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Abstract

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General applicability of chicken egg yolk antibodies: the performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1α

GIERI CAMENISCH, MAURO TINI, DMITRI CHILOV, IVICA KVIELIKOVA, VICKRAM SRINIVAS,* JAIME CARO,* PATRICK SPIELMANN, ROLAND H. WENGER, AND MAX GASSMANN

Institute of Physiology, University of Zürich-Irchel, CH-8057 Zürich, Switzerland; and *Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania 19107–5099, USA

ABSTRACT Avian embryos and neonates acquire passive immunity by transferring maternal immunoglobulins from serum to egg yolk. Despite being a convenient source of antibodies, egg yolk immunoglobulins (IgY) from immunized hens have so far received scant attention in research. Here we report the generation and rapid isolation of IgY from the egg yolk of hens immunized against the α subunit of the human hypoxia-inducible factor 1 (HIF-1α). Anti-HIF-1α IgY antibodies were affinity purified and tested for their performance in various applications. Abundant HIF-1α protein was detected by Western blot analysis in nuclear extracts derived from hypoxic cells of human, mouse, monkey, swine, and dog origin whereas in hypoxic quail and frog cells, the HIF-1α signal was weak or absent, respectively. In electrophoretic mobility shift assays, affinity-purified IgY antibody was shown to recognize the native HIF-1 (but not the related HIF-2) complex that specifically binds an oligonucleotide containing the HIF-1 DNA binding site. Furthermore, IgY antibody immunoprecipitated HIF-1α from hypoxic cell extracts. Immunofluorescence experiments using IgY antibody allowed the detection of HIF-1α in the nucleus of hypoxic COS-7 cells. For comparison, the application of a mouse monoclonal antibody raised against the identical HIF-1α fragment was more restricted. Because chicken housing is inexpensive, egg collection is noninvasive, and IgY isolation is fast and simple, and the applicability of IgY is widespread, immunization of hens represents an excellent alternative for the generation of polyclonal antibodies.

More than a century ago, Klemperer (1) observed that immunized hens transfer immunoglobulins (Ig)2 from the serum to the egg yolk. Despite being classified as IgG-like immunoglobulins, the structure of chicken antibodies differs considerably from that of mammalian IgG. For example, the molecular mass of mammalian IgG heavy chain is about 50 kDa, whereas from chicken it is 67–70 kDa (reviewed in ref 2). Based on their well-defined structural differences compared to mammals, IgG-like antibodies from birds (as well as from amphibians and reptiles) were termed IgY (3). There are several advantages of this technology (termed IgY technology, according to ref 4) over conventional antibody production that uses rabbits and other mammals: chicken housing is inexpensive, egg collection is noninvasive, and IgY isolation is fast and simple. Moreover, due to the phylogenetic distance, conserved mammalian proteins often are more immunogenic in birds than in mammals (5–8). Another advantage is that very low quantities of antigen are required to obtain high and longlasting IgY titers in the yolk from immunized hens (5, 9, 10). Despite being an excellent method of antibody production, chicken IgY antibodies represent an underused resource. This may be due to the lack of information concerning the application of IgY antibodies or because IgY antibodies do not bind to proteins A or G. The aim of the present work was to provide a rapid protocol for isolation and immune purification of chicken antibodies as well as to test the performance of IgY antibodies in a variety of applications. For comparison,

Key Words: monoclonal antibody · affinity purification · Western blotting · EMSA · immunoprecipitation · immunofluorescence

1 Correspondence: Institute of Physiology, University of Zürich-Irchel, Winterthurerstrasse 190, 8057 Zürich, Switzerland. E-mail: labbauer@physiol.unizh.ch

2 Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; ATCC, American Type Culture Collection; ELISA, enzyme-linked immunosorbant assay; EMSAs, electrophoretic mobility shift assays; GST, glutathione S-transferase; HIF-1α, hypoxia-inducible factor 1α; Ig, immunoglobulin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.
we generated a mouse monoclonal antibody against the same antigen.

Hens and mice were immunized against the α subunit of the human hypoxia-inducible factor 1 (HIF-1α). The heterodimeric transcription factor HIF-1, consisting of the two subunits α and β, represents the master regulator of oxygen homeostasis (reviewed in ref 11). Null mutant mice lacking either the HIF-1α (12, 13) or the HIF-1β subunit (14, 15) die shortly after midgestation. Unexpectedly, whereas HIF-1α represents a newly detected protein, HIF-1β was found to be identical to the heterodimerization partner of the dioxin receptor/aryl hydrocarbon receptor (AhR), termed AhR nuclear translocator (ARNT) (16). Upon hypoxic exposure, HIF-1 induces mRNA expression of a variety of oxygen-dependent genes including erythropoietin, vascular endothelial growth factor, and transferrin (11). Transactivation occurs by binding to an HIF-1 DNA binding site (CGTg) present in the regulatory regions of such hypoxically regulated genes. In contrast to these genes, HIF-1 is not regulated at the mRNA but at the protein level (17).

Here we demonstrate that the affinity-purified anti-HIF-1α IgY antibody allowed detection of HIF-1α protein in Western blots, electrophoretic mobility shift assays (EMSAs), immunoprecipitation, and immunofluorescence experiments using hypoxic cells from different species. In contrast, the application of the mouse monoclonal antibody was more restricted.

**MATERIALS AND METHODS**

**Antigen preparation**

A polymerase chain reaction-amplified EcoRI-Hpal fragment of the HIF-1α cDNA spanning the carboxyl-terminal amino acids 530 to 825 of human HIF-1α (16) was cloned into the EcoRI-Smal site of the bacterial overexpression vector pGEX4T1 (Pharmacia, Piscataway, N.J.). The resulting plasmid overexpressed HIF-1α as a fusion protein with glutathione S-transferase (GST) in the bacterial strain BL21 (DE3) plyss (Novagen, Madison, Wis.) after induction with isopropyl-β-D-thiogalactopyranoside. The bacteria were pelleted, resuspended in phosphate-buffered saline (PBS) containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, and lysed in a French press. The GST-HIF-1α fusion protein was purified using glutathione Sepharose 4B (Pharmacia) according to the manufacturer’s instructions. For further purification, the GST-HIF-1α fusion protein was electrophoresed through a preparative sodium dodecyl sulfate (SDS) polyacrylamide gel and the corresponding fractions from four eggs were pooled (8 ml), brought to 50 ml with PBS, and loaded overnight at 4°C at a flow rate of 30 μl/min. The column was washed with 50 ml PBS, and bound IgY antibodies were eluted with 8.5 ml of elution buffer (0.15 M NaCl, 0.1 M NaHCO₃, pH 8.5) and added to the resuspended Sepharose. After end-to-end rotation for 2 h at room temperature and centrifugation at 40 × g for 5 min, the pellet was incubated with 10 ml of blocking agent (0.2 M glycine, pH 8.0) at 4°C for 16 h to inactivate the remaining active groups of the resin. The resin was poured into a column and unspecifically bound proteins were washed out by repeating five times an alternating washing procedure consisting of a washing step with 5 ml coupling buffer, followed by one with 5 ml acetate buffer (0.5 M NaCl, 0.1 M ammonium acetate, pH 4.0). After rinsing the column with 50 ml PBS, isolated IgY fractions from four eggs were pooled (8 ml), brought to 50 ml with PBS, and loaded overnight at 4°C at a flow rate of 30 μl/min. The column was washed with 50 ml PBS, and bound IgY antibodies were eluted with 8.5 ml of elution buffer (0.15 M NaCl, 0.2 M glycine, pH 2.2). For immediate neutralization, the eluted fractions (2 ml) were poured into 15 ml tubes containing 0.8 ml of 1 M Tris-HCl buffer (pH 8.0). The column was neutralized with Tris buffer, washed with PBS, stored in PBS containing 0.05% NaN₃ at 4°C, and washed with PBS before re-use. Prior to storage of the affinity-purified IgY antibody (+ 4°C or −80°C), 0.1 mg/ml bovine serum albumin was routinely added as a carrier.

**Generation of the monoclonal antibody mgc3**

HIF-1α (100 μg) antigen was resuspended in 0.5 ml complete Freund’s adjuvant and injected subcutaneously into mice. Additional aliquots of 100 μg of antigen were resuspended in incomplete adjuvant and administered 21 and 42 days later. After decapitation, isolated splenocytes were fused to myeloma X63Ag8 cells, as described (19), and supernatants from the resulting hybridoma cell lines were screened for the presence of specific antibodies against HIF-1α by enzyme-linked immunosorbent assay (ELISA) using standard methodology. The positive clone was isotype (using IsoStrip from Boehringer Mannheim, Mannheim, Germany), revealing a monoclonal IgG₁ antibody (named mgc3), and affinity purified using a protein A Sepharose column (Pharmacia).
Cell culture and hypoxic exposure

The human epithelial carcinoma cell line HeLa and the African green monkey cell line COS-7 were obtained from American Type Culture Collection in Rockville, Md. (ATCC numbers CCL-2 and CRL-1651, respectively). The mouse hepatoma cell line Hepa1 (also termed HepaClc1c7) was a kind gift of L. Poellinger (Stockholm), the porcine kidney cells LLC-PK1 (ATCC CL-101) and the canine kidney cells MDCK (ATCC CCL-34) were kindly provided by J. Forgo (Zürich), and the quail sarcoma cell line QT6 (ATCC CRL-1708) and the Xenopus laevis kidney cell line A6Cl (ATCC CCL-102) were obtained from L. Vogt and B. Spindler (Zürich), respectively. All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, high glucose, Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated fetal calf serum (Boehringer-Mannheim), 1% minimal essential medium nonessential amino acids, 2 mM L-glutamine, 1 mM natrium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin (all Gibco-BRL). Except for frog A6Cl, the cell lines were cultured in a humidified atmosphere containing 5% CO₂ at 37°C. Oxygen tensions in the incubators (Forma Scientific, model 3519) were either 140 mm Hg (20% O₂ v/v, normoxia) or 7 mm Hg (1% O₂ v/v, hypoxia). Routinely, cells were subjected to hypoxic induction for 4 h at a density of approximately 1–2 × 10⁶ cells/cm².

The kidney cell line A6Cl derived from X. laevis was cultured at 28°C in 5% CO₂ in air. To mimic hypoxia, cells were exposed to 130 m deferoxamine mesylate (Sigma, St. Louis, Mo.) for 6 h.

HeLaS3 cells (ATCC CCL-22), a subline of the HeLa cell line that was adapted to grow in suspension, were cultured in Ham’s F-12 medium supplemented as described above. Hypoxic induction of HeLaS3 cells was performed as previously described (20). In brief, approximately 10⁶ cells/ml were continuously stirred in a tonometer (Instrumentation Laboratory, model II-237) at 37°C using gas mixtures of either 20% O₂, 5% CO₂, and 75% N₂ (normoxia) or 0.5% O₂, 5% CO₂, and 94.5% N₂ (hypoxia) for 4 h at a flow rate of 500 ml/min.

Cellular fractionation, in vitro translation, Western blot analysis, and EMSA

Nuclear extracts from hypoxic and normoxic cells were isolated as previously described (20). For total protein lysates, the culture medium was removed and the cells were washed twice with ice-cold PBS. Ice-cold lysis buffer consisting of 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.5 µM Dithiothreitol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin (all from Sigma). The lysate was sonicated and centrifuged at 10,000 × g for 20 min at 4°C. Total cell lysate (1 mg) was incubated with 50 ng of affinity-purified IgY antibody overnight on ice. A rabbit anti-chicken antibody (Promega, C2691) was added to the mixture for 2 h on ice. Protein A Sepharose (Pharmacia) was added and incubation was continued by rotation for 30 min at 4°C. The precipitate was centrifuged at 15,000 × g the pellet was washed three times with total cellular lysis buffer (see above) and twice with 10 mM Tris–HCl (pH 7.5). Finally, the precipitates were analyzed by Western blotting using IgY antibody.

Similarly, 350 µg of total cellular lysate from HeLa cells was incubated with 50 µl hybridoma mcg3 supernatant overnight on ice. Protein A/G Sepharose (30 µl) (Santa Cruz) was allowed to bind for 3 h at 4°C and the precipitates were analyzed by Western blot, as described above, using the monoclonal mcg3 antibody for HIF-1α detection.

Indirect immunofluorescence microscopy

Hypoxic COS-7 cells were fixed with 4% formaldehyde (pH 8.0) for 10 min at room temperature, washed three times with PBS, permeabilized with 0.5% Triton X-100 (Fluka) in PBS for 5 min, and rinsed three times with PBS. To block non-specific binding, PBS containing 10% fetal calf serum was added for 30 min. Cells were then incubated with or without the affinity-purified IgY antibody (diluted 1:10 in PBS) at 37°C for 1 h. HeLa cells were fixed as described above and then incubated with or without affinity-purified mcg3 antibody diluted 1:10 in PBS.

Primary antibodies were detected using either an FITC-conjugated rabbit anti-chicken antibody (Promega, G2691) or Cy3-conjugated donkey anti-mouse antibody (Jackson Immunoresearch Laboratory, West Grove, Pa.). Secondary antibodies were incubated at room temperature for 30 min, washed extensively in PBS and mounted in DABCO solution.
RESULTS

White leghorn hens were immunized with human GST-HIF-1α fusion protein. Isolation of IgY antibodies from individual eggs was performed by a simple two-step procedure that consisted of a chloroform extraction of the egg yolk, followed by a single polyethylene glycol precipitation step (see Materials and Methods). Using this procedure, IgY antibodies were purified to better than 90% homogeneity (data not shown). Western blot analysis revealed the presence of specific IgY antibodies in eggs collected 10 days after the last boost injection. Because strong unspecific signals were obtained with nuclear fractions derived from HeLaS3 cells, the IgY antibodies were affinity purified. The same fusion protein used for immunization was bound to a resin and specific IgY antibody was eluted using an acidic buffer (pH 2.2). Pooled polyethylene glycol fractions from four egg yolks yielded 14–16 ml affinity-purified IgY antibody, which detected HIF-1α in nuclear extracts from hypoxic HeLaS3 cells at a dilution as high as 1:1000 (Fig. 1). In addition to nuclear fractions, the affinity-purified IgY antibody also allowed detection of HIF-1α protein in total cellular extracts from hypoxic HeLaS3 cells (data not shown). We also generated a mouse monoclonal antibody termed mgc3, which was raised against the same human HIF-1α fragment as that used for hen immunization. Like the IgY antibody, mgc3 recognized HIF-1α protein in nuclear and total cellular extracts from human HeLaS3 cells previously exposed to hypoxia (data not shown).

The stability of IgY antibodies to acidic denaturation has been described to be lower than that of rabbit IgG (10, 23), implying that acidic elution from the affinity column (see above) might be harmful to the IgY antibodies. To test their tenacity toward low pH conditions, affinity-purified IgY were exposed to pH 2.35. Western blot analysis using nuclear extracts from hypoxic HeLaS3 cells showed only slight reduction of HIF-1α signals when IgY antibodies that underwent acidic exposure for up to 3 h were used (Fig. 1).

Next, we analyzed whether the affinity-purified IgY antibody was capable of recognizing native HIF-1α protein in electrophoretic mobility shift assays (EMSAs), using as probe an HIF-1 DNA binding oligonucleotide derived from the erythropoietin gene, as described (20). After incubation of the probe with nuclear extracts from normoxic or hypoxic HeLa cells, the expected nonspecific, constitutive, and hypoxia-inducible factors (20) were detected (Fig. 2A). Addition of the chicken polyclonal antibody partially abolished binding of the HIF-1 heterodimer to the HIF-1 DNA binding site and partially supershifted the HIF-1 complex, whereas the constitutive and the nonspecific factors were not affected (Fig. 2A). On the other hand, addition of the monoclonal antibody mgc3 recognized HIF-1α protein in the same hypoxic HeLa extract and resulted solely in a supershifted
HIF-1 complex. Analogous results were obtained when adding IgY antibody or mgc3 to hypoxic nuclear extracts from the mouse hepatoma cell line Hepa1 (data not shown). Thus, the carboxyl-terminus HIF-1α fragment contains residues that are necessary for functional formation of the HIF-1 complex.

Recently, four groups independently isolated a novel endothel-specific transcription factor that is similar to HIF-1α and was hence termed HIF-2α (reviewed in ref 11). HIF-2α, like HIF-1α, forms a functional heterodimer with ARNT (termed HIF-2), is activated by hypoxia, and transactivates oxygen-dependent genes by utilizing the same DNA binding site as HIF-1 (21). To rule out cross-reactivity of our IgY and IgG antibodies between both hypoxia-inducible transcription factors, we performed EMSAs using in vitro synthesized HIF-1α, HIF-2α, and ARNT. As shown in Fig. 2B, the monoclonal mgc3 and the polyclonal IgY antibody were able to recognize in vitro translated HIF-1α but not HIF-2α protein. These results were confirmed by Western blot analysis with deletion constructs of bacterially expressed fusion proteins. As shown in Fig. 3, both antibodies were specific for HIF-1α and did not cross-react with HIF-2α. Even though the polyclonal IgY antibody was affinity purified using the GST-HIF-1α fusion protein, its cross-reactivity with GST alone was very weak, indicating that the GST protein per se displays a low immunogenicity in chicken. In contrast to the IgY antibody, mgc3 failed to react with the 550–653 HIF-1α fragment, indicating that the mgc3 epitope is localized between amino acids 654 and 825.

As mentioned above, both antibodies recognized native HIF-1α protein present in hypoxic extracts derived from human HeLa or mouse Hepa1 cell lines. Unexpectedly, and in contrast to the EMSA results described in Fig. 2A, the monoclonal antibody mgc3 repeatedly failed to react with denatured HIF-1α protein in Western blot analysis when using extracts from hypoxic mouse Hepa1 cells (Fig. 4A). Probably the specific mouse HIF-1α epitope was altered upon protein denaturation by SDS. Thus, the different mammalian, avian and amphibian cell lines were examined by the affinity-purified chicken IgY antibody only. To this end, human (HeLaS3), monkey (COS-7), swine (LLCPK), dog (MDCK), and quail (QT6) cells were exposed to hypoxia (1% oxygen) for 4 h. In the case of the X. laevis cell line A6C1, which requires a culture temperature of 28°C, hypoxic conditions were mimicked by addition of deferoxamine (130 μM for 6 h), an iron chelator known to induce hypoxically regulated genes including HIF-1α (24). Western blot analysis using affinity-purified IgY and nuclear extracts derived from induced cells detected abundant HIF-1α protein in all mammalian cells tested. In contrast, the HIF-1α protein signal in induced quail and frog cells was very weak or absent, respectively (Fig. 4B). That HIF-1-like DNA binding activity has been observed in induced Drosophila me-
Figure 4. The polyclonal but not the monoclonal anti-HIF-1α antibody recognizes HIF-1α from a variety of species. A) Nuclear extracts (25 μg) from mouse hepatoma (Hepa1) and human cervical carcinoma (HeLaS3) cells were subjected to 7.5% SDS-PAGE; HIF-1α was detected in Western blots using either polyclonal or monoclonal antibodies. Whereas polyclonal anti-HIF-1α (IgY) antibody recognized HIF-1α in human and mouse cells, the monoclonal antibody mgc3 repeatedly detected denatured HIF-1α in human cells only. B) Anti-HIF-1α IgY antibodies recognize HIF-1α protein in hypoxic extracts from human cervical carcinoma (HeLaS3), African green monkey kidney (COS-7), pig kidney (LLCPK1), dog kidney (MDCK), and quail sarcoma (QT6) but not in *Xenopus laevis* kidney (A6C1) cell lines. Total cell lysates (50 μg) were resolved by 7.5% SDS-PAGE and Western blots were analyzed with affinity-purified IgY antibodies at a dilution of 1:100. Overexposure of the Western blot confirmed the presence of a faint band in quail but not in frog cells (data not shown).

Figure 5. Immunoprecipitation of HIF-1 by polyclonal (IgY) and monoclonal (mgc3) anti-HIF-1α antibodies. HIF-1α protein was induced in COS-7 and HeLa cells by exposure to 1% O₂ for 4 h. Total cell lysates from COS-7 and HeLa cells were incubated with anti-HIF-1α (IgY) antibody or hybridoma mgc3 supernatant, respectively. The immunoprecipitates were analyzed by Western blot analysis using the same antibodies as used for immunoprecipitation.

Figure 6. Polyclonal and monoclonal anti-HIF-1α antibodies localize HIF-1 to the nucleus of hypoxic COS-7 and HeLa cells, respectively. After hypoxic stimulation (1% O₂ for 4 h), cells were incubated with either polyclonal anti-HIF-1α or protein A affinity-purified monoclonal mgc3 antibodies, as indicated and visualized by confocal laser scanning microscopy.

Our data imply that the HIF-1α carboxyl-terminal part (used as antigen) is highly conserved within the mammalian species. Chicken IgY antibodies do not bind protein A or G. To circumvent this disadvantage in immunoprecipitation experiments, we made use of a commercially available rabbit anti-chicken IgY antibody. Total cellular lysates from hypoxic COS-7 cells were incubated with IgY antibody (30 ng) overnight, followed by a 2 h incubation with rabbit anti-chicken antibodies. Immunoprecipitation was performed by adding protein A Sepharose to the mixture and the precipitates were analyzed by Western blotting. As shown in Fig. 5, the affinity-purified IgY antibody was able to immunoprecipitate HIF-1α. We also demonstrated that, despite some background signals, the monoclonal antibody mgc3 also immunoprecipitated the HIF-1α protein present in total cellular lysates from human HeLa cells.

*lanogaster* SL2 cells (25), however, suggests that an HIF-1-like mechanism is widespread throughout the animal kingdom. Assuming that this notion is correct, our data imply that the HIF-1α carboxyl-terminal part (used as antigen) is highly conserved within the mammalian species.
Finally, the affinity-purified IgY antibody was tested for its ability to detect HIF-1α in the African green monkey cell line COS-7 by immunofluorescence. As shown in Fig. 6, HIF-1α protein was localized in the nucleus of COS-7 cells that were previously exposed to hypoxia (1% oxygen) for 4 h. No signal was obtained when omitting the primary antibody. Similar results were obtained when immunofluorescence was performed with hypoxic HeLa cells using the monoclonal antibody mgc3. This data demonstrates that, upon hypoxic exposure, HIF-1α is translocated to the nucleus.

DISCUSSION

Based on its simplicity and rapidity, we optimized a modified chloroform polyethylene glycol protocol (18) that very efficiently enriched for chicken immunoglobulins. Note, however, that there are several alternative extractions methods to separate the water soluble proteins including IgY from lipids that are present in high concentrations in the egg yolk (26, 27). As shown recently (28), the use of a crude IgY fraction in Western blot analysis might generate non-specific staining. Unspecific reactions in immunohistochemical studies might also occur using crude IgY fractions (29). Nonspecific staining was circumvented by affinity purification of the chicken antibodies using a GST-HIF-1α affinity column. Apart from GST-based fusion protein, a synthetic peptide (26 amino acids) has also been used successfully to affinity purify IgY antibodies, which in turn allowed purification of human α2-antiplasmin (30).

Unlike mammals, the Fc fragment of hens and most birds does not bind protein A or G (31). Therefore, after incubation of the extracts with IgY antibodies, we added commercially available rabbit anti-chicken antibodies, which allowed immunoprecipitation of HIF-1α with protein A beads. Similarly, the use of a crude IgY fraction and protein G-Sepharose beads preabsorbed with goat anti-chicken antibodies allowed immunoprecipitation of neurofibromatosis type 3 protein (32). It is noteworthy that duck immunoglobulins have been reported to bind to protein A or G; however, this high affinity was not attributed to the Fc fragment but to unique histidine residues occurring predominantly in the CH1 domain (31). Nonetheless, immunization of ducks is not recommended since they lay eggs during a short period of the year and since duck-specific housing is demanding.

Apart from the methods used in this work, IgY antibodies have been successfully applied in a variety of assays such as radioimmunoassay (RIA), ELISA, indirect hemagglutination assay, and immunodiffusion (7, 33–36), as well as in immunogold labeling experiments (37). From a therapeutic point of view, egg yolk from immunized hens has been proposed to be a convenient vehicle for oral passive immunization against enteral germs (10, 38, 39) or even against dental plaque formation in humans (40). Egg yolk antibodies from hens immunized with rattlesnake or scorpion venom have been used to generate antivenoms that neutralize the lethal effects of these venoms in vivo (41).

When comparing different adjuvants for chicken immunization, the use of complete Freund’s adjuvants was reported to yield the highest IgY titers (42). In view of its tissue-damaging property, which is not necessarily observed macroscopically, the use of alternative adjuvants has been proposed recently (42). Considering animal welfare, it should be pointed out that chicken can be kept under conventional conditions without special veterinary support. If required, chicken housing and immunization can be outsourced. Despite their general applicability, IgY antibodies are scarcely used. We hope that the protocols presented in this work will help to increase the acceptance of the IgY technology.

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