Aus dem Institut für Pharmakologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

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DISSERTATION

Development of assays for the detection of agents with bioterroristic potency using the IgY-technology (Avian antibody)

Zur Erlangung des akademischen Grades

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<u>Attachments: publications during Ph.D study</u>

1. Publication 1 **2. Publication 2**

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Publications with relevance for publications doctorate

This dissertation is based on the following papers and contributions:

1. Application of high-titred IgY antibodies in orthopox virus diagnostics
ZHANG Xiao-Ying, Andreas Kurth, Diana Pauly, Georg Pauli, Rüdiger Schade, Heinz Ellerbrok. *Journal of Chinese Pharmaceutical Sciences*. 17(3). 2008. 183-191.
Contribution: 60%.
Experimental work, manuscript preparation, literature investigation

2. **Apparent dose dependent antibody response in chickens immunized with orthopox virus antigens. ZHANG Xiao-Ying**, Rüdiger Schade, Heinz Ellerbrok. *Journal of Chinese Pharmaceutical Sciences*. 17(4). 2008.
Contribution: 60%. Experimental work, manuscript preparation

3. Monitoring of laying capacity, Immunoglobulin Y-concentration, and antibody titer development in chickens immunized with ricin and botulinum toxins over a two-year period. Pauly D, Dorner M.B., **ZHANG X.Y.**, Hlinak A, Dorner B.G., Schade R. *Poultry Science*, 2009. 88: 281-290
Contribution: 20%.
Preparation of egg laying data and preparation of corresponding diagrams.

4. Use of IgY-antibodies in human and veterinary medicine
Rüdiger Schade, **Xiao-Ying Zhang** and Horacio Raul Terzolo
In Book: Bioactive Egg Compounds, chapter 25 (ed. by Rainer Huopalahti, Rosina López-Fandino, M. Anton and R. Schade). *Springer-Verlag Berlin Heidelberg* 2007
Contribution: 20%. Literature investigation

5. Trends and applications of IgY –antibodies in medical research
ZHANG Xiao-Ying, Zheng Li, Ruediger Schade.
Chin Tradit Herb Drugs 2006 July (37): 8-12.
Contribution: 70%. Literature investigation and manuscript preparation

6. Technological aspects of egg yolk immunoglobulin,
ZHANG Xiao-Ying, ZHENG Li , Rüdiger.Schade，Horacio Raul Terzolo
Hanibal Pablo Chacana, Joanna Porankiewicz-Asplund, E. Gutierrez Calzado.
China pharmacological Bulletin 2004 Oct; 20(10):1102-6.
Contribution: 60%. Literature investigation and manuscript preparation

7. IgY-technology and its applications in medicine: theoretical background,
Rüdiger.Schade, **ZHANG Xiao-Ying**, ZHENG Li
China pharmacological Bulletin, 2004 May 20(5): 491-5.
Contribution: 70%. Literature investigation and manuscript preparation

Abstract

The IgY-technology (the non-invasive production of polyclonal Ab in chicken) is a highly innovative and growing branch of biotechnology due to several advantages that IgY-Ab has compared with mammalian Ab.

- Animal welfare. IgY Ab can be easily sampled based on simple action of egg collection and preparation of IgY from egg yolk, instead of the stressful bleeding of animals.
- High amount of IgY (more than tenfold compared to an animal of similar size like a rabbit).
- No cross-reactivity with rheumatoid factors.
- No cross-reactivity with human anti-mouse Ab (so-called HAMA).
- No activation of the mammalian complement system.
- High resistance to immunisation with toxins.

In the study presented polyclonal Ab was produced against different agents with bioterroristic background like OPV and different toxins (*Ricinus communis* and C*lostridium botulinum*). The immunisations resulted generally in Ab with partly extremely high titres and other different specificities (concerning the different chains of ricin as well as botulinum toxins). It is important to notice that stable and high Ab titre could be achieved by injecting only 20 µg of antigen per immunisation. Neutralizing properties of the Ab under investigation could be shown in vitro and in vivo. It could be proved that the Ab with specificity against OPV is suited for detection of viruses from strongly diluted samples. The procedure is based on a combination of an enrichment technique (binding of virus particles with IgY-coupled magnetic beads) followed by a PCR. A result rather by chance was the observation that there is an apparent correlation between the number of virus particles used for immunisation and the resulting Ab-titre. A more detailed study using the same antigen but with changed concentrations (formerly high concentrated now diluted, and formerly low concentrated now higher concentrated, respectively). The results obtained confirmed the expectations and are also in line with observations of other working groups.

Furthermore, it could be demonstrated that the laying hens can lay eggs continuously during a two years period. The laying capacity diminishes approximately during the second half of the second year (2-3 eggs per week compared to 5-7 eggs at the beginning). However, since the IgY concentration is higher in the second year the mean yield of IgY is similar if both years are

compared. It could be demonstrated by that hens produce IgY at a rate of about 20 g per year. Interestingly, the IgY concentration follows a complex biological oscillation in a range of a circaseptan rhythm and a multiple of seven.

In summarizing, due to the advantages mentioned above chickens are excellently suited for the production of Ab against agents with bioterroristic background and other substances.

Zusammenfassung

Title: Entwicklung von Testsystem zum nachweis von Agenzien mit bioterroristischem Potential auf der Basis der IgY-Technologie (aviären Antikörpern)

Die IgY-Technologie (die nicht-invasive Produktion von polyklonalen Antikörpern in Legehennen) ist ein hoch innovativer und wachsender Zweig der Biotechnologie, zurückzuführen auf verschiedene Vorteile, die diese Technologie gegenüber der Gewinnung von Ak in Säugern hat:

Die Methode gilt als Alternative im Sinne des Tierschutzes, da IgY unblutig aus dem Eidotter gewonnen wird.

- Große Menge an IgY (mehr denn das Zehnfache, das von einem Säuger vergleichbarer Größe [Kaninchen] erhalten werden kann).
- Keine Kreuzreaktion mit rheumatoiden Faktoren.
- Keine Kreuzreaktion mit humanen anti-Maus Ak (sogenannten HAMA).
- Keine Aktivierung des Säuger-Complementsystems.
- Erstaunliche Resistenz gegen Immunisierung mit Toxinen.

In der vorgelegten Studie wurden Ak gegen Substanzen mit bio-terroristischem Hintergrund wie OPV und verschiedene Toxine ((*Ricinus communis* und C*lostridium botulinum*) produziert. Die Immunisierungen führten generell zu Ak mit teilweise extrem hohen Titern. Ein wichtiges Ergebnis ist auch, dass Antigenmengen von nur 20 µg pro Immunisierung zu hohen und stabilen Ak-Titern führte. Bezogen auf die Reaktionen mit den unterschiedlichen Ketten der genannten Toxine war die Spezifität der generierten Ak unterschiedlich. Darüber hinaus hatten die jeweiligen Ak neutralisierende Eigenschaften in vitro und in vivo. Mit den anti-Orthopxvirus Ak konnte eine Virus-Anreicherung zur Detektion von Viren in stark verdünnten Proben entwickelt werden. Die

Methode basiert auf der Bindung von Viren an IgY-gekoppelte magnetic beads, gefolgt von einer PCR. Ein eher zufälliges Ergebnis war die Beobachtung, dass die Anzahl der für die Immunisierung eingesetzten Viruspartikel offensichtlich die Höhe des resultierenden Ak-Titers beeinflusst. Eine gezielte Untersuchung unter Verwendung derselben Antigene (das vorher hochkonzentrierte jetzt verdünnt, das vorher niedrigkonzentrierte jetzt aufkonzentriert) bestätigte diesen Zusammenhang. Dieses Ergebnis entspricht auch Beobachtungen durch andere Arbeitsgruppen.

Im Rahmen der Studie konnte gezeigt werden, dass die verwendeten Legehennen-Rassen kontinuierlich Eier über einen Zeitraum von ca. zwei Jahren legten und damit eine Immunisierung der Tiere über diesen Zeitraum möglich ist. In der zweiten Hälfte des zweiten Jahres reduzierte sich die wöchentliche Legeleistung (2-3 Eier im Unterschied zu 5-7 zu Beginn der Immunisierung). Da aber die IgY-Konzentration im zweiten Jahr deutlich gestiegen ist, sind die IgY-Mengen in beiden Jahren vergleichbar. Die pro Jahr erzielte IgY-Menge liegt in der Größe von 20 g pro Huhn. Überraschenderweise konnte gefunden werden, dass die IgY-Konzentration einer komplexen biologischen Oszillation unterliegt. Es konnte ein circaseptaner Rhythmus gefunden werden, dem Rhythmen mit dem Vielfachen von sieben überlagert sind. Zusammenfassend lässt sich feststellen, dass Legehennen aufgrund der oben genannten Vorzüge hervorragend auch zur Produktion von Ak gegen Substanzen mit bioterroristischem Hintergrund geeignet sind.

Abbreviations

Ab	Antibody
BoNT/A	*Clostridium botulinum* neurotoxin type A
BoNT/B	*Clostridium botulinum* neurotoxin type B
BSA	Bovine serum albumin
CEF	Chicken embryofibroblast
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EEV	Extracellular enveloped form
ELISA	Enzyme-linked-immunosorbent-assay
IEM	Immunoelectron microscopy
IFA	Immunofluorescence antibody test
IgG	Immunoglobulin G
IgY	Immunoglobulin Y, egg yolk antibody
OPV	Orthopox virus
PBS	Phosphate-buffered saline, pH 7.4
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
$PRNT_{50}$	50% plaque reduction neutralization test
RT	Room temperature
SARS	Severe Acute Respiration Syndrome
VACV	Vaccinia virus
WB	Western blot
w/v	weight/volume

Introduction

In 1893, Klemperer (1) first described an experiment in which he demonstrated that the immunization of a hen caused the transfer of specific Abs into the egg yolk. However, for a long time there was no scientific application of this knowledge. When animal welfare became a serious ethical concern for the scientific community, the results of Klemperer′s experiment began to attract public interest. In particular this development was initiated by the work of Russel and Burch in 1959 (The principles of human experimental technique, 2). Over the next 20 years more and more researchers recognized the importance of Klemperer's results. Since the 1980s IgY-Abs found a broader application, possibly due to availability of commercial secondary reagents like IgY-purification kits, IgY-standards and labelled Abs (FITC, AP, and POD) with specificity against IgY. Since 1996 (3) IgY-technology (Dr. C. Staak in 1995) has been the internationally accepted term for the production and use of IgY-Ab. In 1996 ECVAM (European Centre for the Validation of Alternative Methods) recommended the use of IgY instead of mammalian IgG in order to minimize pain due to invasive Ab-sampling. This ECVAM workshop also gave information about practical aspects of the rearing of laying hens, chicken immunization, use of adjuvants, IgY extraction methods, etc. 4). Today the IgY-technology is a highly innovative and expanding branch of biotechnology which has several advantages. Compared with the most common mammalian Ab, IgY Abs activate neither mammalian complement nor cross-react with mammalian rheumatoid factor. Further aspects of IgY technology increasingly attract the interest of the scientific community. For example, IgY-Ab can be easily sampled non-invasively based on the simple action of egg collection, instead of the stressful bleeding of animals to obtain serum. Due to the so-called phylogenetic distance between the animal classes *Mammalia* and *Aves*, chicken are able to react with an Ab response whereas the rabbit immune system remains silent despite of an identical immunization (5). From an economical point of view, the Ab production of a hen roughly corresponds to that of a large mammal, such as a sheep or a goat but only needs the keeping costs of a small animal. Thus, an extraordinary amount of Ab can be produced from only one hen, approximately 17–35g of total IgY/chicken/year, of

which 1–10% can be expected to be antigen-specific (6). This huge quantity of available Abs opens the door for new fields of application for IgY technology, such as in immunotherapy and in immunoprophylaxis for several viral and bacterial infections in veterinary and human medicine and recently also for defense against bioterrorism (BT). In case of a bioterroristic attack it is important to have enough specific Abs with neutralizing properties in order to protect affected people. For example, LeClaire *et al.* (7) produced IgY Abs against the highly toxic staphylococcal enterotoxin B (SEB). The authors demonstrated the prophylactic and therapeutic application of anti-SEB IgY. Complete protection of mice and rhesus monkeys against a lethal SEB aerosol challenge has been observed when applied twenty minutes before or four hours after challenge.

Objectives

The aim of the study was to demonstrate the advantages of IgY-technology in the context of Ab-production against agents with bioterroristic background in particular against different OPV and toxins from *Ricinus communis* and *Clostridium botulinum*. The focal points of the study were the following:

- To produce Abs against different OPV and to characterize sensitivity and specificity of the Abs obtained.
- To develop an enrichment procedure for detection of OPV also in small quantities (combination of enrichment and PCR).
- To study the laying capacity of hens during a two-year period and to check the amount of IgY obtainable from one hen per year.
- To monitor the dynamic of IgY-concentration during the observation period.
- To prepare an IgY review in Chinese language in order to attract the interest of the Chinese scientific community for IgY-technology.

Methods

Animals: 16-week-old egg-laying white leghorn hens kept in separate cages and treated in accordance with the applicable law and guidelines.

1. Immunization of laying hens

Laying hens are immunized with inactivated OPV (VACV strain Lister-Elstree, 7.2×10^8 PFU/ml, cowpox virus isolate 81/02, 1.0×10^6 PFU/ml and calpox virus isolate 2443, 5.3×10^6 PFU/ml). Five boosters had been followed within 13 months. The virus was multiplied in primary CEF cells and harvested from the supernatant by ultra-centrifugation.

2. Extraction of IgY from egg yolks by improved PEG precipitation.

The yolk of a single egg was diluted 1:2 with PBS. To eliminate lipids and lipoprotein, 3.5% PEG was added. After 10 min of gentle shaking at RT followed by centrifugation (10 000 g for 20 min at 4°C), the supernatant was harvested, PEG was added to a final concentration of 12% and the mixture was centrifuged again under the conditions described above. The precipitate was dissolved in 10 ml PBS, PEG was again added to 12% and centrifuged once more. Finally, the precipitate was dissolved in 1.2 ml PBS, transferred into a micro dialyser (QuixSep®, Roth) and dialysed 3 times against 1 L PBS overnight at 4°C.

3. Monitoring of IgY titre development by indirect IFA

Hep2 cells were infected with virus at multiplicity of infection of 1 and cultured for 24 h at 37°C in a 5% CO_2 atmosphere. Cells were fixed with acetone. Ten-fold serial dilutions of IgY from 1:10 to 1: 10^6 were prepared in PBS containing 2% BSA and 2% Na-acid, added to the fixed cells (20 µl per well) and incubated for 1 h. After washing with PBS, cells were incubated with 20 µl of fluorescein isothiocyanate-conjugated AddiniPure donkey-anti-chicken IgY (1:80 dilution) for 1 h and analysed under a fluorescence microscope. Uninfected Hep2 cells were used as negative control.

4. Neutralisation activity of anti-OPV IgY by $PRNT_{50}$.

120 µl of virus (approx. 500 PFU/ml of VACV, 600 PFU/ml of calpox virus and 600 PFU/ml of cowpox virus) was incubated for 1 h at 37°C with 120 µl of two-fold serial

dilutions from 1:10 to 1:1 280 of IgY in DMEM medium in 96-well plates. 100 µl of the virus–IgY mixtures was added to 100 µl of VeroE6-7 cell suspension (1.2×10^6 cells/ml) growing in 48-well tissue culture plates and incubated for 4 h at 37°C. 200 µl of 1.6% carboxyl methyl cellulose medium was added and incubated for further 96 h at 37ºC. The plates were fixed with 4% formaldehyde in PBS for 20 min and stained by 0.4% (w/v) naphthalene blue black solution. Plaques were counted and $PRNT_{50}$ were calculated.

5. Observation of IgY binding site by negative staining IEM

400-mesh copper grids covered with Pioloform F (Wacker, Germany) and carbon were floated on virus-containing drops for 5 min, followed by two washings on drops of 1% BSA-PBS for 10 min each. Grids with adsorbed virus were then floated on drops of different dilutions of specific IgY Ab in 1% BSA-PBS (ten-fold dilutions from 1:10 to $1:10^5$) for 1 h at RT, followed again by two washings on drops of 1% BSA-PBS. Goat-anti-chicken IgG-gold 5 nm (1:100 in 1% BSA-PBS) was applied to the samples for 30 min at RT, followed by washing with PBS and with distilled water. The grids were negatively stained with 1% uranyl acetate (20 g/L, pH=4). The specimens were examined in IEM.

6. Protein analysis.

Specific binding of IgY to virus proteins was shown by WB. OPV samples and uninfected Hep2 cells were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis using 10% gels. After gel electrophoresis and transfer to nitrocellulose membranes, the membranes were soaked in blocking buffer, then incubated with 1:15 000 diluted IgY and 1:10 000 diluted rabbit-anti-chicken IgY peroxidase conjugate for 1 h at RT. Signals were visualised by SuperSignal® West Dura substrate and exposure to Kodak X-OMAT AR film.

7. Cross-reactivity of two IgY abs with cells infected with the different strains of the

OPV was also investigated by different methods (IFA, $PRNT_{50}$ and WB).

8. Concentration of OPV with IgY coupled to magnetic beads

According to the recommendation of the manufacturer's manual (Dynabeads™ M-280), a ratio of 3µg ab/10^7 Dynabeads was chosen. Purified anti-VACV IgY (IFA titre 1:10^5) was incubated overnight with Dynabeads™ M-280 at 37°C on a roller. Constant amounts of virus (Table 1) were diluted into different volumes of 0.1% BSA-PBS and each sample was incubated with 20 µl IgY-coupled magnetic beads for 30 min while rotating at RT. The magnetic beads were washed twice with 0.1% BSA-PBS containing 0.5% Tween. Finally, DNA was released from bound viral particles with 100 µl 30% chelex. Magnetic beads were pelleted by centrifugation. 90 µl of the supernatant containing the viral DNA was used for PCR. 0.1% BSA-PBS and anti-botulinum IgY coupled to magnetic beads was used as negative controls.

Extraction of VACV DNA from virus samples was performed with the QIAGEN DNA kit. Viral genome equivalents were determined with a quantitative real-time PCR in duplicate in a 7700 Sequence Detection System. Samples were analysed with the SDS 2.2.2 software and quantified by comparison with a serial dilution of a quantified standard plasmid containing the viral target sequence. The average Ct values and mean quantities were calculated. In order to assess the recovery, viral genome equivalents of VACV DNA were extracted in parallel and determined by PCR and used as standard groups to calculate the recovery.

9. Analysis of biological oscillation (laying capacity of immunized chickens and total IgY-concentration).

4 chickens were immunized and boosted with ricin and BoNT for 2 years (Table 2). Specific IgY were analyzed by biological methods similar to OPV research. The eggs produced per chicken per week and total IgY-concentration of each egg were registered. We used the moving average as a first mathematical method to roughly analyse the IgY-concentration curves on biological oscillations. We used the Chronos-Fit program to analyse the data more in detail (8). The GraphPad Prism (3.0)

program was used for preparation of the IgY-concentration curves.

Results

Anti-OPV IgY titer development

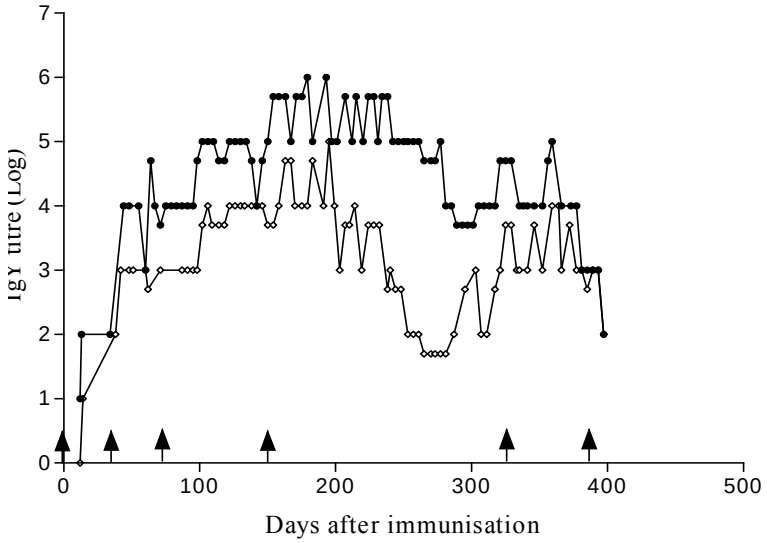

Fig. 1 Monitoring of IgY Ab titres by IFA. Chickens were immunized and boosted (arrow) with VACV (●) and calpox virus (□).

Anti-VACV IgY and anti-calpox virus IgY were positive even in very high dilutions in IFA (titres up to $1:10^6$ and $1:10^5$, respectively) and persisted on a plateau over 10 months after four booster injections.

The neutralisation titres of anti-VACV IgY and anti-calpox virus IgY paralleled to some extent the development of IFA titres. After the 2nd booster injection, anti-VACV IgY and anti-calpox virus IgY exhibited maximum neutralisation titres of 1:320 and 1:80, respectively.

Observation of ab sensitivity and specificity

Both, anti-VACV IgY and anti-calpox virus IgY recognized virus proteins from VACV, calpox virus and cowpox virus, with the main reactivity against two proteins with molecular weights of 100 kDa and 55 kDa.

The binding of IgY to viral particles was analysed by IEM. The gold-labelled anti-VACV IgY bound to the surface of VACV in dilutions ranged from 1:10 to $1:10^5$

with an optimal dilution of $1:10^3$. The range of specific binding of anti-calpox virus IgY to calpox virus was from 1:10 to $1:10^4$ with an optimal concentration of $1:10^3$.

IgY as virus concentration tool

In order to improve the sensitivity of PCR assays for the detection of low numbers of OPV particles in diluted samples, anti-VACV IgY was coupled with magnetic beads and used to concentrate VACV out of large sample volumes prior to DNA extraction.

Constant amounts of virus (100 µl of 10^3 PFU/ml or 100 µl of 10^2 PFU/ml) were added to different volumes of buffer, yielding a total sample volume from 200 µl to 2 ml (Table 1). IgY-coupled magnetic beads were added to the different solutions. Magnetic beads were harvested and, after DNA isolation, the number of genome equivalents was determined by PCR. The percentage of virus particles bound to the magnetic beads versus total virus in the sample was calculated.

The binding efficiency was expressed by genome equivalents bound to magnetic beads versus total number of genome equivalents present in the assay sample. In these experiments it could be shown that the OPV-specific IgY coupled with magnetic beads were able to effectively concentrate OPV particles from large volumes and in consequence increase the sensitivity of OPV diagnosis (Table 1). It was also found that an increase in the amount of magnetic beads-IgY complex in the sample also increased virus recovery.

Table 1 Determination of VACV genome equivalents by qPCR after reaction of IgY-coupled beads with diluted VACV samples

Assay volume[a] (µl)	Virus concentration (PFU/ml)	PFU/1µl beads[b]	Average Ct	Mean quantity[c]	Recovery (%) [d]
200	500	5	34.1	134.0	79.0
200	50	0.5	38.1	15.3	68.5
500	200	5	34.9	75.1	44.3
500	20	0.5	37.9	10.3	46.2
1000	100	5	34.6	94.0	55.5
1000	10	0.5	39.5	6.4	28.8
2000	50	5	35.2	64.5	37.8
2000	5	0.5	38.4	8.0	35.4

[a]constant amounts of virus (100 PFU and 10 PFU, resp.) were diluted in different

assay volumes. The number of viruses in the suspension per µL beads was calculated; [b]20µl of beads were used for each assay; [c] Genome equivalents; [d]compared to initial number of genome equivalents in the assay

Cross-reactivity

Strong cross-reactivity between OPV was observed. In both IFA and $PRNT_{50}$ assays the titres against the heterologous OPV were reduced to no more than one order of magnitude compared with reactivity with homologous virus. By WB, similar protein recognition patterns (100 kDa and 55 kDa) were seen with both the homologous virus and the heterologous virus.

Table 2 Monitoring of laying capacity, IgY-content and periodic fluctuation of the anti toxin IgY-concentration

	Chicken 21 (Ricin)	Chicken 22 (Ricin)	Chicken 19 (BoNT/A)	Chicken 23 (BoNT/B)
number of eggs laid	625	626	545	608
% of max. possible egg number[1]	86	86	75	84
processed eggs	565	620	283	401

Table 2. Two years statistics of egg laying capacity of chickens immunized with ricin or BoNT antigens.
[1]The max. possible egg number per year is calculated according to the formula: 52 week X 7=364 eggs.

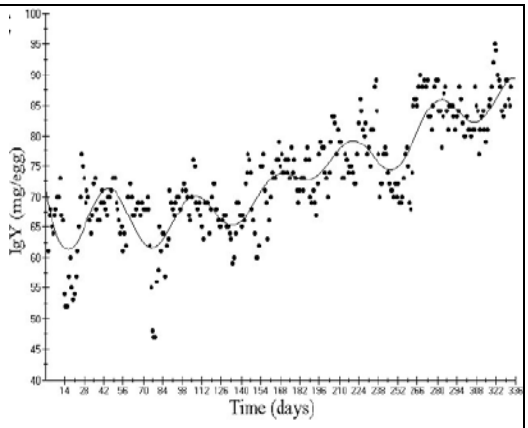

Fig. 2. Time dependent development of total IgY-concentration of chicken 22.

Table 2 shows the total numbers of eggs laid. Chickens usually laid 7 eggs per week and productivity started to decrease between the 70th-80th weeks to 2-5eggs per week. The weekly mean IgY-content increased from 38-45 mg during the first 10 weeks to 53-60 mg and remained constant for approx. 1 year.

By analysing the data of anti-ricin IgY (chicken 22) with the Chronos-fit program, a significant rhythm (rhythm 73.5%, $P<0.001$) with a period length of app. 60 days was identified (Fig.2). A significant rhythm (96%, $P<0.0001$) with a period length of 7 days was found when we analysed a part of the data in more

detail. Similar findings were obtained for other chickens.

Apparent dose dependent antibody response

As a comparative analysis, dose-dependent IgY antibody response to different amounts of OPV antigen has been studied in immunized chickens for two different OPV strains (Table 3). The antibody responses to different immunizations were tested and compared by IFA.

Table 3 Relation between OPV antigen used for immunization and resulting Ab-titer

Virus	PFU/mL	Ab titer (IFA) [a]
VACV[b]	7.2×10^{8}	$1:10^{6}$
VACV (this study)	7.0×10^{6}	$1:10^{1}$
Cowpox virus[b]	1.0×10^{6}	$1:<10^{1}$
Cowpox virus (this study)	9.2×10^{7}	$1:10^{4}$

[a]max. titer observed [b]data from *Zhang et al* (9, see: Results- Anti-OPV IgY titer development).

Discussion

IgY-technology is increasingly used worldwide in different fields of bio-medical research. Besides the application as a diagnostic-analytical tool, IgY-Ab is also used for therapeutic/prophylactic purposes in human- and veterinary medicine to prevent intestinal diseases in infant calves and piglets caused by enterotoxic *E.coli* and rotaviruses, respectively (10). Also in aquaculture, the most expanding branch of food technology today, IgY -technology is being tested in the prevention and treatment of fish diseases. The application of IgY-technology attracted more interest in the context of bio terrorism due to the enormous IgY-production of laying hens (7). For the same reason Chinese researchers produced Ab against SARS in order to have a tool for passive immunisation (11). Abs specific against different influenza serotypes were

also successfully produced in chickens (12). There are only a few reports about immunisation of chickens with toxins like ricin or botulinum toxins (13) probably due to danger of such substances. Surprisingly, it has been known for more than a hundred years and also confirmed by our results that chicken are amazingly resistant to toxins (e.g. tetanus toxin, 1).

The objective of the production of different anti-OPV Ab has been to eventually develop tools to differentiate between different OPV strains on the one hand and to have enough material for *in vivo* and *in vitro* neutralization experiments on the other.

It could be shown that anti-VV IgY and anti-calpox virus IgY were positive even in very high dilutions in IFA (titres up to $1:10^6$ and $1:10^5$, respectively) and persisted on a plateau over 10 months after four booster injections. However, the *in vitro* neutralizing activity of the Ab was lower than expected. Probably, that is due to the use of inactivated virus samples. According to the experience of colleagues from Argentina (P. Chacana, personal communication) gathered in producing Ab against bovine rotavirus, the neutralizing activity of IgY-Ab was much higher when living virus samples were used instead of inactivated samples. As expected, there was a high cross-reactivity between the different OPV strains.

Our study show that immunisation with the same antigen but in varying concentrations can result in different Ab-titres. Our results, together with data from the literature, show that the antigen dose used for immunization plays an important role for the production of specific Abs. An increase in antigen concentration may achieve higher Ab titers but, dependent on the immunogenicity of the antigen, it can also result in an immune depression. However, in this study we found for OPV a positive correlation between antigen concentration and Ab-titer.

Immunization of hens with ricin or botulinum toxins led to comparable results if the Ab titres were taken into consideration. It is worth to notice that a stabile titre could be achieved only after at least three immunisations (in one case after 11 immunisations). It could also be shown, that it is possible to immunise hens for more than two years. In addition, it is also highly interesting that these results could be achieved by injecting antigen doses in the range of 10 to 20 µg. As far as we know,

there is only one reference (14) demonstrating an Ab response by injecting a dose as small as 0.1 µg BSA as a potent immunogen in contrast to insulin (a minimum of 10 µg was necessary).

According to data from literature the theoretical outcome of total IgY/hen/year is in the range of 40 to 100 g (6, 15). However, such data are mostly projections comprising two values: the maximum number of eggs laid by a chicken per year and the mean total IgY of one egg in mg as published by several authors (e.g. 15). The data presented here are in the range of 20 g total IgY per year and so corresponds roughly with the theoretical values. In view of our findings it seems to be very optimistic to assume that 100 g total IgY can be obtained from one hen per year. Interestingly, there are no significant differences in the amount of IgY compared with the first and second laying year. The reason for this is that in the first year the IgY concentration is lower combined with a high laying frequency in contrast to the second year with a lower laying capacity but combined with a higher IgY concentration per egg.

A result obtained rather by chance is the observation that the development of total IgY during the two- year period follows complex significant biological oscillations. Interestingly, the smallest rhythm observed is a seven-day rhythm, a so-called circaseptan rhythm. This was confirmed earlier by data published in 2000 (16). The other period lengths obtained (21/24 days [data not shown] and 60 days) roughly can be considered as multiples of seven. There are contradictory points of view as to weather or not the IgY rhythm is significant. The contra argument is that the hen has to produce a constant IgY concentration to supply all the offspring with the same amount of IgY (16). However, according to Kowalczyk *et al.* (17) the fate of more than 90 % of yolk IgY is uncertain. This means- that approx. 10% of the yolk IgY is of importance for the passive immunity of the newly hatched chicken. Thus, it seems that a biological rhythm of IgY would not be a risk for the passive immunity of the hen's offspring.

In summary, our data are useful in order to optimize the cost/benefit ratio when producing quantitative amounts of polyclonal IgY. The total and specific IgY content

remains on a high level for about two years: roughly the same total amount of specific Ab was raised in the second as in the first year.

References

1. Klemperer, F. (1893).Über natürliche Immunität und ihre Verwerthung für die Immunisirungstherapie. *Archiv für Experimentelle Pathologie und Pharmakologie* **31**, 356-382.
2. Russell, W.M.S. & Burch, R.L. (1959). The principles of human experimental technique. Methuen, London.
3. Schade R. & Hlinak A. (1996). Egg yolk antibodies, state of the art and future prospects. *ALTEX* **13** (Suppl.), 5-9.
4. Schade R., Staak C., Hendriksen, C., Erhard MH., Hugel H., Koch G., Larsson A., Pollmann W., van Regenmortel M., Rijke E., Spielmann, H., Steinbusch, H. & Straughan, D. (1997). The production of avian (egg yolk) antibodies: IgY. The report and recommendations of ECVAM workshop 21. *ATLA* **24**, 925-934.
5. Larsson A. & Sjöquist J. (1990). Chicken IgY: Utilizing the evolutionary difference. *Comparative Immunology, Microbiology and infectious Diseases* **13**, 199-201.
6. Narat M., (2003). Production of antibodies in chickens. Food Technol Biotechnol, 41:259-267.
7. LeClaire R.D., Hunt R.E. & Bavari S. (2002). Protection against bacterial staphylococcal enterotoxin B by passive vaccination. *Infection and Immunity* **70**, 2278-2281.
8. Zuther and Lemmer, 2004, www.ma.uni-hedelberg.de/inst/phar/forschungLemmer.htm
9. ZHANG X.Y., Kurth A., Pauly P., Pauli P., Schade R., Ellerbrok H.(2008). Application of high-titred IgY antibodies in orthopox virus diagnostics *J Chinese Pharmaceutical Sciences*. 17(3). 183-191.
10. Schade R. Zhang X. and Terzolo H. R. (2003). Use of IgY-antibodies in human and veterinary medicine. In Book: Bioactive Egg Compounds, chapter 25 (ed. by Rainer Huopalahti, Rosina López-Fandino, Marc Anton and Rüdiger Schade). Springer-Verlag Berlin Heidelberg 2007
11. Fu C., Huang.H, Wang X., Liu Y., Wang Z., Cui S., Gao H., Li Z., Li J. and Kong X. (2006). Preparation and evaluation of anti-SARS coronavirus IgY from yolks of immunized SPF chickens, *J of Virol Methods*, **133**:112-115.
12. Döller P.C., Döller G. and Gerth H.J., (1987) Subtype-specific identification of influenza virus in cell cultures with IFTC labelled egg yolk antibodies. *Med Microbiol Immunol.* **176**: 27-35.
13. Gomez C.M., Biamonte S., Barragan J., Babusci M., Garavano J., Guillen G., Schade. R. (2006). Advances in agricultural Sciences. *Adv. Agric. Sci*. **10**: 25-32.

14. Larsson, A., Carlander, D. & Wilhelmsson, M. (1998). Antibody response in laying hens with small amounts of antigen. *Food and Agricultural Immunology* **10**, 29-36.
15. Boudjeroua K., Jacques M. and Dubreuil J.D. (2000). Purification of IgY by affinity chromatography using an immobilized Streptococcus suis serotype 2 immunoglobulin-bingding protein. *Int.J.Bio-Chromatogr*. **5**: 97-110.
16. Schade R., Behn I., Erhard M., Hlinak A. and Staak C, (2001). Chicken egg yolk antibodies, production andapplication: IgY technology. Springer, Berlin 1-255.
17. Kowakczyk K., Daiss J., Halpern J., Roth T.F., (1985). Quantitation of maternal-fetal IgG in the chicken. *Immunology* **54**(4):755-762.

Erklärung

Ich, ZHANG xiaoying, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema:

“Development of assays for the detection of agents with bioterroristic potency using the IgY-technology (Avian antibody) “

(In German: Entwicklung von Testsystem zum nachweis von Agenzien mit bioterroristischem Potential auf der Basis der IgY-technologie (aviären Antikörpern))

Selbst verfasst und keine anderen als die angegebenen Quellen und Hifsmittel benutzt, ohne die Hilfe Dritter verfasst und auch in Teilen keine Kopie anderer Arbeiten dargestellt habe.

Datum Unterschrift

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Lebenslauf

Mein Lebenslauf ird aus datenschutzrechtlichen Gruenden in der elektronischen Version meiner Arbeit nicht veroeffentlicht.