

# Preparation of Anti-*Aspergillus fumigatus* Antibodies in Egg Laying Hens and Their Protective Efficacy in BALB/c Mice

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**KEY WORDS:** *Aspergillus fumigatus*, BALB/c mice, IgY

## ABSTRACT

The aim of this study was to prepare anti-*Aspergillus fumigatus* antibodies in egg-laying hens and test their protective efficacy in BALB/c mice. Heat-killed *A. fumigatus* was emulsified with Freund's complete and incomplete adjuvant. Egg-laying hens were injected subcutaneously with each emulsion, with a 15-day interval between the former and the latter preparation. IgY-rich extracts were obtained from the yolk of eggs laid pre- and post-immunization using the EGGstract IgY system (Promega), pooled, dialyzed, and lyophilized. The presence of IgY anti-*A. fumigatus* antibodies in the post-immunization extracts was confirmed by ELISA. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of 2-mercaptoethanol (2-ME)-treated post-immunization extract revealed heavy and light chain bands. A 190-KD band representing IgY was obtained when the extract that was not treated with 2-ME was subjected to

PAGE in the absence of SDS. Less intense bands appeared with pre-immunization extract or post-immunization extract absorbed with heat-killed *A. fumigatus*. Only the post-immunization extract protected mice against lethal challenge with *A. fumigatus*. Death due to *A. fumigatus* in controls was confirmed by tissue homogenates culture and lung gross morphology. It appears that antibodies produced in egg-laying hens directed against *A. fumigatus* are protective in mice when given in proximity of a lethal fungal dose. A clinical trial to test their potential efficacy in immunocompromised patients might be considered.

## INTRODUCTION

*Aspergillus fumigatus* is a cosmopolitan and ubiquitous fungus, the body of which is made up of a branching network of hyphae with emerging conidia. Once these are released into the environment, they could be inhaled or ingested via contaminated food or water.<sup>1</sup> Infection with this fungus could lead to allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, or

**Table 1.** Protocol for the assessment of the protective efficacy of IgY anti-*Aspergillus fumigatus* antibodies.

	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Injected with:	A <i>fumigatus</i> + PBS	A <i>fumigatus</i> + pre-extract (24 hrs later)	A <i>fumigatus</i> + pre-extract (at the same time)	Pre-extract + A <i>fumigatus</i> (24 hrs later)	A <i>fumigatus</i> + post-extract (24 hrs later)	A <i>fumigatus</i> + post-extract (at the same time)	Post-extract + A <i>fumigatus</i> (24 hrs later)

Six mice per group; challenge dose of *A fumigatus* = 2 × LD50 = 2 × 26.3 Units = 52.6 Units; pre-extract = yolk extract from pre-immunization egg; post-extract = yolk extract from post-immunization egg; protocol was done twice: once using 5 mg protein/mouse injected intraperitoneally and the other using 2.5 mg protein/mouse.

invasive aspergillosis (IA).

The incidence of aspergillosis in immunocompromised patients has been steadily increasing over the past decades.<sup>2,3</sup> Available treatment and preventive measures are not adequate, and morbidity and mortality rates associated with this infectious disease are high, especially with the emergence of resistant *A fumigatus* strains.<sup>2,4,9</sup>

Innate immunity in the immunocompetent host, accompanied by the acquired immune response, plays a major role in clearing conidia.<sup>10</sup> It is believed that a predominant Th2 response is non-protective in aspergillosis, while a Th1 response is.<sup>11</sup> In as much as specific antibodies are concerned, those belonging to the IgG, IgA, and IgE classes are produced<sup>12</sup>; however, none of the reports dealt with the protective efficacy of these antibodies, in particular those that belong to the IgG class.

Active vaccination against *A fumigatus* might not produce an adequate immune response in immunocompromised patients. In such a situation, one would resort to passive immunization. The latter is prepared by actively immunizing an immunocompetent animal, subsequently withdrawing its blood, collecting the serum, and purifying the immunoglobulin fraction. Chick egg yolk is an alternative, inexpensive antibody source, providing passive immunization with egg yolk IgY (IgG equivalent).<sup>13-18</sup> IgY can be easily extracted in high yield

and in relatively pure form.<sup>18</sup> IgY antibodies have been reported to have prophylactic and therapeutic value against a number of infections.<sup>19,22</sup> In an earlier study done in our laboratory, IgY anti-*Candida albicans* antibodies that were produced proved to be protective in mice against lethal challenge with *C albicans*.<sup>22</sup>

Because the efficacy of immunoglobulins might vary among hosts and against different microorganisms, the aims of this study were to prepare IgY anti-*A fumigatus* antibodies in egg-laying hens, confirm their presence by ELISA, test the purity of the preparation by polyacrylamide gel electrophoresis (PAGE), and then test the protective efficacy of the IgY antibodies in mice. If proven to be protective, it would be tempting to test the effect in a clinical trial.

## MATERIALS AND METHODS

### Preparation of IgY Anti-*Aspergillus fumigatus* Antibodies

An isolate of *A fumigatus* was obtained from the diagnostic microbiology laboratory at the American University of Beirut Medical Center and cultured on Sabouraud dextrose agar at 25°C for 3 days. A suspension containing 10<sup>-3</sup> units (100 unit/mL; suspension having an absorbance of 1 at a wavelength of 480 nm) was heated at 80°C for 1 hour. The heat-killed suspension was emulsified with an equal volume of Freund's complete adjuvant; 2 mL were injected sub-

**Table 2.** Absorbances obtained in ELISA for determination of IgY anti-*Aspergillus fumigatus* antibody levels in pooled egg yolk extract.

Dilution	Undiluted	1/10	1/20	1/40	1/80	1/160	Sum of Absorbances
Absorbance (pre-hen 1)	0.554 ± 0.03	0.704 ± 0.061	0.4395 ± 0.014	0.1645 ± 0.036	0	0	1.862
Absorbance (pre-hen 2)	0.3195 ± 0.08	0.735 ± 0.202	0.1865 ± 0.046	0	0	0	1.241
Absorbance (post-hen 1)	3 ± 0	2.706 ± 0.09	1.3385 ± 0.014	0.481 ± 0.216	0.1535 ± 0.111	0	7.679
Absorbance (post-hen 2)	2.187 ± 0.610	2.547 ± 0.32	1.763 ± 0.343	0.8795 ± 0.37	0.1395 ± 0.04	0	7.516

Wavelength = 450 nm.

cutaneously into each of 2 egg-laying hens. Fifteen days later, the hens were injected subcutaneously with the same dose of the heat-killed suspension emulsified with Freund's incomplete adjuvant. Eggs laid post-primary and -secondary immunization were collected and stored at 4°C until used. Eggs collected pre-immunization served as controls. IgY was extracted from each egg yolk using EGGstract® IgY Purification System (Promega Corporation, Wisconsin, USA) according to the manufacturer's protocol. This procedure was applied to 5 post-immunization and 1 pre-immunization egg from each hen. IgY rich-yolk extracts from 5 eggs laid by hen 1 post-immunization were pooled, 5 post-immunization extracts from eggs laid by hen 2 were pooled, and IgY rich-yolk extracts from pre-immunized eggs were pooled. Each preparation was dialyzed against distilled water for a period of 4 days, lyophilized using the Freeze Dry/Shell Freeze System (Labconco, Missouri, USA), and dissolved in phosphate buffered saline (PBS, pH 7.2) to contain 10 mg/mL protein.

### Detection of IgY Anti-*Aspergillus fumigatus* Antibodies by ELISA

Fifty microliters of the heat-killed suspension of *A. fumigatus* was added to each well of an ELISA plate. The plate was then left at 4°C overnight after which wells were washed 3 times with

PBS. Fifty microliters of 1% bovine albumin in PBS was then added to each well and the plate was allowed to stand for 1 hour at room temperature. After washing, 25 µL of the yolk extract to be tested was added to each of the coated wells. The plate was then incubated at 37°C for 30 minutes and then washed. Twenty five microliters of peroxidase conjugated rabbit anti-IgY antibody was added to each well, and the plate was then incubated for 30 minutes at 37°C. After washing, 25 µL of 33'55' tetramethylbenzidine (TMB) was added to each well and the plate was placed in the dark at room temperature for 30 minutes. Finally, 25 µL of 3M NaOH was added to each well. Absorbances were determined at a wavelength of 450 nm using an ELISA reader (Automated Microplate Reader ELX 800, Bio-Tek Instruments, Vermont, USA). Absorbances were compared to those obtained when no yolk extract was added to the wells. All samples were tested in duplicates and means were calculated.

### Absorption of IgY-Rich Extracts

One milliliter of heat-killed *A. fumigatus* suspension (10<sup>-3</sup> units/mL) or 1 mL of PBS was mixed with 1 mL of IgY-rich extract (10 mg/mL) and incubated at 37°C for 1 hour. The mixture was centrifuged at 4,500 rpm for 5 minutes. The supernatants were used in PAGE. Both

**Table 3.** Protocol for the assessment of the protective efficacy of IgY rich-yolk extract (5 mg protein/ mouse).

	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Number of mice/group	6	6	6	6	6	6	6
IgY extract (mL)	0	0.5	0.5	0.5	0.5	0.5	0.5
Number of dead mice							
Day 1	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0
Day 3	2	1	1	2	0	0	0
Day 4	2	0	1	0	0	0	0
Day 5	1	2	0	2	0	0	0
Day 6	1	2	2	0	0	0	1
Day 7	0	0	0	0	0	0	0
Day 8	0	1	1	0	0	0	0
Day 9	0	0	0	0	0	0	0
Day 10	0	0	0	0	0	0	0
Total	6	6	5	4	0	0	1

Table 1 outlines group injections.

pre- and post-immunization IgY-rich extracts were absorbed.

### **Polyacrylamide Gel Electrophoresis (PAGE) of IgY Extracts**

The procedure was performed according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, California, USA).

### **Sodium Dodecyl Sulfate (SDS)-PAGE Using 2-Mercaptoethanol (2-ME) Treated-IgY Extracts**

One tenth of a milliliter of 10% SDS was added to each of the resolving gel and the stacking gel. Twenty five microliters (10 mg/mL concentration) of the yolk sac extract (pre or post) were mixed with 25 µL of loading buffer (152 µL laemmli sample buffer and 8 µL 2-ME). Thirty three microliters of this mixture were applied to the gel. A pre-stained ladder (consisting of 7 uniquely colored proteins with molecular weights ranging from 6,500–200,000 Da) was also loaded on the gel.

Electrophoresis was run using Mini-PROTEAN® 3 Cell device at 200 volts for 35-45 minutes. After the run was completed, the gel was removed from

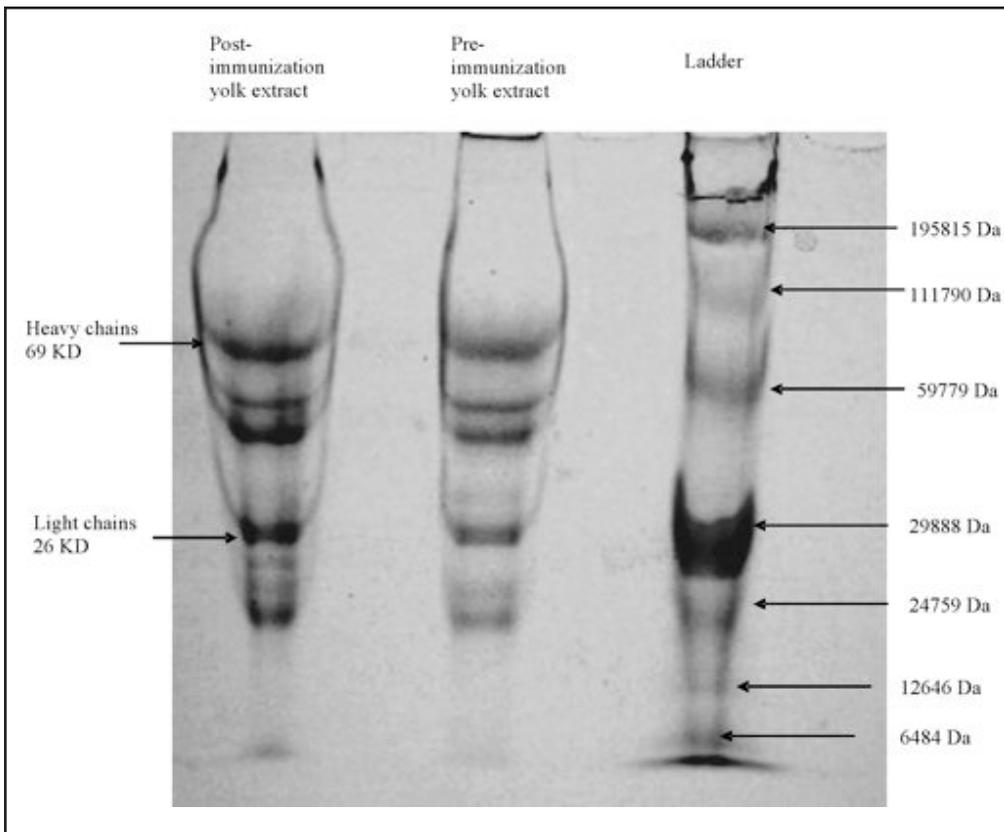
the cassette and immersed in a solution of 1% naphthalene black in acetic acid for 1 hour. The gel was destained in 7% acetic acid.

### **PAGE Using Absorbed IgY-Rich Extracts**

Fifty microliters of the supernatant was diluted with 50 µL of loading buffer. Lyophilized albumin (5 mg/mL) was used as a control and was mixed with the loading buffer (1:2 dilutions). The same procedure described above was followed for electrophoresis, except that SDS and 2-ME were not used.

### **Evaluation of the Protective Efficacy of IgY Anti-*Aspergillus fumigatus* Antibodies in Mice**

The LD50 of the *A. fumigatus* isolate was determined by the method described by Nowotny.<sup>23</sup> Forty two mice were randomly divided equally into 7 groups (A, B, C, D, E, F, and G). The protocol for the treatment of mice with an intraperitoneal injection of 5 mg per mouse of pre- or post-yolk extracts in relation to time of an intraperitoneal challenge with 2 LD50 (26.3 units × 2) per mouse of *A. fumigatus* is given in



**Figure 1.** SDS PAGE electrophoresis of mercaptoethanol treated yolk extracts. Lane 1: Post-immunization yolk extract; Lane 2: Pre-immunization yolk extract; Lane 3: Ladder. When SDS and 2-ME treatment were included, a heavy chain band having a molecular weight of about 69 KD and a light chain band having a molecular weight of about 26 KD were obtained. It can be noted that the bands obtained with post-immunization extract were more intense than those obtained with pre-immunization extract.

Table 1. The experiment was repeated using 2.5 mg per mouse.

**Gross Morphology of Lungs and Culture of Homogenized Lung Tissue from Dead Mice That Were Challenged With *Aspergillus fumigatus***

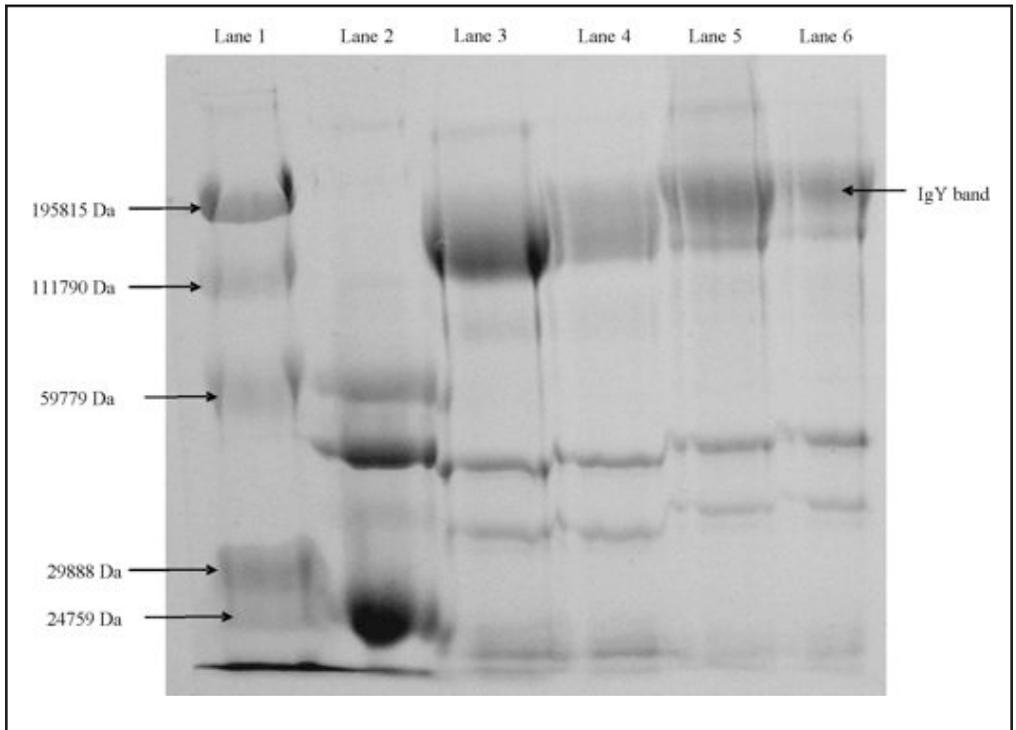
Lungs of non-immunized dead mice were removed surgically and compared to lungs obtained from sacrificed immunized mice. Moreover, lung homogenates were cultured on Sabouraud agar to confirm that death was due to *A. fumigatus*. Note that all procedures involving the animals included in this study conformed to the ILAR

Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council.

**RESULTS**

**Anti-*Aspergillus fumigatus* Antibody Levels in IgY-Rich Extracts**

The ELISA pre-immunization titers of the IgY-rich extract obtained from hen 1 and hen 2 were 1:40 and 1:20, respectively, while hen 1 and 2 post-immunized yolk extracts each had a titer of 1:80. When the sum of the degree of positivity obtained for each dilution was calculat-



**Figure 2.** PAGE electrophoresis of yolk extract. Lane 1: Ladder; Lane 2: Albumin; Lane 3: Post-immunization yolk extract; Lane 4: Post-immunization yolk extract treated with heat-killed *Aspergillus fumigatus*; Lane 5: Pre-immunization yolk extract; Lane 6: Pre-immunization yolk extract treated with heat-killed *A. fumigatus*. When SDS and 2-ME were excluded, the heavy and light chains were not dissociated and an IgY band with a molecular weight of about 190 KD was obtained. Moreover, when the absorbed post immunization extracts were subjected to electrophoresis a less intense IgY band was obtained.

ed as described by Nowotny,<sup>23</sup> about a 6-fold increase was observed when post- and pre-immunization yolk extract from both hen 1 and 2 were compared (Table 2).

### PAGE

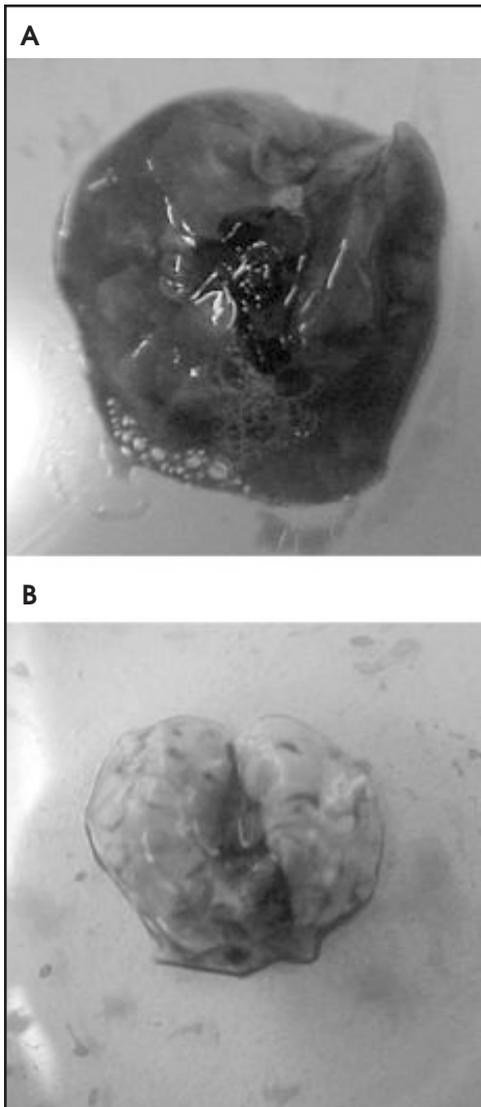
When SDS and 2-ME treatment were included, a heavy chain band having a molecular weight of about 69 KD and a light chain band having a molecular weight of about 26 KD were obtained (Figure 1). It can be noted that the bands obtained with post-immunization extract were more intense than those obtained with pre-immunization extract.

When SDS and 2-ME were excluded, the heavy and light chains were not dissociated and an IgY band with a molec-

ular weight of about 190 KD was obtained (Figure 2). Moreover, it can be observed that when the absorbed post immunization extracts were subjected to electrophoresis, a less intense IgY band was obtained. Other bands appeared in both SDS PAGE and PAGE patterns.

### Evaluation of the Protective Efficacy of IgY Anti-*Aspergillus fumigatus* Antibodies in Mice

Survival of mice treated with 5 mg protein/mouse of pre- or post-immunization yolk extract at different times with respect to challenge with 2LD50 of *A. fumigatus* is given in Table 3. All mice that were given post-immunization yolk extract with challenge or 24 hours after challenge survived, and 5 of 6 mice sur-



**Figure 3.** Lungs obtained from dead mouse challenged with *Aspergillus fumigatus* (A) and lungs obtained from normal mouse (B) The lungs from dead mice were greenish, hemorrhagic, and necrotic compared to healthy lungs. *Aspergillus fumigatus* grew when lung homogenates from dead mice were cultured on Sabbouraud agar. No growth was obtained when lung homogenates from immunized mice were cultured.

vived in the group that received post-immunization extract 24 hours prior to challenge. On the other hand, most mice treated with PBS or pre-immunization yolk extract did not survive the chal-

lenge dose. When the dose of yolk extract was decreased to 2.5 mg/mouse, no protection was observed.

### **Gross Morphology of Lungs and Culture of Homogenized Lung Tissue From Dead Mice That Were Challenged With *Aspergillus fumigatus***

The lungs from dead mice were greenish, hemorrhagic, and necrotic compared to healthy lungs (Figure 3). *Aspergillus fumigatus* grew when lung homogenates from dead mice were cultured on Sabbouraud agar. No growth was obtained when lung homogenates from immunized mice were cultured.

### **DISCUSSION**

Earlier IgY anti-human lymphocyte antibodies and IgY anti-*C albicans* antibodies were prepared in our laboratory. The former preparation was shown to be active in vitro and the latter protected mice against lethal challenge with *C albicans*.<sup>22,24</sup> The increased incidence of *A fumigatus* infections in immunocompromised patients and the limited efficacy of anti-fungal drugs, led to the preparation of IgY anti-*A fumigatus* antibodies and testing their protective efficacy in mice.

The egg-laying hen was used as the antibody producer because a considerable amount of IgY (equivalent to human IgG) produced by a hen ends up in the yolk sac of the egg and could be easily extracted in relatively pure form.<sup>25</sup> The yield is high compared to the IgG yield from serum of an immunized rabbit.<sup>25,26</sup> No other immunoglobulin class appears in the yolk sac. Moreover, hens are easier to handle and less expensive to maintain than the usual animal antibody sources such as rabbits or horses.

To demonstrate the presence of IgY anti-*A fumigatus* antibodies in the post-immunized yolk extracts, an ELISA was established and antibodies were demonstrated. There was a 2- to 4-fold increase

in the titer of post-immunization IgY-rich extract when compared to the pre-immunization extract titer. Rather than expressing results as the highest dilution of extract that is positive, Nowotny<sup>23</sup> uses the sum of the degree of positivity of each dilution to represent an estimate of the amount of antibody present. Using this approach, there was about a 6-fold increase in antibody level. Comparing the Anti-*Aspergillus* titer obtained (1:80) to those in the literature, other investigators have obtained higher titers. In fact, Chen et al<sup>27</sup> reported anti-*Aspergillus* exoantigens titers of 1:8,000 to 1:10,000. In addition, Wang et al<sup>28</sup> reported titers of 1:6,000 to 1:12,000 against *Candida albicans*. These differences might be attributed to the nature of the antigen, the strain of the chicken, the immunization protocol, and the time of egg collection.

The SDS-PAGE patterns obtained confirmed that the yolk extracts contained mainly IgY. 2-Mercaptoethanol disrupts the disulfide bonds connecting the polypeptide chains and SDS prevents their re-association. When 2-ME-treated IgY extract was subjected to SDS-PAGE, a band representing the heavy chain and another representing the light chain were obtained. Similar patterns were obtained by Hensel et al.<sup>29</sup> The fact that more intense bands were obtained with the post-immunization extract as compared to pre-immunization extract suggested that the former contains more IgY. When IgY-rich extract that was not treated with 2-ME was subjected to PAGE in the absence of SDS, a band representing IgY was obtained. A fainter band was obtained when the absorbed IgY-rich extract was used, indicating that specific IgY was removed.

Five milligrams of yolk extract per mouse, but not lower doses, protected mice against a lethal dose of *A fumigatus*. If the mechanism by which the anti-

bodies control the infection is to be speculated, the involvement of the complement system is to be ruled out because it was reported that IgY does not activate the mammalian complement system<sup>15</sup>. It was more likely that IgY behaves as an opsonin facilitating the uptake of the fungus by phagocytes. It is worth noting that protection was observed when the extract was given either 24 hours before or after challenge. This might indicate that timing might be an important criterion. It is possible that antibodies present at the time of exposure or shortly after, would be protective. On the other hand, antibodies produced late after infection might not be effective.

The current work used an artificial model of invasive aspergillosis, delivering the *Aspergillus* conidia intraperitoneally. The administration of *Aspergillus* conidia by inhalation was not carried out due to the difficulty of handling the fungal preparation for murine inhalation as well as the difficulty of mice handling during the procedure. However, invasiveness and death of mice due to the fungus occurred when the animals were injected intraperitoneally.

An important matter to raise is the fact that IgY itself is an antigen that elicits IgG and IgM production in mice.<sup>30</sup> In a study analyzing a pool of 5% human IgG for intravenous use from a large number of individuals, the amount of human IgG against chicken IgY was 1.27 mg/mL.<sup>31</sup> Hence, when IgY immunotherapeutics are considered, attention should be paid concerning risks of unwanted reactions and/or decreased efficiency in populations with a high intake of poultry meat and eggs.

In conclusion, production of protective antibodies in egg-laying hens was successful. The IgY rich-extract did not appear to contain other immunoglobulin classes but contained other proteins as

apparent in the PAGE patterns obtained. To be considered for a human clinical trial, care must be taken to avoid unwanted reactions to IgY<sup>31</sup> and the IgY must be purified in order to cut down on risks of allergic reactions in some individuals allergic to eggs.<sup>32</sup>

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