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Production, Characterization and Use of Monoclonal Antibodies Recognizing IgY Epitopes Shared by Chicken, Turkey, Pheasant, Peafowl and Sparrow

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Summary

Chicken antibodies are not only a part of immune defense but are more and more popular commercial products in form of chicken polyclonal, monoclonal or recombinant antibodies. We produced and characterized mouse monoclonal antibodies (mAbs) that recognize epitopes located on heavy or light chain of chicken immunoglobulin Y (chIgY) shared also by some other *Phasianidae* birds. The use of mAbs 1F5 and 2F10 that recognize heavy chain on chIgY common epitopes was demonstrated on immunoglobulins of turkey, pheasant and peafowl. Chicken IgY light chain specific mAb 3E10 revealed the presence of common epitopes on immunoglobulins of turkey, pheasant and sparrow. Monoclonal antibody clone 1F5/3G2 was used to prepare horseradish peroxidase (HRP) conjugate and immunoadsorbent column. Conjugated mAbs were demonstrated to be excellent secondary antibodies for diagnostics of certain infections in different avian species. Since they do not react with mammalian immunoglobulins using our mAbs as secondary antibodies in human serodiagnostics would minimize background staining that appears when using mouse detection system. In dot immunobinding assay (DIBA) and immunoblot assay they recognized specific IgY antibodies against *Mycoplasma synoviae*, *Mycoplasma gallisepticum* and *Newcastle disease virus* in sera of infected or vaccinated birds. Immunoadsorption as a method for removal of IgY from samples in which *Mycoplasma synoviae* specific IgY was predominant immunoglobulin class enabled more exact demonstration of specific IgA and IgM antibodies.

Herein we are presenting effective mAbs useful in diagnostics of avian and mammalian infections as well as in final steps of detection and purification of chicken antibodies and their subunits produced *in vivo* or *in vitro* as polyclonal, monoclonal or recombinant antibodies.

Key words: monoclonal antibodies, IgY, avian, *Phasianidae*

Introduction

Avian IgY is a counterpart to mammalian IgG molecule and is considered as evolutionary ancestor of mammalian IgG and IgE (*1*). Some classifications of avian immunoglobulin molecules distinguish serum antibodies also called avian IgG, IgM and IgA (*2*) and antibodies transferred into the egg yolk, therefore are named IgY (*3*,*4*). In spite of some profound differences in structure leading to different terminology (*5*), the IgY molecule has, in general, the same physiological function in birds as IgG in mammals (*1*).

Of all avian immunoglobulins chIgY was most frequently studied, best characterized and also the most interesting one for application in medicine and biotechnol-

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ogy. IgY molecule is composed of 2 heavy chains (HC) with molecular mass of 67–70 kDa each and 2 light chains (LC) with molecular mass of 25 kDa each. Compared to mammalian IgG, chIgY has one additional constant region (Cυ2) in HC (Cυ1–Cυ4) leading to higher molecular mass, which is about 180 kDa (*1*).

There is limited number of data about the structure of IgY molecules in other avian species. Beside IgY, a truncated form IgY (ΔFc) with molecular weight of 120 kDa was found in ducks and geese, some reptiles and lungfish. IgY (ΔFc) lacks two C-terminal domains (Cυ3 and Cυ4) in H chain, is structurally close to F $(ab')_2$ fragment of IgG and is not transferred to egg yolk (*1*).

Despite the advances in the diagnosis and treatment of infectious diseases, pathogenic microorganisms remain the most important threat to poultry production. Diagnosis usually includes serologic tests that detect pathogen specific antibodies. At present, enzyme immunoassays (different ELISA kits) are the most widely used in poultry pathogen diagnostics. They all include secondary antibodies that recognize chIgY and can be enzyme-conjugated. Secondary anti-IgY antibodies are polyclonal rabbit or goat antibodies raised against chicken (*6–9*) or turkey IgY (*10,11*). In certain studies ELISA with mAbs to chicken immunoglobulins was utilized but its use remained limited to research work (*12–14*).

Due to their specific characteristics different from mammalian IgG (*15*) chIgY antibodies are getting more and more important also as research and diagnostic, and even therapeutic tool (*16*). They can be produced either as polyclonal antibodies, monoclonal or recombinant antibodies. For further use they have to be isolated from serum, egg yolk or culture supernatant. Many methods have been described so far (*17,18*), from standard methods using precipitation to remove lipids from yolk first and then precipitation of protein fraction including IgY, to more sophisticated methods as preparative electrophoresis (*19*), ligand (*20*) or antigen specific immunoaffinity chromatography (*21*) and many other variants of chromatography.

We report herein about the production and characterization of monoclonal antibodies reacting with different epitopes on IgY molecule of five avian species and demonstrate their potential use.

Methods

Chicken IgY

Chicken IgY (chIgY) was isolated from egg yolk using Gamma Yolk kit (Pharmacia) or with simple extraction with chloroform (*22*). As a source of chicken serum samples of IgY sera were used (diluted 1:20 with PBS). For detection of cross reactions sera from turkey, peafowl, pheasant, parrot, sparrow, pigeon and egg yolk (diluted 1:50 with PBS) from duck, goose and quail were used. In addition, samples of sera from human, rabbit, mouse, pig, horse and cow were used as a source of non-avian IgG.

Chicken IgY light and heavy chains were separated by reducing IgY with 2 % β-mercaptoethanol before applying to electrophoresis gel.

Production of mouse monoclonal antibodies (mAbs)

Monoclonal antibodies were produced according to standard method (*23*) with slight modifications routinely used in our lab. Eight-week-old Balb/c mouse was immunized four times in 3-week intervals, each time with 80 μg of isolated chIgY. Antigen was applied intraperitoneally three times, and the last time intravenously. First immunization was carried out with a mixture of chIgY and complete Freund's adjuvant (Sigma) (volume ratio 1:1); second and third immunization mixtures were made of chIgY and incomplete Freund's adjuvant (Sigma) (1:1). In the last immunization only chIgY was applied three days before the fusion. Fusion between spleen cells and NS-0 myeloma cell line was done using 50 % PEG (Sigma) in DMEM (Sigma). After screening hybridomas for antibodies to IgY, selected hybridomas were cloned twice using the method of limiting dilution. Selected clones were finally cultured in serum-free medium (HyClone) and culture supernatants were collected. Fifty mL of culture supernatants were applied on 10 mL (1 column volume) of HA ultrogel (Biosepra, USA or Sigma) column and washed first with 3 column volumes of washing buffer (10 mM NaH_2PO_4, pH=6.8) and then with 3 column volumes of 50, 100, 150, 200, 250 and 300 mM NaH_2PO_4, respectively (pH=6.8). Ten-mL fractions were collected and tested for protein presence by measuring absorption at 280 nm and for antibody activity using dot immunobinding assay. Active fractions were collected and concentrated with ultrafiltration using Centrisart®-C30 (Sartorius) ultrafiltration tubes. Monoclonal antibodies were stored at 4 °C until use.

Dot immunobinding assay (DIBA)

One-step and two-step DIBA were used for detection of specific mAb – antigen reactions according to methods described elsewhere (*24*). Briefly, for the detection of IgY from egg yolk and IgY in fractions, eluted from immunoaffinity column containing mAb to IgY, one-step method was used. Chicken IgY samples were applied to NTC membrane strips with drawn squares (2 μL per 5 mm x 5 mm square). After blocking in 0.5 % Tween 20/PBS for 30 min strips were incubated for 30 min in peroxidase conjugated rabbit anti-chIgY antibodies (Sigma, A-9046). After washing in PBS substrate True Blue (Kirkegaard & Perry Lab.) was applied. When a distinct blue spot appeared at the position of positive control, the reaction was stopped by rinsing strips in distilled water and the results were evaluated.

Two-step DIBA was used for checking the mouse immune response to IgY (mouse IgG anti-chIgY in mouse immune serum) for screening of hybridomas for mAbs or for detection of mAbs in fractions, eluted from HA ultrogel. The same assay was used for checking the mAb specific, nonspecific and cross reactions. Chicken IgY and/or sera or egg yolk samples of tested birds (2 μL per 25 mm^2) and mammals were applied to the membranes (Figs. 1 and 2). After blocking, membrane strips were incubated in particular sample containing mouse IgG to IgY (immune serum, culture supernatant or HA ultrogel fractions) for 30 min, washed and then incubated in secondary antibodies *i.e.* peroxidase conjugated

goat anti-mouse IgG (Sigma, A-5278). Final steps were the same as described above.

For the analysis of cross-reactions of mAb M1 that recognize chicken IgM (obtained from J. Davison, Institute for Animal Health, Compton, UK) with avian IgM molecules, two-step DIBA was performed. Chicken and other avian sera or yolk were applied to membrane strips and incubated in mAb M1, established by Mocket in 1986 (*25*). After washing and incubation in secondary anti-mouse peroxidase conjugated antibodies, substrate was added and reaction was finished as described above.

To determine optimal and extreme working conditions for 1F5/3G2 mAb some parameters in DIBA were changed. To determine optimal and extreme pH conditions, strips with applied chIgY were incubated in 1F5/3G2 mAb diluted in PBS with different pH values, from 3 to 12, increasing each time by 0.5. To define optimal and minimal incubation time in DIBA, individual strips were incubated in 1F5/3G2 mAb from 5 to 45 min (increasing time interval was 5 min).

To test a carbohydrate nature of epitopes on chIgY, strips with the applied chIgY were incubated in 10 mM periodic acid in 50 mM Na-acetate, pH=4.5 for 1 h before incubation in mAbs 1F5/3G2 mAb, 4E4/E5, 3C10/F2 and 2F10/D2.

For isotyping of mAbs immunoenzyme assay using mouse mAb, isotyping reagent ISO-2 (Sigma) was used according to manufacturer's instructions.

SDS-PAGE and immunoblotting

To determine mAb binding sites on chIgY molecule, SDS-PAGE followed by Western blotting was carried out according to the previously described procedures (*26,27*). Chicken IgY samples were treated with 2 % β-mercaptoethanol to release light chains (LC) from heavy chains (HC) and applied to the gel. After the proteins were transferred to the membrane, the membrane was cut into strips. Each strip was then incubated in appropriate mAb solution and immunoenzyme reactions were carried out as described for DIBA.

Conjugation of horseradish peroxidase to 1F5/3G2 mAb and use of conjugate for detection of infections in chickens

Monoclonal antibody 1F5/3G2 was conjugated to horseradish peroxidase (HRP) using one-step glutaraldehyde method (*28*). Briefly, 2 mg of antibody 1F5/3G2 diluted in 0.1 M Na_2CO_3, pH=9.2 (in concentration of 10 mg/mL) were incubated for 24 h with 4 mg of enzyme (Sigma P 8125; previously diluted in 160 μL glutaraldehyde, incubated over night and dialyzed against 0.1 M Na_2CO_3, pH=9.2). After blocking the remaining reactive groups with 0.2 M lysine, HRP conjugated antibodies 1F5/3G2 were dialyzed against PBS.

Indirect immunoenzyme assays (IIPA) were used to determine whether mAbs can detect specific antibodies in chicken sera. IIPA using native colonies in agar blocks for detecting antibodies (IgY, IgA and IgM) to *Mycoplasma synoviae* or *Mycoplasma gallisepticum* was performed as described elsewhere (*29*). Briefly, agar blocks with native colonies of *Mycoplasma synoviae* or *Mycoplasma gallisepticum* were incubated in the tested samples (sera, tracheal washings, amniotic and allantoic fluids) for 45 min and washed with PBS. In one-step method for detecting IgY, HRP-conjugated 1F5/3G2 mAbs were used, while in two-step method mAbs to IgY (40 min incubation) were used first, followed by incubation with HRP-conjugated goat anti-mouse IgG antibodies. When chIgA and chIgM were examined, mAb A62 that recognizes HC on chicken IgA (*30*) and mAbs M1 (*25*) were used followed by the incubation in conjugate. After washing in PBS, the blocks were drained and treated with substrate containing DAB (Sigma D 5905). The reactions were examined by microscope and scored.

Additionally, HRP-conjugated 1F5/3G2 mAbs were used in immunoblots to determine which proteins of *Mycoplasma synoviae, Mycoplasma gallisepticum* and *Newcastle disease virus* were recognized by chIgY antibodies. SDS-PAGE, Western blotting and immunoenzyme reactions on membranes were conducted as described before (*29*). The reactions obtained with HRP-conjugated 1F5/3G2 mAb were compared with those obtained with commercial HRP-conjugated rabbit IgG to chIgY (Sigma P9046), both used in the same dilution (1:1000).

Immunoadsorption of IgY

Monoclonal antibody clones 1F5/3G2 were coupled to CNBr Sepharose 4B as previously reported (*16*) and used as an immunoadsorbent to remove IgY from the samples of chicken serum and allantoic or amniotic fluid of chicken embryos. Undiluted or diluted (serum, 1:10) samples were mixed within the appropriate volume of CNBr sepharose coupled to 1F5/3G2 mAb. The reaction took place at room temperature for 1 h. After centrifugation the supernatants were collected and assayed for total IgY, as well as for *Mycoplasma* specific IgY, IgA and IgM antibodies by the IIPA.

Results

Monoclonal antibodies

Ten days after the fusion, cell culture supernatants from 53 wells containing hybridomas were tested for the presence of specific anti-chIgY antibodies using indirect DIBA. Fifteen of them reacted with IgY isolated from chicken egg yolk as well as with chicken serum. In addition, some of them reacted also with serum samples from turkey and pheasant, while none of them reacted with mammal sera included in the test. Hybridomas from four wells were selected and sub-cloned to obtain mAbs specific for chIgY and/or mAbs that would cross-react also with serum proteins, presumably IgY, of other avian species. After cloning, individual clones produced mAbs that had the same pattern of reactivity as antibodies produced by their parental hybridomas. Clones 4E4/G5, 4E4/G7 and 4E4/G11 secreted mAbs that reacted with chIgY only (Fig. 1, line A). Clones 3C10/F2, 3C10/F6 and 3C10/F11 produced mAbs that gave strong reactions with chIgY and pheasant serum, whereas reactions with turkey and sparrow sera were weaker (Fig. 1, line B). Clones 2F10/D2, 2F10/D10 and 2F10/E9 secreted mAbs that reacted strongly with chIgY and peafowl serum, whereas moderate reactions were observed with turkey and pheasant sera (Fig. 1, line D). Clone 1F5/3G2

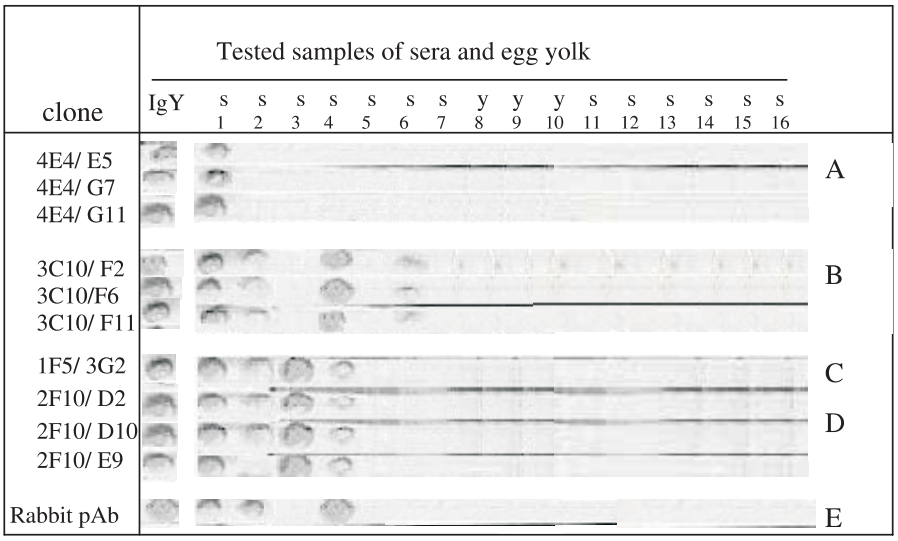

Fig. 1. Reactions of mAbs with chIgY, isolated from chicken egg yolk (IgY) and with avian sera (s, 1–7) or egg yolk (y, 8–10) and with sera of some mammals (s, 11–16). Samples are marked as follows: 1 chicken, 2 turkey, 3 peafowl, 4 pheasant, 5 parrot, 6 sparrow, 7 pigeon, 8 duck, 9 goose, 10 quail, 11 rabbit, 12 pig, 13 cattle, 14 horse, 15 mouse, 16 human. Line A: clones grown from 4E4 well; line B: clones grown from 3C10 well; line C: clone grown from 1F5 well; line D: clones grown from 2F10 well; line E: reaction of the same sera and yolk samples with commercial polyclonal HRP-conjugated rabbit anti-chIgY antibodies

derived from 1F5 maternal hybridoma produced mAbs which reacted similarly to mAbs of clones that arose from 2F10 hybridoma (Fig. 1, line C).

Mouse mAb isotyping kit showed that all the produced mAbs belong to the IgG1 isotype.

Monoclonal antibody M1 specific for chicken IgM was used in DIBA to obtain more data about common epitopes on IgM of different avian species. As expected, mAb M1 reacted with serum samples of chicken, turkey, pheasant and peafowl, as well as with the egg yolk sample of a duck. Moreover, mAb M1 revealed relatively strong reactions with sparrow serum and egg yolk of Japanese quail, whereas it did not react with samples from goose, pigeon or parrot (Fig. 2).

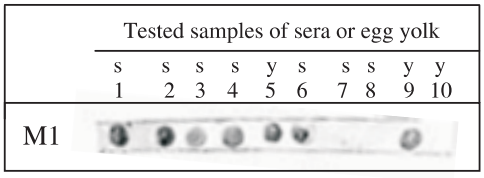

Fig. 2. Reactions of mAb M1 to HC of chicken IgM in DIBA with avian sera (s) or egg yolks (y). Samples are marked as follows: 1 chicken, 2 turkey, 3 peafowl, 4 pheasant, 5 Japanese quail, 6 sparrow, 7 pigeon, 8 parrot, 9 duck, 10 goose

IgY epitope mapping

Immunoblot analyses of denatured and reduced forms of chIgY were used to determine the location of epitopes for produced mAbs. As shown in Fig. 3 (panel B, lanes 1 and 2), mAbs 1F5/3G2 and 2F10/D10 reacted with HC of about 67 kDa. Similar molecular weight proteins were recognized by these two mAbs also in the serum samples of pheasant and turkey, whereas protein recognized in the serum of peafowl was a few kDa smaller (data not shown in Fig. 3). Monoclonal antibody clone 3C10/F6 reacted with a similar-size protein as a reference commercial mAb to chicken LC (Fig. 3, panel B, lanes 3 and 4). In addition, mAb 3E10 recognized a protein of similar size, presumably LC, also in pheasant serum (not shown in Fig. 3). Monoclonal antibody clone 4E4/G11 reacted weakly with proteins of about 67 and 20 kDa (not presented in Fig. 3).

Treatment of chIgY with periodic acid did not have visible effect on the reactivity of mAbs with IgY. This may suggest that carbohydrate moieties present on LC and HC were not essential components of epitope for mAb recognition.

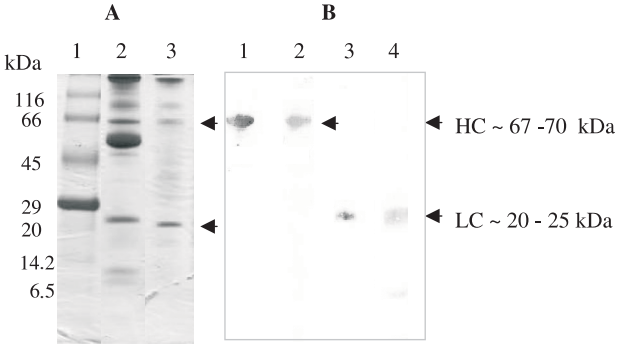

Fig. 3. Mapping of IgY epitopes for mAbs using reducing conditions and fragmentation of IgY in HC and LC. Panel A: SDS-PAGE after Coomassie Blue staining of proteins; lane 1, molecular markers (on the left side are molecular weights in kDa); lane 2, chicken serum; lane 3, chIgY from egg yolk. Panel B: immunoenzyme reaction on membrane after transfer of fragmented IgY from egg yolk; lane 1, 1F5/3G2 mAb; lane 2, 2F10/D10 mAb; lane 3, 3C10/F6; lane 4, commercially available reference antibody reacting with chicken LC (CH 31, Sigma). Arrows on the right indicate reactions at the positions of heavy chain (HC, 67–70 kDa) and light chain (LC, 20–25 kDa)

Conditions for optimal binding of mAbs

All mAbs showed relatively high affinity of binding onto native as well as onto denatured and reduced IgY molecules in the immunoblots. They remained bound to IgY in rather harsh washing conditions *i.e.* repeated washings with buffers containing 0.5 % Tween 20. Monoclonal antibody clone 1F5/3G2 showed particularly high affinity for chIgY. The extreme pH values where mAbs 1F5/3G2 were stable and still reacted with IgY were pH=3 and pH=10.5, while pH optimum was shown to be from 6.5 to 7. In DIBA performed at room temperature a visible reaction was detected after 15 min of incubation but the intensity of signal visibly increased up to 30 min of incubation. This mAb was also conjugated to HRP and tested in different immunoenzyme assays that detect chIgY. Conjugate retained specificity, affinity and binding at pH optimum.

Use of mAbs in serology and research

Produced mAbs can be used in research and in diagnostic assays that detect specific IgY antibodies to poultry pathogens. We used mAb 1F5/3G2 and its HRP conjugate in a number of studies of chicken antibody response against pathogenic *Mycoplasma* species. As shown in Fig. 4, the use of HRP-conjugated 1F5/3G2 mAb as secondary antibody enabled a demonstration which shows that locally synthesized chIgY antibodies recognize variably expressed antigens on the surface of *Mycoplasma gallisepticum* colonies. Variable expression of certain immunodominant proteins, like haemagglutinin of *Mycoplasma gallisepticum*, named pMGA, results in sectorial staining of colonies *i.e.* staining of parts where particular protein is expressed (Fig. 4, arrow 2) and no-staining (Fig. 4, arrow 1) of parts where this protein is not expressed or in stronger (Fig. 4, arrow 3) or weaker (Fig. 4, arrow 2) immuno-staining of one colony.

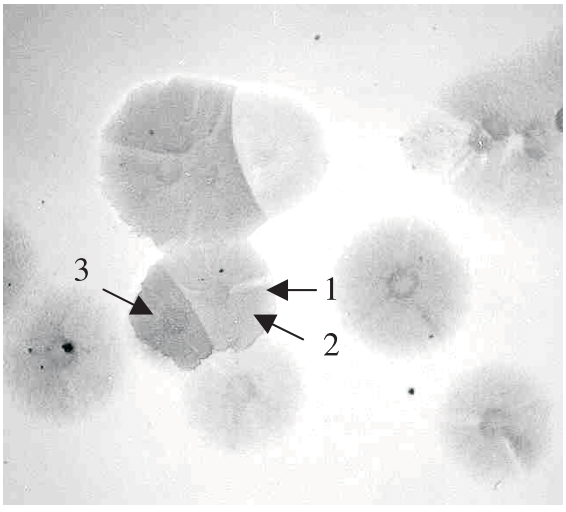

Fig. 4. Detection of IgY antibodies specific for *in vivo* expressed *Mycoplasma gallisepticum* antigens using HRP-conjugated 1F5/3G2 mAbs. In IIPA agar blocks with *Mycoplasma gallisepticum* colonies were incubated in tracheal washing of an infected chicken. As secondary antibody HRP-conjugated 1F5/3G2 mAbs were used. Arrows indicate various (2 and 3) and sectorial (1 and 2) staining depending on variably expressed antigens recognized by local antibodies

Additionally, HRP-conjugated 1F5/3G2 mAb was used in the immunoblot to detect and define protein antigens recognized by IgY antibodies of experimentally and naturally infected chickens, turkeys and pheasants. In immunoblot analyses, shown in Fig. 5, HRP-conjugated 1F5/3G2 mAb (lane 2) was compared with commercial conjugate (lane 1) concerning their capability to detect chIgY specific for particular protein antigens of *Mycoplasma gallisepticum* (panel A), *Mycoplasma synoviae* (panel B) and *Newcastle disease virus* (panel C). The patterns of specific reactions between protein antigens, specific IgY and secondary antibodies were very similar for both conjugates. However, HRP-conjugated 1F5/3G2 mAbs gave less background staining, particularly in the case of *Newcastle disease virus* immunoblotting (Fig. 5, panel C, lane 2).

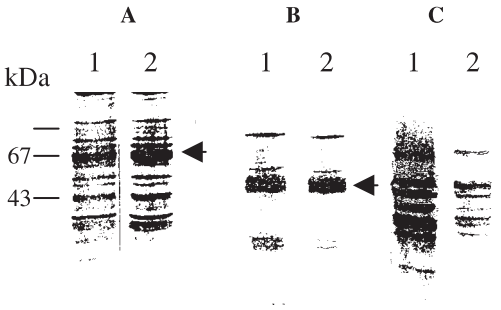

Fig. 5. Comparison of commercial conjugate and HRP-conjugated 1F5/3G2 mAb for detection of specific IgY antibodies against protein antigens of three major poultry pathogens using immunoblotting. Panel A, *Mycoplasma gallisepticum;* panel B, *Mycoplasma synoviae*; panel C, *Newcastle disease virus*. After incubation in sera of infected chicken membrane strips were incubated in secondary antibodies: lanes 1, peroxidase conjugated rabbit anti-chIgY antibodies; lanes 2, HRP-conjugated 1F5/3G2 mAb. Molecular mass is indicated on the left side (in kDa); arrows indicate major immunogenic proteins *i.e.* haemagglutinins pMGA (panel A) and haemagglutinis of *M. synoviae*, named MSPB (panel B). Note: HRP-conjugated 1F5/3G2 mAb gave much less background staining, particularly in panel C

As we previously described (*16*), mAb 1F5/3G2 was coupled to CNBr Sepharose 4B that was used for IgY isolation from chicken serum and egg yolk. Now we used this immunoaffinity column to study the presence of maternal antibodies to *Mycoplasma synoviae* in the allantoic and amniotic fluids of chicken embryos. We assumed that predominant IgY antibodies can compete with IgA and IgM antibodies in the indirect immunoperoxidase assay, where titers of IgA and IgM were only 1:8 and 1:0, respectively. Using mAb 1F5/3G2 coupled to CNBr sepharose it was possible to eliminate almost all IgY antibodies from samples of embryonic fluids. Consequently, IgA and IgM antibodies gave more intense immunostaining and could be detected in 2-fold (1:16 for IgA) or even 8-fold (1:8 for IgM) lower titers than before the elimination of IgY. This indicates that mAb 1F5/3G2 is specific for IgY class and that competition of IgY antibodies with IgA and IgM antibodies in binding to corresponding antigen can result in wrong interpretation of results.

Discussion

Chicken antibodies are not only indicators of infections but are more and more popular as an alternative to antibodies of mammal origin (*16*). In this paper we have presented a set of new monoclonal antibodies that recognize epitopes on avian IgY molecule and their multipurpose use in research, diagnostic and biotechnology.

We produced mAbs that recognize different epitopes on the chIgY molecule. Monoclonal antibody 4E4 reacted with IgY of chicken only, while other mAbs recognized epitopes on immunoglobulins, presumably on IgY of certain other avian species. Monoclonal antibody 3C10 recognized epitopes common to chicken, turkey, pheasant and sparrow. After treatment of chIgY with β-mercaptoethanol, which is known to release LC even in the absence of dissociating agents (*31*), our mAb 3C10 and reference mAb to chLC reacted with a protein of similar size, *i.e.* about 25 kDa. This indicates that mAb 3C10 recognizes an epitope on LC of chicken. It is more likely that the epitope is located in constant part of LC than in variable part. Most of the chLC are of the mammalian λ type and are common to all immunoglobulin classes, *i.e.* IgY, IgA and IgM (*31,32*). Therefore, it can be expected that mAb 3C10 will recognize also LC on IgA and IgM subclasses in chicken, pheasant, turkey and sparrow sera. Actually, it was confirmed by immunoblot analysis that this mAb reacted with LC of chIgM (not shown in the results). Monoclonal antibody 3C10 has a considerable potential to be used in research of immunoglobulins in different avian species, in serologic diagnostics as well as in biotechnology.

Two of our mAbs, 1F5 and 2F10, apparently detect same epitopes that are common to IgY of chicken, turkey, pheasant and peafowl and are located in HC of IgY molecule. Shared epitopes on HC of IgY are not surprising since these birds belong to the same family, *i.e. Phasianidae*. Previous studies have already shown that chicken, turkey and pheasant share epitopes for different polyclonal and monoclonal antibodies produced to chicken and/or turkey IgY (*14,25,33,34*). Suzuki *et al.* (*35*) reported that due to the specific type of glycosilation Concanavalin A (Con A) binds nonspecifically to the Fc part of chIgY. In the immunoblot, Con A and our mAbs 1F5 and 2F10 bind to proteins of the same sizes (not shown in the results), indicating that epitope for these two mAbs is located on HC belonging to Fc part of molecule. Except for ducks and chickens, where some cross-reactions were also observed (*36–38*), there is not much data about amino acid sequences of avian immunoglobulins, therefore we can not look for shared epitopes at amino acid sequence identity level.

Monoclonal antibodies to chIgM M1 established by Mocket (*25*) recognized the epitope on HC of chIgM shared by chicken, turkey, duck, pheasant and peafowl (*34*). Present study showed the presence of the same epitope in sparrow and Japanise quail. Based on the allotypic and isotypic analysis of 7S Ig antigenic determinants in chickens and turkeys, Ch'ng and Benedict (*39*) described two putative phylogenetically ancient determinants. Thus, sharing the same epitopes on IgY and IgM molecule with chickens, turkeys, pheasants and peafowl is in accordance with their evolutionary connection that is also reflected in the classification of these birds in the same family *Phasianidae* (order *Galliformes*). Therefore, it is also not surprising that determinants recognized by mAbs to chIgY are not present in IgY of birds from other orders like ducks and geese (order *Anseriformes*), parrots (order *Psittaciformes*) or pigeon (order *Columbiformes*).

It was reported that the type of glycosilation can influence some biological functions of immunoglobulines, such as immunogenicity. Suzuki and Lee (*35*) described site-specific N-glycosilation of chIgY. Since treatment of IgY with 10 mM periodic acid had no influence on mAbs recognition of epitopes we can assume that the epitopes do not contain carbohydrate.

At present, different immunoenzyme reactions and kits detecting specific antibodies are used for the serologic diagnosis of viral and bacterial infections in poultry flocks as well as after their vaccinations. Most of these kits use HRP-conjugated polyclonal rabbit (*2,9,14*) or goat (*6–8,10*) antibodies to chIgY as secondary antibodies that can detect only chicken and turkey IgY. Our HRP-conjugated mAb 1F5 has wider potential use since it can detect specific antibodies in chicken, turkey, pheasant and peafowl with less background. With practically unlimited production of mAbs we are able to overcome the species-specific platform and produce only one antibody for multipurpose use.

Further on, 1F5 mAb and 2F10 mAb recognize epitopes in their native state as well as when they are partially denatured, which is not true for some commercially available mAbs (CG-25, Sigma). Knowing that 1F5 mAb binds to IgY in the pH range from 3 to 10.5 allows us to use the immunoaffinity column with 1F5 to capture the IgY via Fc and also potentially to capture the IgY-antigen complexes. When such column is used to remove mainly IgY from the samples where it competes with low amounts of IgA and IgM, these two subclasses can be detected correctly.

None of our mAbs reacted with the tested mammalian sera. Jefferis *et al.* (*40*) reported about murine monoclonal antibodies against human IgG that cross-react with 7S chicken immunoglobulin, while data about the vice versa reaction, where anti-chIgY would recognize human or other mammalian immunoglobulins, could not be found. Therefore, in human serologic diagnosis, where instead of mouse mAbs chicken monoclonal IgY are used to detect human immunoglobulins, our HRP-conjugated mAbs to chIgY can be used. In contrast to mouse antibodies, where cross-reactions between polyclonal HRP-conjugated antibodies to mouse mAbs and human Ig are often troublesome, here, with chicken antibodies, no cross-reactions are expected.

Because of many advantages of chicken antibodies (*16,41*), chicken polyclonal, monoclonal and recombinant antibodies are becoming more and more interesting as a substitute for mouse or other mammalian antibodies (*16*). They effectively replaced mouse monoclonals in many basic researches, diagnostic kits and even in therapy. In any kind of chicken antibody production, isolation of IgY or their fragments such as L or H chains, Fc or Fab, ScFv, *etc.* from egg yolk, serum or culture supernatant should be performed. Many different methods

are in use for isolation of chIgY. We have already reported that 1F5/3G2 mAb was coupled to CNBr activated Sepharose 4B and used for immunoaffinity isolation of chIgY from different sources (*16*). By combining our mAbs of different specificities we are able to isolate distinct parts of chIgY molecule.

Using the immunoaffinity column prepared, for example from mAb 3C10, to isolate an appropriate Ig subunit containing L chain would be possible. In this way, the separation of Fab from Fc or LC from HC would be simple, one-step method, no matter how these IgY subunits were produced, as product of enzyme digested polyclonal or monoclonal chicken antibodies or as chicken recombinant antibodies.

We believe that we have developed monoclonal antibodies that can be widely used in basic research, diagnostics of avian and human diseases as well as in biotechnology whenever chIgY molecule or its subunits are involved.

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Proizvodnja, karakterizacija i korištenje monoklonskih antitijela koja prepoznaju IgY epitope u pilićima, puranima, fazanima, paunima i vrapcima

Sažetak

Antitijela pilića ne sudjeluju samo u imunološkoj obrani već se sve više koriste kao komercijalni proizvodi u obliku poliklonalnih, monoklonalnih ili rekombinantnih antitijela. U radu su proizvedena i okarakterizirana monoklonska antitijela miša (mAbs) koja prepoznaju epitope smještene na teškim ili laganim lancima pilećeg imunoglobulina Y (chIgY) što se nalaze i u nekim pticama vrste *Phasianidae*. Pokazalo se da mAbs 1F5 i 2F10, koji prepoznaju teški lanac na zajedničkom epitopu chIgY, djeluju i na imunoglobuline purana, fazana i pauna. Monoklonsko antitijelo mAb 3E10, specifično za lagani lanac chIgY, otkrilo je prisutnost zajedničkih epitopa na imunoglobulinima purana, fazana i vrabaca. Antitijelo mAb1F5/3G2 upotrijebljeno je za pripravu konjugata s peroksidazom hrena i za punjenje imunoadsorpcijske kolone. Konjugirani mAbs pokazali su se izvrsnim sekundarnim antitijelima pri dijagnosticiranju određenih infekcija raznih vrsta ptica. Budući da oni ne reagiraju s imunoglobulinima sisavaca, primjena naših mAbs kao sekundarnih antitijela u ljudskoj serodijagnostici smanjuje obojenost pozadine koja se pojavljuje korištenjem mišjeg sustava detekcije. Primjenom točkastog imunovezanja (DIBA) i postupka »imunoblotting« konjugirani mAbs prepoznaju IgY antitijela protiv *Mycoplasma synoviae, Mycoplasma gallisepticum* i *Newcastle disease virus* u serumima inficiranih ili vakciniranih ptica. Imunoapsorpcija, kojom se uklanja IgY iz uzoraka u kojima je specifična IgY *Mycoplasma synoviae* predominantna imunoglobulinska vrsta, omogućava puno egzaktnije određivanje specifičnih IgA i IgM antitijela.