Short Technical Report

Eliciting Antigen-Specific Egg-Yolk IgY with Naked DNA

BioTechniques 31:670-675 (September 2001)

Marco Romito, Gerrit J. Viljoen, and Dion H. Du Plessis
Onderstepoort Veterinary
Institute, Onderstepoort,
South Africa

INTRODUCTION

Methods for the isolation of chicken egg yolk antibodies (IgY) and the benefits of using them in immunoassays are well established (11,17). Eggs are produced five to seven times weekly, and 50–100 mg IgY can be extracted from the yolk of a single egg. IgY does not cross-react with mammalian antibodies or bind to mammalian complement, F_c receptors, or rheumatoid factor (8,10), and its judicious use can often reduce unwanted background in immunoassays. Immunizing with naked DNA as opposed to purified antigens or recombinant proteins also has many potential advantages (1,4,13). For instance, it is not necessary to produce and purify recombinant proteins, a process that is often tedious and costly. A further advantage is that, unlike with proteins, the identity and quality of the immunogen can readily be ascertained by simple methods such as agarose gel electrophoresis and sequencing. DNA immunization has been used to generate high-affinity polyclonal antibodies in rabbits (3), and in chickens it can protect against various avian diseases [e.g., influenza (14), Newcastle disease (16), and infectious bronchitis (19)].

To avoid raising antibodies to unwanted contaminants, it is usually necessary to immunize an animal with purified antigen. Many expression vectors have been specifically designed to facilitate protein purification, but some of the reagents used (e.g., high concentrations of guanidine HCl and urea) can deleteriously affect the tertiary structure of the protein, while unwanted antibodies to epitope or histidine tags can

be formed. Moreover, cross-absorption of immune serum to remove unwanted specificities is not always completely successful. Injecting hens directly with the appropriate DNA obviates all protein expression and purification steps, while precipitating the IgY from their eggs eliminates the need to bleed animals. DNA immunization of laying hens may provide a way to obtain useful quantities of avian antibodies to pathogenic or toxic antigens, when antigen is not readily available, or to help identify viral proteins that possess neutralizing epitopes. It can also serve as a means of evaluating constructs for DNA immunization, instead of in vitro cell culture, because the presence of IgY in egg yolk would imply that the promoter or gene is functional. In principle, antibodies produced by DNA immunization could then also be used to purify the encoded protein. Accordingly, taking into account the inherent advantages of DNA immunization together with those associated with the use of chicken IgY, we investigated whether eggs from DNA-immunized hens could be regarded as a practical source of specific antibodies.

MATERIALS AND METHODS

Chickens

Amberlink hybrids (Golden Lay, Johannesburg, South Africa), approximately 20 weeks old, and/or specific pathogen free (SPF) Leghorn layer hens (Avimune, Centurion, South Africa) were used. SPF hens were used to evaluate immune responses following

ABSTRACT

Immunization with naked DNA was used to elicit chicken egg yolk antibodies (IgY). Layer hens were inoculated with plasmid DNA encoding the enhanced green fluorescent protein, the fusion protein of Newcastle disease virus, and VP2 of African horse sickness virus. IgY was extracted from egg yolks by polyethylene glycol precipitation. Specific antibodies were present in the yolks of eggs from hens immunized with each of the three different plasmids. This approach to raising polyclonal antibodies obviates the need to produce and purify large quantities of proteins for immunization and can potentially yield large amounts of diagnostically or therapeutically useful reagents.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

immunization with the plasmid pCI-F containing the gene encoding the F protein of Newcastle disease virus (NDV) because commercial chickens are routinely vaccinated with attenuated NDV.

Antigen and Plasmid Constructs

DNA immunization was with the 4008-bp eukaryotic expression plasmid vector pCI (Promega, Madison, WI, USA) containing the 723-bp enhanced green fluorescent protein gene (pEGFP-C1; Clontech Laboratories,

Palo Alto, CA, USA), cDNA of the 1256-bp fusion protein (F) gene of the Onderstepoort strain of Newcastle disease virus (pCI-F) (unpublished data), or cDNA of the 3221-bp VP2 gene of African horse sickness virus (AHSV) serotype 3 (15,22). Recombinant plasmids were propagated in *E. coli* DH5 α , and large-scale plasmid purification was done by anion exchange column chromatography (Qiagen, Hilden, Germany). AHSV serotype 3 was used as a positive control antigen for inoculation and immunodetection. It was propagated

in cell cultures and purified by sucrose density gradient centrifugation (7). NDV was propagated in embryonated chicken eggs. Allantoic fluid was harvested and centrifuged at 54 000 $\times g$ for 60 min to yield a virus-containing pellet (5). EGFP was from Clontech Laboratories.

Immunization

Chickens were inoculated at several sites in the pectoral and upper thigh muscles on three separate occasions with either AHSV or plasmid DNA (pCI-VP2, pCI-F, and pEGFP-C1). About 100 μ g plasmid DNA were administered in 500 μ L PBS, followed by two booster inoculations of 50 μ g each approximately three weeks apart. Chickens were inoculated at seven-day intervals with 80, 40, and 40 μ g AHSV, respectively. Virus was suspended in PBS (500 μ L) together with an equal volume of Montanide[®] ISA50 adjuvant (Seppic, Paris, France). Chickens are routinely vaccinated against NDV; hence, such a non-SPF chicken was used as a positive control without further immunization. Recombinant EGFP was not injected into any bird because of limited availability.

IgY Extraction

Eggs were processed seven to 10 days after immunization and, in some cases, also at varying additional time intervals. IgY was purified as described by Polson et al. (12). Briefly, 3.5% (w/v) polyethylene glycol (PEG 6000; Merck, Darmstadt, Germany) was added to yolk diluted in four volumes buffer (PBS) to remove yolk solids and lipophilic substances. This was followed by precipitation of IgY with 8.5% (w/v) PEG 6000. The reconstituted pellet was then precipitated with 12% (w/v) PEG 6000. Protein concentration was determined by spectrophotometry ($\epsilon_{280} = 1.33 \text{ cm}^2/\text{mg}$) (20).

Detection

Indirect ELISA (2) or immunoblotting was used to determine antibody responses. For ELISA, microplates were coated with 1–10 μ g antigen/well. IgY samples were added at a 1:100 dilution,

Figure 1. SDS-PAGE gel (A) and an immunoblot (B) using IgY from a layer inoculated with pCI-EGFP. Lanes 1, molecular weight markers (kDa); lanes 2, EGFP; and lanes 3, CER cell lysate used as unrelated antigen.

Figure 2. SDS-PAGE gel (A) and immunoblot (B) using IgY from a layer inoculated with pCI-F. Lanes 1, molecular weight markers (kDa); lanes 2, CER cell lysate; and lanes 3, NDV.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

and bound antibody was detected using sheep anti-chicken peroxidase-labeled antibody (The Binding Site, Birmingham, UK). For immunoblotting, proteins were separated by SDS-PAGE in 10% or 12% gels (9) under reducing conditions (Figure 1A). Separated proteins were transferred electrophoretically to a polyvinylidene fluoride transfer membrane (Immobilon™-P; Millipore, Bedford, MA, USA) without methanol (21) or to a nitrocellulose membrane (Hybond-C™ Pure; Amersham Life Science, Little Chalfont, UK). Purified egg yolk IgY was diluted 1:100 in blocking buffer and incubated with membrane overnight at 4°C. Peroxidase-conjugated sheep anti-chicken antibody (The Binding Site) and 4-chloro-naphthol (Sigma-Aldrich, St. Louis, MO, USA) or chemiluminescence substrate (Pierce, Rockford, IL, USA) was used to detect membrane-bound antibodies. Finally, 50% plaque reduction virus neutralization assays (6) were done using IgY from AHSV- and pCI-VP2-immunized hens.

RESULTS AND DISCUSSION

A DNA construct (pEGFP-C1) in which the EGFP gene was placed under the control of the immediate early enhancer-promoter of cytomegalovirus was used to immunize an SPF Leghorn hen. After three injections, IgY that bound strongly in immunoblotting to a M_r 27 000 band representing recombinant EGFP was isolated from yolks of the eggs laid by the immunized hen (Figure 1B, lane 2). There was no binding to any of the proteins in a CER cell lysate used as a negative control (Figure 1B, lane 3). The presence of egg-yolk antibodies that recognized EGFP was further confirmed in ELISA. Absorbance values that were approximately five times that obtained with IgY purified from pre-immune eggs were obtained. Since viral antigens are expressed *in vivo* following immunization with the relevant DNA vaccine, the conformation and post-translational modifications of such antigens are likely to closely resemble those of proteins synthesized during actual infection by the pathogen. To determine whether antibodies could be raised against a viral

glycoprotein in the same way, a similar construct (pCI-F) containing the 1256-bp insert coding for the F protein of NDV was used to immunize an SPF hen. IgY that bound specifically in immunoblotting to a cleaved M_r 53 000 component of the F glycoprotein (F_1) (18) from purified NDV used was shown in eggs from this hen (Figure 2B, lane 3). This confirmed that a viral glycoprotein synthesised *in vivo* fol-

lowing DNA immunization could elicit antibodies that recognized chicken embryo-cultured virus.

AHSV is an orbivirus causing severe disease in horses. Its most important neutralizing epitopes are located on VP2, a M_r 123 078 outer capsid protein. The ability to elicit neutralizing antibodies is often important in viral diagnosis, for instance in determining serotypes by means of viral neutraliza-

Figure 3. ELISA absorbance readings. IgY isolated from eggs of a hen inoculated with pCI-VP2. Eggs laid at different time intervals and IgY diluted 1:100 were used. AHSV and CER cell lysate were used as coating antigens. Inoculation times are indicated with arrows.

Figure 4. SDS-PAGE gel (A) and immunoblots obtained after using IgY from a layer inoculated with AHSV (B) and pCI-VP2 (C). Lanes 1, molecular weight markers (kDa); lanes 2, AHSV; and lanes 3, CER cell lysate.

tion (6). Accordingly, in addition to ELISA and immunoblotting, IgY resulting from the immunization of hens with the gene encoding VP2 was tested for its ability to neutralize viral infectivity.

Indirect ELISA showed the presence of egg-yolk antibodies that recognized VP2. After three injections of plasmid DNA (pCI-VP2), antibody titres increased until 30–40 days after the first injection. There was a decline until 90 days after the last booster injection when levels reached pre-immunization levels (Figure 3). Immunoblotting using antibodies from a yolk obtained after three injections resulted in a band (Figure 4B, lane 2), which corresponded with the migration of the target protein in a 10% polyacrylamide gel. IgY from a hen immunized with intact virus bound all the major AHSV proteins (VP2, VP3, VP5, and VP7) (Figure 4B), in contrast to the DNA-immunized hen where only VP2 (M_r 123 078) was recognized (Figure 4C). The virus neutralization titre of IgY from the hen inoculated with plasmid was 1:40. This neutralization titer was, however, approximately 80-fold less than that found with IgY from a hen immunized with purified AHSV (not shown).

These results show that it is possible to generate egg-yolk IgY against three different antigens of varying sizes by using DNA to immunize hens. For some applications, this approach could provide a useful alternative to producing antibodies by traditional methods. If titers from DNA are not as high as with protein immunizations, this could be offset by the large amounts of IgY obtainable and the ability to dilute concentrated IgY pellets as desired. Antibodies are widely used for diagnostic purposes in various immunoassays. In addition, they can be used for immunotherapeutic or even industrial purposes. IgY produced by the method mentioned would thus potentially have a wide range of applications.

ACKNOWLEDGMENTS

We acknowledge the assistance given by Dr. Hannes Swart and Prof. Louis Coetzee, Avimune, Centurion, South Africa, for supplying SPF leghorns and SPF facilities.

REFERENCES

1. **Beard, C.W. and P.W. Mason.** 1998. Out on the farm with DNA vaccines. *Nat. Biotechnol.* 16:1325-1328.
2. **Du Plessis, D.H.** 1992. Serological differentiation of five bluetongue virus serotypes in indirect ELISA. *Onderstepoort J. Vet. Res.* 59:119-122.
3. **Gårdsvoll, H., H. Solberg, K. Danø, and G. Høyer-Hansen.** 2000. Generation of high-affinity rabbit polyclonal antibodies to the murine urokinase receptor using DNA immunization. *J. Immunol. Methods* 234:107-116.
4. **Giese, M.** 1998. DNA-antiviral vaccines: new developments and approaches—a review. *Virus Genes* 17:219-232.
5. **Hamaguchi, M., T. Yoshida, K. Nishikawa, H. Naruse, and Y. Nagai.** 1983. Transcriptional complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* 28:105-117.
6. **Huismans, H. and B.J. Erasmus.** 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort J. Vet. Res.* 41:51-58.
7. **Huismans, H., N.T. Van Der Walt, M. Cloete, and B.J. Erasmus.** 1987. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology* 158:172-179.
8. **Jensenius, J.C., I. Andersen, J. Han, M. Crone, and C. Koch.** 1981. Eggs: conveniently packed antibodies. Methods for purification of yolk IgG. *J. Immunol. Methods* 46:63-68.
9. **Laemmli, U.K.** 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:608-658.
10. **Larsson, A. and J. Sjoquist.** 1988. Chicken antibodies: a tool to avoid false positive results by rheumatoid factor in latex fixation test. *J. Immunol. Methods* 108:205-208.
11. **Polson, A. and B. Von Wechmar.** 1980. Isolation of viral IgY antibodies from yolks of immunized hens. *Immunol. Commun.* 9:475-493.
12. **Polson, A., T. Coetzer, J. Kruger, E. Von Maltzahn, and K.J. Van Der Merwe.** 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol. Invest.* 14:323-327.
13. **Ramsay, A.J., I.A. Ramshaw, and G. Ada.** 1997. DNA immunization. *Immunol. Cell Biol.* 75:360-363.
14. **Robinson, H.L., L.A. Hunt, and R.G. Webster.** 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid. *Vaccine* 11:957-960.
15. **Romito, M., D. H. Du Plessis, and G.J. Viljoen.** 1999. Immune responses in a horse inoculated with the VP2 gene of African horsesickness virus. *Onderstepoort J. Vet. Res.* 66:139-144.
16. **Sakaguchi, M., H. Nakamaru, K. Sonoda, F. Hamada, and K. Hirai.** 1996. Protection of chickens from Newcastle disease by vaccination with a linear plasmid DNA expressing the F protein of Newcastle disease virus. *Vaccine* 14:747-752.
17. **Schade, R., C. Staak, C. Hendriksen, M. Erhard, H. Hugl, G. Koch, A. Larsson, W. Pollman et al.** 1996. The production of avian (egg yolk) antibodies—IgY. *Altern. Lab. Anim.* 24:925-934.
18. **Scheid, A. and P.W. Chopin.** 1977. Two disulfide-linked polypeptide chains constitute active F protein of paramyxoviruses. *Virology* 80:51-66.
19. **Seo, S.H., L. Wang, R. Smith, and E.W. Collisson.** 1997. The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. *J. Virol.* 71:7889-7894.
20. **Tenenhouse, H.S. and H.F. Deutsch.** 1996. Some physical-chemical properties of chicken-globulins and their pepsin and papain digestion products. *Immunochemistry* 3:11-20.
21. **Towbin, J., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedures and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
22. **Vreede, F. and H. Huismans.** 1994. Cloning and characterisation of the gene that encodes the major neutralization-specific antigen of African horsesickness virus serotype 3. *J. Gen. Virol.* 75:3629-3633.

Received 9 April 2001; accepted 21 May 2001.

Address correspondence to

Marco Romito
Biotechnology Division
Onderstepoort Veterinary Institute (OVI)
Private Bag X5
Onderstepoort 0110, South Africa
e-mail: marco@moon.oivi.ac.za

For reprints of this or
any other article, contact
Reprints@BioTechniques.com