Kagaya et al., 1989). The Th2 cytokines include interleukin (IL)-4, -5, and -10, which can help to mature the B cell response so that efficient antibodies are made to deal with extracellular pathogens, such as streptococci (Lebman and Coffman, 1988). Whether an immune response is dominated by antibody production or inflammation can make a remarkable difference in the severity of a given infectious disease (Kim et al., 1985; Stevens et al., 1988; Heinzel et al., 1991). Therefore, controlling the Th1/Th2 balance is of fundamental interest for vaccine design. Our goal was to explore ways to differentially modulate Th1/Th2 immune responses by delivering antigen to receptors on antigen-presenting cells (APC). We and others have shown that such strategies generate higher magnitudes and more Th1-oriented responses in mice (Abraham et al., 1995; Pasare et al., 1998; Singh et al., 1998).

Before mammalian CD4 T cells can recognize their target antigens, APC must digest these antigenic proteins into peptide fragments and bind them to MHC class II proteins for T cell presentation at the APC surface (Babbitt et al., 1985; Unanue and Allen, 1987; Guagliardi et al., 1990; Lanzavecchia, 1990; Amigorena et al., 1994). This APC presentation is limited by the ability to express MHC class II molecules. B cells, macrophages, and dendritic cells are the dominant APC for CD4 T cells in mammalian and avian systems. For complete T cell acti-

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Abbreviation Key: APC = antigen presenting cell; DTH = delayed type hypersensitivity; IFNγ = interferon-γ; IL = interleukin; polyG = poly-guanylic acid; PCR = polymerase chain reaction; RT = reverse transcriptase; SR = scavenger receptor; Th = T helper cell.

vation, secondary costimulatory signals that are antigen independent, consisting of cell surface molecules and secreted cytokines from these same APC, are also necessary (Mueller et al., 1989; Jenkins et al., 1990; Liu and Janeway, 1992; Schwartz, 1992; Freeman et al., 1993).

Modulation of the costimulatory signals can lead to alteration of the Th1/Th2 balance in mice (Liu and Janeway, 1992; Kuchroo et al., 1995). Costimulatory signals such as IL-12, secreted by macrophages and dendritic cells, predispose the generation of a Th1 response (Hsieh et al., 1993; Afonso et al., 1994). Some costimulatory molecules expressed by B cells appear to help generate a Th2 response from CD4 T cells (Swain et al., 1990; Hathcock et al., 1994; Macatonia et al., 1995; Sabin and Pearce, 1995; Stockinger et al., 1996). The peptide-MHC complex density also plays an important role in regulating Th1/Th2 responses in CD4 T cells. Thus, we (Pasare et al., 1998) and others (Pfeiffer et al., 1995; Singh et al., 1998) have shown that increasing the density of peptide-MHC complexes on APC can lead to selective enhancement of Th1 responses in mice.

Although chicken immune responses depend on CD4 T cells (Arstila et al., 1994), little is known about how such Th1/Th2 choices are controlled in domestic fowl. In chickens, the delayed type hypersensitivity response (DTH) represents the Th1 immune response in vivo. By measuring local swelling upon antigen injection into the wattle, wing-web, or toe web of immunized birds, the DTH response can be assessed generally (York and Fahey, 1990) or specifically (Corrier, 1990). Chicken lines with low DTH responses appear to show better resistance to Marek's disease (Afraz et al., 1994). The maintenance of the DTH response has been demonstrated to be more important than antibodies for resistance against *Cryptosporidium baileyi* (Hatkin et al., 1993). Moreover, although antibody responses were not correlated with resistance to *C. baileyi*, specific inhibition of DTH, but not of antibody responses, by cyclosporin A increased susceptibility (Hatkin et al., 1993). Thus, Th1/Th2-like pathways appear to be present in avian species. Chicken IFNγ has been purified, functionally characterized (Kaspers et al., 1994; Lowenthal and Digby, 1995; Schultz et al., 1995), cloned and sequenced (Digby and Lowenthal, 1995; Weining et al., 1996), and shown to be analogous to mammalian IFNγ. Recently, IFNγ responses have been shown to be key in Eimeria infections (Lowenthal et al., 1997; Lillehoj, 1998; Lillehoj and Choi, 1998; Lowenthal et al., 1998; Choi et al., 1999). A system of immunization in which Th1/Th2 response balances can be reliably modulated in avian species, especially outbred birds, would thus be useful to extend the mechanistic analysis of avian immunity as well as for design of more efficient vaccines.

So far, few immunization protocols preferentially generating Th1 or Th2 response in the same individual have been described, although it is a prerequisite for vaccine design strategies. Cytokines, such as IL-12, have been included in vaccine preparations to induce Th1 or Th2 response in this connection (Scott and Trinchieri, 1997). Our work with receptor-mediated immunogen delivery for generating Th1-dominated responses was extended to the avian model. The scavenger receptor (SR) family of proteins is expressed by the APC lineages (Pearson, 1996). Scavenger receptors can bind a broad specificity of structurally unrelated ligands with high affinity and deliver the endocytosed ligands efficiently to antigen processing compartments in mammalian macrophages (Zhang et al., 1993). Recently, SR-delivered immunogens have been used to enhance the magnitude and the Th1 component of immune responses in mice (Abraham et al., 1995; Singh et al., 1998). Scavenger receptors, conserved in evolution (Resnick et al., 1994), have not yet been characterized in avian species, but their existence has been indirectly suggested (Yancey and Jerome, 1998). Therefore, SR were used as a ligand delivery system for avian macrophages.

MATERIALS AND METHODS

In Vitro Study

Cell Lines. A macrophage cell line, MQ-NCSU, (gift of M. Qureshi, North Carolina State University, Raleigh, NC 27695), (Qureshi et al., 1990), was grown in complete L-glutamine-fortified LM-HAN medium with 8% fetal bovine serum, 10% chicken serum,[3] 1% sodium pyruvate, 5% tryptose phosphate, 5×10^{-5} *M* 2-mercaptoethanol,[4] and antibiotics. For use in assays, adherent MQ-NCSU cells were dislodged, harvested, and kept at 4 C briefly prior to the assay.

SR Ligands. The RIA-grade BSA[3] (96% pure; A4503) was maleylated at alkaline pH by using maleic anhydride[5] as described earlier (Haberland and Fogelman, 1985). The maleylated protein was subjected to extensive dialysis against PBS, and the degree of maleylation was estimated from the loss of active ε-amino groups (Habeeb, 1966). For staining purposes, biotinylation was performed on a single batch of BSA, and maleylation done subsequently on half of it, hence the degree of biotin substitution is the same between the two reagents, and comparisons could be made directly.

Fluorescent Reagents. To assess the effect of antigen maleylation on binding to a macrophage cell line, cell markers were fluorescently stained. The staining reagents were R-phycoerythrin conjugated mouse monoclonal anti-chicken MHC class II[6] (Chen et al., 1991) and streptavidin-phycoerythrin,[7] which was used in conjunction with biotinylated BSA and maleyl-BSA. The known mammalian SR ligands, fucoidin and poly-guanylic acid (poly-G),[5] were used as binding competitors with maleyl-BSA when preincubated with MQ-NCSU

[3]Pel-Freez Biologicals, Rogers, AR 72757.
[4]Life Technologies, Grand Island, NY 14072.
[5]Sigma, St. Louis, MO 63178.
[6]Southern Biotechnology, Birmingham, AL 35226.
[7]Caltag, Burlingame, CA 94010.

cells. Ligand binding to SR was assessed by flow cytometry.

Flow Cytometry. MQ-NCSU cells were immunofluorescently stained by incubation on ice for 45 min with 100 μg of competitors/mL of staining buffer (PBS containing 0.1% sodium azide and 0.2% gelatin[3]). Next, the cells were washed three times with ice-cold PBS and stained with primary reagents, followed by an indirect third staining step with streptavidin-phycoerythrin[7] (3 μg/mL), if required, in the same fashion. After staining, cells were fixed in 0.05% paraformaldehyde in PBS and stored until analysis. Flow cytometric analysis was carried out using a FACSort[8] instrument. The instrument used a 488 nm laser, and the filter parameters were as follows: forward scatter = 488/10, side scatter = 488/10, fluorescence 1 = 530/30, fluorescence 2 = 585/42, fluorescence 3 = 650 long pass. Data were analyzed with CellQuest[8] and FlowJo[9] software packages. Gates were set using isotype controls stained directly or with the addition of a secondary staining agent. All data are representative of at least three independent experiments.

In Vivo Study 1

Chickens. MHC-matched egg-type chickens were used. All bird experimentation followed protocols in accordance with the Institutional Animal Care and Use Committee. Fertilized eggs from M. Qureshi (North Carolina State University) were hatched. Ten Cornell K Strain Leghorns (*B*15*B*15) were raised in the John Kirkpatrick Skeeles, Jr. Poultry Health Laboratory (biosecurity level 2), Fayetteville, AR. The chickens were 6 wk old at the beginning of the experiment and included seven males and three females. Birds were fed diets that met or exceeded NRC requirements. Feed and water were provided ad libitum.

Antigens. The RIA-grade BSA[3] (96% pure; A4503) was maleylated as described above. Only maleyl-BSA with over 80% substitution was used for all immunizations.

Experimental Protocol. Groups of 6-wk-old Cornell K strain Leghorns were immunized with 2.5 mg (0.5 mL) of maleyl-BSA or BSA in PBS as a single dose into the breast muscle (five birds per group). Antigen dosages were based on preliminary experiments that indicated peak responses resulted from dosages between 1 and 3 mg. Heparinized blood was collected from the brachial vein immediately before and at Days 7, 10, and 21 post-antigen injection. Plasma was frozen and stored at –20 C until ELISA determinations of specific IgG and IgM levels were made. At 22 d postimmunization, both groups were challenged s.c. with 100 μL (0.5 mg) of BSA into one wattle and 100 μL of PBS into the other wattle (vehicle control). Wattle thickness was measured with a dial metric caliper[10] at 12- to 24-h intervals for 4 d postchallenge. Wattle swelling was expressed as the difference in thickness (millimeters) before and after injection. Birds were killed on Day 28 by CO_2 inhalation, and spleens were removed for splenocyte activation and RNA isolation.

Antibody Assays. ELISA were used to estimate antigen-specific plasma Ig. Polyvinyl chloride microtiter plates[7] were coated with antigen (10 μg/mL at 50 μL per well) (BSA or maleyl-BSA) overnight and blocked overnight, and then sample plasma was titrated. This plasma was incubated on the plate for 30 min. The bound plasma Ig was detected by adding biotinylated anti-chicken Ig[6] (0.13 μg/mL) and then streptavidin-peroxidase[6] (1:10,000 dilution of manufacturer's stock), each for 30 min., followed by the revealing agents 2,2′-azinodi [3-3-ethylbenzthiazoline-6-sulfonate] (ABTS) and hydrogen peroxide.[11] A plasma dilution of 1:300 was judged to be within the linear range for IgM and IgG assays for anti-BSA immunized test samples. Because at this dilution the preinjection, PBS control samples, and sometimes maleyl-BSA immunized samples were below the linear range of assay sensitivity, the titer of these samples was determined separately with doubling dilutions of plasma. To be considered positive, a sample was required to be two times the standard error (George et al., 1994), and this endpoint determined the titer of a given sample. To estimate antigen-specific IgG, biotinylated mouse anti-chicken IgG monoclonal antibody (clone G1[12]) at 0.5 μg/mL constituted the detection system. To estimate antigen-specific IgM, biotinylated mouse anti-chicken IgM[12] at 0.13 μg/mL was used. Absorbance was read at 492 nm in a microplate reader.[12]

Detection of IFN-γ Transcripts. Single spleen cell suspensions from BSA or maleyl-BSA immune chickens were isolated as enriched mononuclear cells 28 d after immunization by FicoLite[13] gradient centrifugation. Cells (5×10^5 cells/well) were cultured in vitro at 41 C with graded doses of each antigen (3 to 1,000 μg/mL) in 96-well, flat-bottom microtiter plates[14] in a total volume of 200 μL of LM-HAN medium per well. Cells were cultured with BSA and maleyl-BSA, regardless of in vivo exposure. After determining in preliminary experiments that 1,000 μg/mL at 48 h gave the best IFNγ signal, RNA from 48-h, 1,000-μg/mL cultures was prepared by resuspending pelleted cells in 2 mL of RNAzol[15] followed by purification with chloroform, isopropanol, and ethanol as described by the manufacturer. The IFNγ mRNA from these antigen-activated cells was detected with reverse transcription (RT) in RT reaction buffer[14] followed by RT-polymerase chain reaction (RT-PCR) as described (Christoph et al., 1994). The sequences used for PCR amplification were 1) sense (direct) primer: 5′-TTGAGAATCCAGCGCAAAG-3′ and 2) antisense (indirect) primer: 5′-GAGCAGGTATGAGTGGGTTTTC-3′. These primers, expected to yield a 446 bp band, were

[8]Becton and Dickinson, Lincoln Park, NJ 07035.
[9]Tree Star, San Carlos, CA 94070.
[10]Bel-Art Products, Pequannock, NJ 07440.
[11]Kirkegaard and Perry Laboratories, Gaithersburg, MD 20879.
[12]Bio-Tek Instruments, Vinooski, VT 05404.
[13]Atlanta Biologicals, Norcross, VA 30093.
[14]Intermountain Scientific Corp., Kaysville, UT 84037.
[15]Tel-test Inc., Friendship, TX 77546.

optimized using software programs: Cprimer v 1.08[16] and Amplify v 1.2.[17] Taq polymerase (2 U) was used for PCR amplification in a reaction containing 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 9.0), 200 μM dNTP, and 50 p*M* primers. The PCR was conducted for 35 cycles as follows: denaturation at 93 C for 45 s, annealing at 53 C for 90 s, extension at 72 C for 60 s, and expansion at 72 C for 10 min. The product was electrophoresed on a 1.5% agarose gel at 200 V for 1 h. Band intensity estimates were made by densitometric quantitation using Alphaease software[18] or the freeware package NIH Image v1.60.[19]

In Vivo Study 2

Chickens. Outbred commercial broiler breeders were used in this study. Twenty-one broiler breeder females (27 wk old), raised and maintained in conventional housing conditions at the University of Arkansas Poultry Farm, were the kind gift of N. B. Anthony, University of Arkansas, Fayetteville, AR 72701. Birds were fed diets that met or exceeded NRC requirements. Feed and water were provided ad libitum.

Antigens. Immunization antigens were prepared as described in Study 1.

Experimental Protocol. Three groups of 27-wk-old broiler breeders (seven birds per group) were immunized with 1.4 mg (0.2 mL) of BSA, maleyl-BSA, or PBS (control) as a single dose into the breast muscle. At 21 d postimmunization, the groups were challenged s.c. with 100 μL (1.0 mg) of the immunizing antigen into one wattle and 100 μL of PBS into the other wattle as the vehicle control. The PBS group was challenged with BSA. These chickens were bled, wattle challenged, measured, and killed as described in Study 1. ELISA were used as outlined in Study 1, and IFNγ levels were determined as stated in Study 1.

Statistical Analysis

Appropriate statistical tests of significance, such as Student's *t*-test or ANOVA, were applied as indicated. Probability levels have been included with each set of data.

RESULTS

In Vitro Study

Effect of Maleylation of Antigens on Binding to a Macrophage Cell Line. MQ-NCSU cells, used to assess binding of biotinylated native BSA or biotinylated maleyl-BSA, expressed MHC class II (Figure 1A) and did not bind BSA (Figure1B). However, binding was demonstrable when maleylated protein was used (Figure 1C). To confirm that the observed binding of maleyl-BSA was SR mediated, fucoidin and polyG (100 μg/mL) were used to inhibit maleyl-BSA binding to MQ-NCSU cells. The competitor concentrations were the same as unlabeled BSA (100 μg/mL). The fucoidin and polyG significantly inhibited the subsequent binding of maleyl-BSA (Figure 2), whereas unlabeled BSA at the same concentration showed no competition (not shown). Results with the MQ-NCSU cell line was confirmed by staining fresh ex vivo chicken spleen cells with comparable results (not shown). After establishing that SR was available for binding on chicken macrophages, our next step was delivering antigen to the SR to attempt to alter the Th1/Th2 balance of the chicken immune response.

In Vivo Study 1

Enhancement of Antigen-Specific DTH by Maleyl-Protein Immunization. In Cornell K strain Leghorns, a far stronger DTH response occurred at 24 h postchallenge in those birds initially injected with maleyl-BSA as compared with those initially injected with BSA (Figure 3).

Modulation of Antigen-Specific Antibody Responses by Maleyl-Protein Immunization. Prior to immunization and in PBS controls, background levels of antigen-specific anti-BSA and anti-maleyl-BSA antibodies were determined to be negligible. Pre-immunization total Ig titers were ≤1:8. The IgM titer for the BSA-treated birds was 1:1,024 and for the maleyl-BSA treated birds was 1:32 (Figure 4A). For comparisons of individual birds, 1:300 was determined to be within the positive linear range of sera for detection of IgM (Figure 4A) and IgG (Figure 4B) in BSA-immunized birds. The IgG titer for BSA-treated birds was 1:256 (Figure 4B), whereas the titer for maleyl-BSA-treated birds was less than 1:16 (Figure 4B) and was determined to be 1:2 (data not shown). Individual birds responded consistently within immunization groups. In Cornell K strain Leghorns immunized with BSA, plasma anti-BSA IgM levels were much greater (75-fold) than in maleyl-BSA-immunized birds (Figure 4C). This BSA-specific IgM antibody did not recognize maleyl-BSA (Figure 4D). Also, no anti-maleyl-BSA IgM response occurred in maleyl-BSA-immunized birds (Figure 4D). Similarly, anti-BSA IgG levels (Figure 4E) were significantly higher (56-fold) in those K strain Leghorns immunized with BSA compared with those immunized with maleyl-BSA. Anti-BSA IgG did not recognize maleyl-BSA, and no anti-maleyl-BSA IgG response was detectable in maleyl-BSA-immunized birds (Figure 4F). Because anti-maleyl titers were low, we confirmed that this result was not an artifact caused by inadequate binding of maleyl-BSA to the ELISA plates. We found well-characterized mouse anti-maleyl-ovalbumin antisera (Abraham et al., 1995; Singh et al., 1998) (gift of Vineeta Bal, National Institute of Immunol-

[16]Department of Medicine and Department of Biochemistry, School of Medicine, University of California, Los Angeles, CA 90095-1361.
[17]Department of Genetics, University of Wisconsin, Madison, WI 53706.
[18]Alpha Innotech Corp., San Leandra, CA 94577.
[19]NIH, Bethesda, MD 20892 http://rsb.info.nih.gov/nih-image.

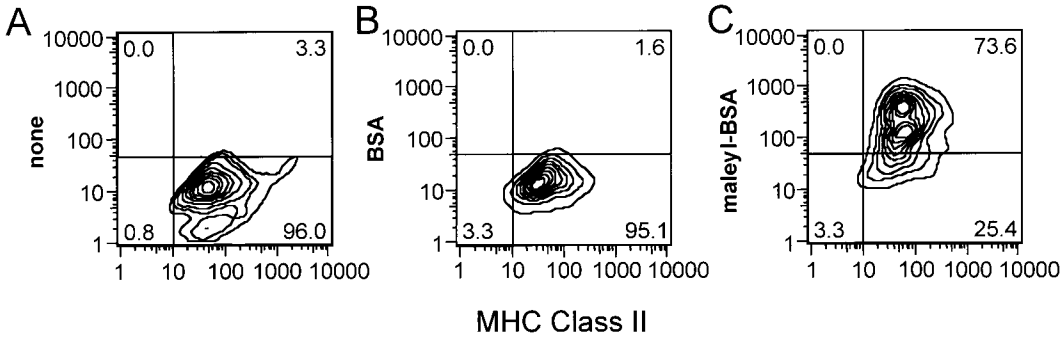

FIGURE 1. Flow cytometric analysis of maleyl-BSA binding to the chicken macrophage cell line MQ-NCSU. The MQ-NCSU cells were directly stained with phycoerythrin-conjugated mouse anti-chicken MHC class II and with streptavidin-Red670 (Panel A), BSA-biotin and streptavidin Red670 (Panel B), or maleyl-BSA-biotin and streptavidin Red670 (Panel C). Cell frequencies shown in each quadrant are percentages of a total of 20,000 cells analyzed using a FACSort flow cytometer.

ogy, Aruna Asaf Ali Road, New Delhi, 110067, India) had the expected titer (data not shown).

Maleyl-Protein Immunization Enhances Production of the T Cell Cytokine, IFNγ. To confirm the altered Th1/Th2 balance suggested by these ex vivo DTH and serological data, molecular studies were necessary to show that the essential Th1 cytokine, IFNγ, is better induced in maleyl-protein-immunized birds than in those immunized with native protein. Therefore, spleen cells from maleyl-BSA- or BSA-immunized Cornell K Strain Leghorns were compared for antigen-induced levels of IFNγ mRNA. Triplicate cell cultures from BSA-immunized birds showed a far lower (eightfold) induction of IFNγ mRNA than cells from maleyl-BSA-immunized birds (Figure 5A). These levels are compared with a constitutively expressed internal control, β-actin, which is produced in equivalent amounts regardless of treatment. The mRNA of concanavalin A-activated cells was used as induced positive controls (Figure 5B), and unstimulated cells were the uninduced controls. The BSA and uninduced levels, when normalized by the β-actin signal, are comparable, and the maleyl-immunized level is about fourfold higher than the concanavalin A-induced control.

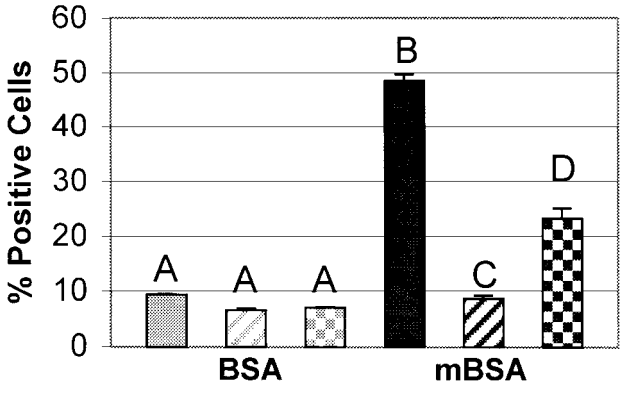

FIGURE 2. Blocking the binding of maleyl-BSA (mBSA) to MQ-NCSU macrophage cells by scavenger receptor (SR) ligands, fucoidin and poly-guanylic acid (poly-G). Cells from the chicken macrophage cell line MQ-NCSU were preincubated with fucoidin (diagonal lines) or poly-G (hatched) and incubated with BSA-biotin (solid light bars) or maleyl-BSA-biotin (solid dark bars) and streptavidin Red670. The percentage of fluorescing cells was determined by analysis with Cell Quest. Data were expressed as the mean percentage ± SEM of fluorescence-positive cells. Different letters denote significant ($P \leq 0.01$) differences between treatments.

In Vivo Study 2

Enhancement of Antigen-Specific DTH by Maleyl-Protein Immunization. For broiler breeders, the peak wattle response was at 24 h. The PBS-immunized control birds had no significant (0.01 ± 0.08 mm) wattle swelling. A strong DTH response was observed in the maleyl-BSA-immunized chickens compared with those immunized with BSA (Figure 6). Thus, the DTH response is enhanced by immunogen maleylation. In maleyl-BSA-immunized chickens, DTH can be evoked by challenge with native BSA or maleyl-BSA (Figure 6).

Modulation of Antigen-Specific Antibody Responses by Maleyl-Protein Immunization. Prior to immunization, background levels of antigen-specific Ig anti-BSA and anti-maleyl-BSA were determined to be negligible. The IgM titer for the BSA-treated birds was 1:1,024 and for the maleyl-BSA treated birds was 1:32 (Figure 7A). The IgG titer for BSA-treated birds was 1:1,024, whereas the maleyl-BSA-treated bird titer was 1:16 (Figure 7B). Preliminary titrations of the pooled anti-BSA sera showed that 1:300 was within the linear range for detection for the IgM (Figure 7A) and IgG (Figure 7B), and the titrations were used for analysis of individual sera. Anti-BSA IgM antibody levels were highest at Day 7 postimmunization in BSA-immunized birds (Figure 7C). These antibodies cross-reacted poorly with maleyl-BSA (Figure 7D). Maleyl-BSA-immunized birds showed low levels (or levels equal to the background) of IgM antibodies against native or maleyl-BSA (Figures 7C and 7D). Similarly, anti-BSA IgG antibody levels were induced well in BSA-immunized birds and poorly in maleyl-BSA-immunized birds (Figure 7E). The anti-BSA IgG antibodies in BSA-immune birds did not recognize maleyl-BSA well, and anti-maleyl-BSA IgG levels were also low in maleyl-BSA-immunized birds

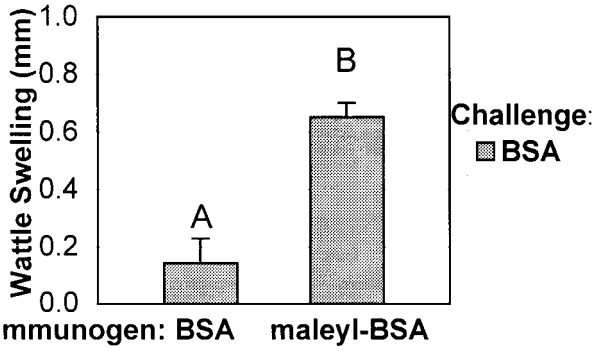

FIGURE 3. Immunization with maleyl-BSA induced a strong delayed type hypersensitivity (DTH) reaction in Cornell K Strain Leghorns. THe DTH responses (mean ± SEM) were measured as an increase in wattle thickness (millimeters) over the normal wattle size 24 h postantigen injection into sensitized individuals. Wattle swelling is shown for Cornell K Strain Leghorn chickens injected s.c. with 2.5 mg (0.5 mL) BSA or maleyl-BSA (five birds per group). The wattle challenge was 22 d after the initial injection. One wattle was injected with 100 μL (0.5 mg) of the challenge antigen, BSA, whereas the other was injected with 100 μL of PBS as the control.

(Figure 7F). Thus, even in outbred chickens, BSA immunization generates better antibody responses than immunization with maleyl-BSA.

Maleyl-Protein Immunization Enhances Production of the T Cell Cytokine, IFNγ. The IFNγ signal from BSA-immunized broiler breeders declined sharply at total mRNA template of less than 3 ng to barely detectable. In the maleyl-BSA-immunized birds, the IFNγ signal was detectable even at 0.1 ng of total RNA (Figure 8). Thus, between the two treatments, at least a 30-fold difference was evident and confirmed the significant difference, in broiler breeders, between IFNγ induced by the two modes of immunization. This finding is confirmed by dilutions of the mRNA for the BSA and maleyl-BSA-immunized birds (Figure 8). The β-actin bands were expressed at equal levels (±2%).

DISCUSSION

CD4 T cells can help generate inflammatory, macrophage-activating Th1 responses or antibody-dominated Th2 responses. This dichotomy is critical to successful resistance against infections. Therefore, the regulation of such differential commitment in chickens has been investigated. Based on previous mammalian experience (Pfeiffer et al., 1995), one way to do this would be to breed or otherwise identify a genetic difference between lines of birds differing in the generation of a Th1 or a Th2 response to an infectious agent. Although such an approach would provide a model system for dissection of Th1/Th2 commitment in genetically defined lines of chickens, it would not provide a way of controlling this commitment in outbred chickens. Our protocols modified the immunogen, rather than the birds, to control the immune choice. In addition to MHC-matched chickens, broilers were examined, so that the data may be directly applicable to real-life situations in the poultry industry.

In both types of chickens examined, immunization with maleyl-BSA resulted in a more Th1-dominant response than immunization with BSA. These findings suggest that Th1/Th2 balances can be modulated in avian immune responses, even in outbred birds, by using immunogen maleylation. The ability of exogenously administered soluble protein antigens to generate an immune response in vivo is usually exceedingly limited. Most soluble antigens must be administered with adjuvants to function as efficient immunogens. Here, the ability of APC to take up antigen efficiently and to present it to avian T cells in an effectively stimulatory fashion has been enhanced. The data demonstrate that maleylation of BSA allows high levels of uniform binding to the chicken macrophage cell line, MQ-NCSU (Figure 1), and that this binding is mediated through surface receptors having the same binding specificities as mammalian SR (Figure 2). This result is evident in the inhibition of maleyl-BSA binding by fucoidin and polyG, two structurally unrelated SR-binding competitor ligands (Majumdar and Basu, 1991; Mukhopadhyay et al., 1992). The

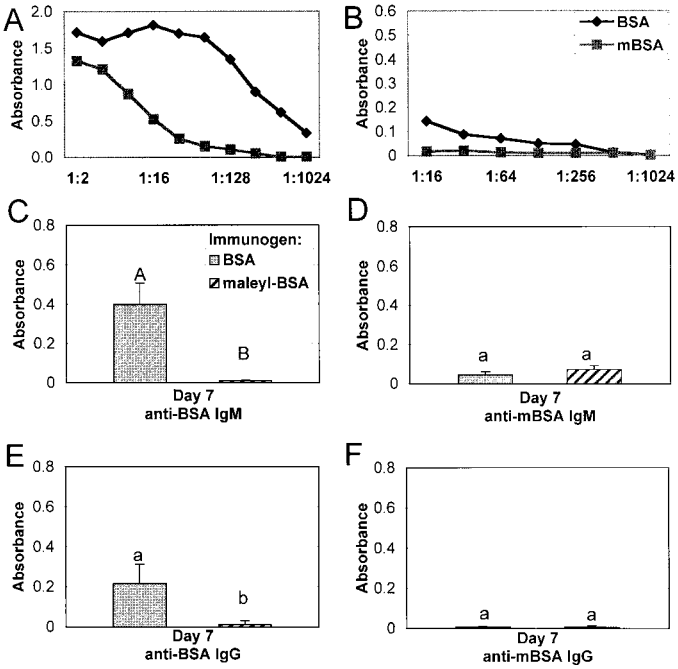

FIGURE 4. Plasma IgM and IgG antibody levels were higher in BSA-immunized chickens than maleyl-BSA-immunized chickens. Titrations of IgM antibody levels at Day 7 after immunization are shown for Cornell K Strain Leghorns treated with BSA (1:1,024) and maleyl-BSA (1:32) (Panel A). Titrations of IgG antibody levels are shown for Cornell K Strain Leghorns treated with BSA (1:256) and maleyl-BSA (mBSA) (<1:16) (Panel B). Specific plasma IgM and IgG antibody levels were higher in BSA-immunized birds than in maleyl-BSA-immunized Cornell K Strain Leghorns. Antibody levels from Cornell K Strain Leghorns at Day 7 after immunization were determined by absorbance values (mean ± SEM) with ELISA at plasma dilutions predetermined to be within the linear range of the titration curves (1:300) for the BSA-immunized birds. Panel C: IgM assayed on BSA-coated plates. Panel D: IgM assayed on maleyl-BSA-coated plates. Panel E: IgG assayed on BSA-coated plates. Panel F: IgG assayed on maleyl-BSA-coated plates. Different letters denote significant differences between groups. Lower case letters indicate statistical differences at $P \leq 0.05$, and upper case letters indicate $P \leq 0.01$.

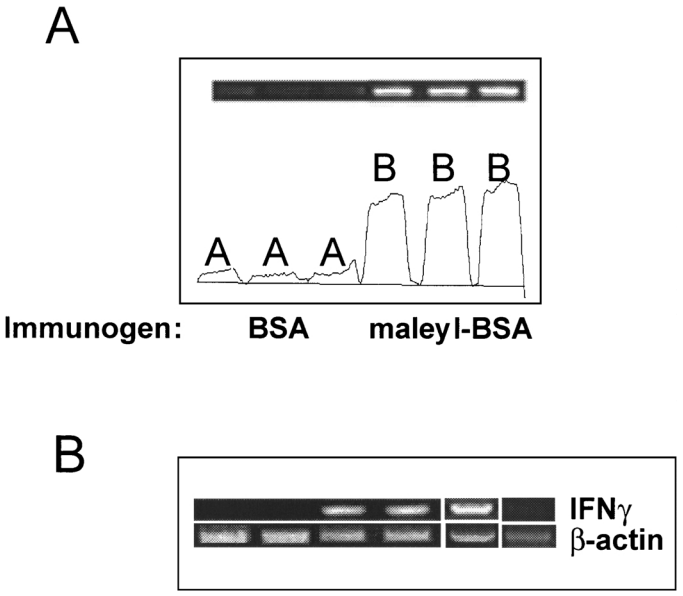

FIGURE 5. Interferon-γ (IFNγ) production was greater in maleyl-BSA-immunized Cornell K Strain Leghorns than in BSA-immunized Leghorns. Panel A. Signals from splenocyte RNA activated by BSA or maleyl-BSA (1,000 μg/mL) after a 48-h incubation are shown. RNA was harvested and IFNγ mRNA was detected as described in the Materials and Methods section. The relative signal intensities were A = 738 ± 85; B = 5,720 ± 221 ($P < 0.01$). Panel B. The IFNγ production is compared with the internal control β-actin. Lanes 1 and 2 contain signals from BSA-immunized chickens, and Lanes 3 and 4 contain signals from maleyl-BSA. The bottom row is the β-actin band. Lanes 5 and 6 are signals from concanavalin A stimulated and vehicle control cells, respectively.

work with cell lines has been confirmed by staining on fresh ex vivo cells with similar results (data not shown). Scavenger receptors are constitutively present on mature mammalian macrophages and to a lesser extent on some endothelial cells (Resnick et al., 1994). Importantly, they are not down-modulated by ligand; they mediate very efficient and rapid internalization of bound ligands followed by degradation in the endo-lysosomal compartments (Zhang et al., 1993), and they repeatedly recycle to the cell surface (Goldstein et al., 1979). Although SR are conserved across many species, some differences are observed between oxidized and acetylated protein handling in avian versus mammalian macrophages (Yancey and Jerome, 1998). Thus, it was possible that the immuno-enhancing properties conferred by SR-mediated antigen delivery in mammalian species might not be found in birds. However, the following data show that SR-mediated immunogen delivery is indeed immunogenic in avian species.

The shift in the immune response toward the Th1 pathway is as clearly and easily induced in avian systems as in mice by maleylated protein because, in vivo, DTH responses are enhanced (Figure 3 and 6) and antibody responses are diminished (Figures 4 and 7) by immunization with maleyl-BSA as compared with native BSA. Thus, maleylation of this protein antigen is qualitatively altering the resultant immune response toward the Th1 pathway. The enhanced induction of DTH with maleyl-BSA immunization in chickens (Figures 3 and 6) correlates well with the mRNA induction for IFNγ (Figure 5 and 8) and with the induction of IFNγ in the mouse system as well (Singh et al., 1998). Thus, in chickens, the same peptide, depending on its peptide-MHC complex density, may trigger a Th1 or a Th2 response, as in mammals (Pfeiffer et al., 1995). The efficiency of peptide generation in chickens, therefore, is of interest in terms of the differences between the postuptake handling of some SR ligands in avian versus murine macrophages (Yancey and Jerome, 1998).

The differences between the antibody levels induced by native versus maleyl-protein immunization are remarkable (Figures 4 and 7). Background anti-BSA levels both by DTH and antibody responses in Cornell K Strain birds were negligible, indicating that primary responses were being studied. Slightly higher backgrounds in antibody levels but not DTH were observed in broiler breeders. In both cases, after immunization, Th1 responses were dominant in maleyl-BSA-immunized birds. The maleyl-BSA antibody responses can switch rapidly to IgG (Figure 7F), suggesting some T cell help. However, the magnitude of the antibody responses is very low in maleyl-BSA-immunized birds. Serological cross-reactivity between BSA and maleyl-BSA is poor, unlike the excellent cross-reactivity observed at the T cell level (Figure 3, 5, 6, and 8), and maleyl-BSA immunization generates poor antibody responses as compared with BSA immunization, in sharp contrast to the data on DTH generation (Figure 3 and 6). Although the Th2 cytokines such as IL-4 or IL-5 are highly efficient at providing help to B cells for making antibodies (particularly IgG_1 and IgE), the Th1 cytokines such as IFNγ can also help B cells in mammalian systems (Collins and Dunnick, 1993), particularly high levels of IgG_{2a} to promote opsonization. Overall, it is noteworthy that the Th1 response in chickens is extremely poor at providing B cell help. This poor response may indicate interesting evolutionary dif-

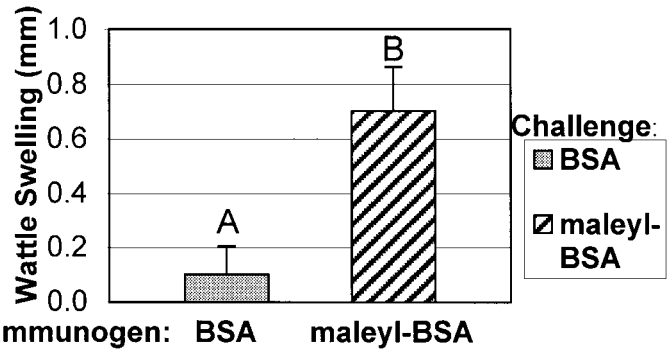

FIGURE 6. Immunization with maleyl-BSA induced a strong delayed type hypersensitivity (DTH) reaction in broiler breeders. The DTH responses (mean ± SEM) were measured as increases in wattle thickness (millimeters) over the normal wattle size 24 h postantigen injection into sensitized individuals. Wattle swelling is shown for broiler breeders injected s.c. with 1.4 mg (0.2 mL) of BSA or maleyl-BSA (seven birds per group). The wattle challenge was 21 d after the initial injection. One wattle was injected with 100 μL (1.0 mg) of priming antigen, whereas the other was injected with 100 μL of PBS as the control. Different letters denote significant ($P \leq 0.01$) differences between groups.

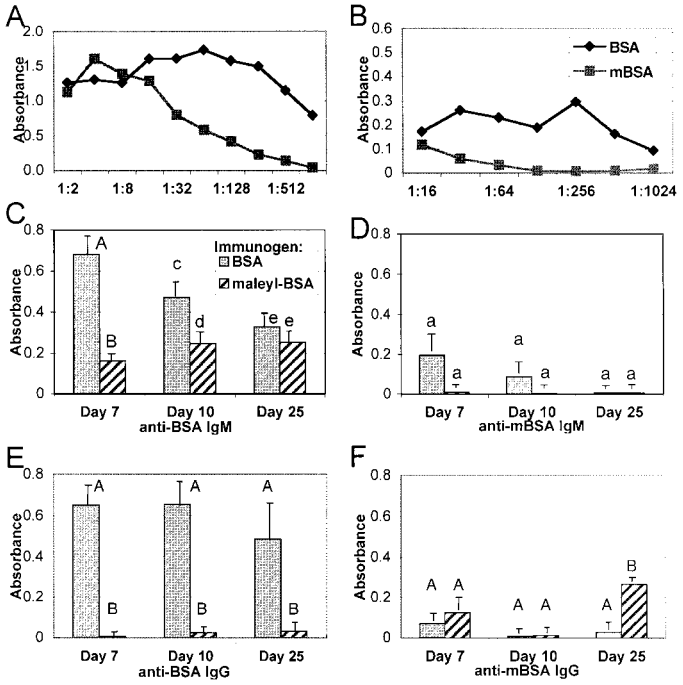

FIGURE 7. Plasma IgM and IgG antibody levels were higher in BSA-immunized chickens than in maleyl-BSA-immunized chickens. Titrations of IgM antibody levels at Day 7 after immunization are shown for broiler breeders treated with BSA (1:1,024) and maleyl-BSA (1:32) (Panel A). Titrations of IgG antibody levels are shown for broiler breeders treated with BSA (1:1,024) and maleyl-BSA (1:16) (Panel B). The BSA-specific plasma IgM and IgG antibody levels were higher in broiler breeders immunized with BSA than in those immunized with maleyl-BSA. Antibody levels from individual broiler breeders at Days 7, 10, and 25 after immunization were determined by absorbance values (mean ± SEM) with ELISA at plasma dilutions predetermined to be within the linear range of the titration curves (1:300) for BSA immunization on BSA plates. Panel C: The IgM assayed on BSA-coated plates. Panel D: IgM assayed on maleyl-BSA (mBSA)-coated plates. Panel E: IgG assayed on BSA-coated plates. Panel F: IgG assayed on maleyl-BSA coated plates. Different letters denote significant differences between groups. Lower case letters indicate $P \leq 0.05$, and upper case letters indicate $P \leq 0.01$.

ferences between regulatory mechanisms in avian and mammalian immune responses. Further, SR in mammals can transduce signals activating macrophages (Johnson et al., 1982; Palkama, 1991), and similar events occurring in avian cells may be possible, thereby modifying the costimulatory functions of chicken APC. In the experiments reported here, maleyl-BSA consistently resulted in Th1-dominated responses, regardless of the initial level of responsiveness.

In the mouse system, maleylation changes the native protein antigenically in addition to making it an SR ligand. At the B cell level, a major new epitope involving maleyl-lysine is introduced so that maleyl groups can be thought of as haptens that may mask some native epitopes recognized by B cells (Abraham et al., 1995). If many lysines were present on a given protein, the result could be a polyvalent T-independent B cell antigen. The preparations used here show over 80% conversion from lysine to maleyl-lysine. However, the data show that low levels of anti-maleyl-BSA antibodies generated by maleyl-BSA immunization (Figure 4 and 7) change to IgG (Figure 7E), suggesting that the antibody response is T cell dependent. The maleyl group on our maleyl-BSA is recognized by anti-maleyl-OVA mouse sera. However, the anti-BSA antibody response clearly does not recognize maleyl-BSA well (Figures 4 and 7), showing that native and maleyl-proteins do not cross-react at the B cell level in the avian immune system. This lack of cross-reactivity might be partly caused by maleyl groups masking native epitopes by virtue of charge, because maleylation confers a net charge of –2 for each residue modified. In contrast, maleylation does not seem to generate T cell epitopes that are dramatically different from those generated from the native protein. Birds immunized with maleyl-BSA mount efficient DTH responses to native BSA, indicating substantial cross-reactivity between the two forms of BSA at the T cell level.

These studies strongly suggest that, similar to mammalian species, a Th1/Th2 dichotomy exists within the T helper cell compartment of birds. Moreover, as in mammals, the Th1/Th2 balance can also be modulated in chickens toward a Th1-dominated response by modifying the immunogen (i.e., maleyl-BSA) and still be protective against native antigen (i.e., BSA). Considering that maleylation of commercially important antigens may lead to similar results, this approach, delivery of antigen specifically to macrophages via SR, will find direct application in the development of vaccines where a Th1-dominated response would be more protective than a Th2-dominated response. Having identified a system allowing us to modify Th1/Th2 balance with the immunogen, we will not only be able to investigate the physiological processes involved but also use these data to provide concrete suggestions for modulation of vaccine design. Our results, as well as mammalian investigations of the differential regulation of Th1 and Th2 responses, support the concept that the delivery of antigen specifically to APC via the SR in the body is likely to trigger a Th1 response. Investigations have been initiated to examine the effectiveness of the SR-directed im-

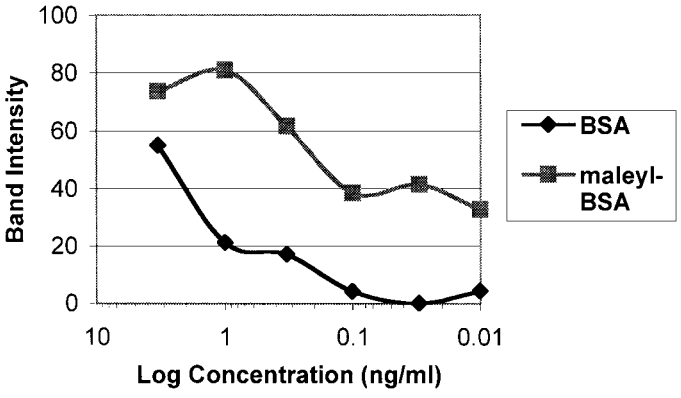

FIGURE 8. Interferon-γ (IFNγ) production was greater in maleyl-BSA-immunized broiler breeders than in BSA-immunized broiler breeders. The graph illustrates reverse-transcriptase-polymerase chain reaction IFNγ signals generated from testing total RNA from splenocytes activated by BSA or maleyl-BSA as in Figure 6. RNA was harvested and IFNγ mRNA was detected as described in the Materials and Methods section. The RNA template concentrations (ng/mL) were titrated as shown.

munogen delivery system in vivo toward altering the Th1/Th2 commitment using antigens relevant to protection against avian diseases.

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