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# The Chicken and Egg System for the Development of Anti-Idiotypic Vaccines

**Angel Alberto Justiz Vaillant[1], Patrick Eberechi Akpaka[1]*, Norma McFarlane-Anderson[2], Monica P. Smikle[3] and Wisdom Brian[4]**

[1]Department of Para-Clinical Sciences, The University of the West Indies, St. Augustine, Trinidad & Tobago
[2]Department of Basic Medical Sciences, Faculty of Medical Sciences, University of the West Indies, Mona, Kingston, Jamaica
[3]Department of Microbiology, Faculty of Medical Sciences, University of the West Indies, Mona, Kingston, Jamaica
[4]School of Biology and BioChemistry, Medical Biology Centre, The Queen's University of Belfast, Belfast, UK

## Abstract

This study investigates the use of the chicken and egg system for the development of an oral HIV vaccine. Brown leghorn chickens were immunized with keyhole limpet hemocynin conjugated with a HIV-gp120 peptide (fragment 254-274). An indirect ELISA for antibodies to HIV-gp120 was used to measure anti-HIV antibody titres in the watery soluble fraction of eggs up to 14 weeks after the second week post-immunization. Over a period of 10 weeks, 3 cats were fed with the eggs from the immunized chickens and 2 cats with eggs from non-immunized chickens. An indirect enzyme linked-immunosorbent assay (ELISA) and a binding inhibition assay were used to assess the antibody response to HIV-gp120 peptide in the cat serum. The most important finding was the development of serum anti-HIV antibodies in cats fed with eggs from chickens that were positive for anti-HIV antibodies. These feline anti-HIV antibodies bound to the original HIV-gp120 peptide and also inhibited the binding of egg yolk anti-HIV antibodies to the HIV gp120 peptide, showing that the anti-HIV antibody raised in cats after feeding, was an anti-anti-idiotypic antibody. The results of this study suggest that eggs from immunized hens could be considered in the management of HIV infections.

**Keywords:** Anti-HIV antibody; gp120 peptide; Chicken egg yolk; ELISA; Anti-idiotypic antibody

## Introduction

The species traditionally chosen for antisera production are mammals especially rabbits but recently there has been a growing use of hens. Immunoglobulin (Ig)-Y is the major antibody produced by birds and have further advantages compared with mammalian IgG. There are no activation of mammalian complement system, no cross-reactivity with HAMA (human anti mouse antibody), rheumatoid factors or human blood group antigens (lack of heteroagglutinins) [1]. According to Jerne's network theory, antibodies contain in their variable region a representation of the 'universe' of antigenic structures, the idiotype. It is possible to produce an antibody against the antigen-binding site of antibodies produced to that antigen [2,3]. It was reported the development of a humoral and mucosal immune response in rabbits fed daily doses of the MOPC-315 murine IgA antibody, supporting the hypothesis that human exposure to xenogeneic antibodies, most commonly bovine milk immunoglobulins, may provoke the production of anti-idiotypic antibodies [4]. We wanted to support the same hypothesis by demonstrating the capacity of animals orally fed hyper immune eggs to induce systemic immune responses against the same idiotype. The first set of study was to demonstrate that immunized chicken with the HIVgp120 peptide produced specific anti-HIV antibodies and the second set of study was to demonstrate that cats fed anti-HIV hyper-immune eggs can develop antibodies that are able to inhibit the binding of egg yolk anti-HIV antibodies to the HIV gp120 peptide (original antigen), showing that the anti-HIV antibody raised in cats after feeding, was an anti-anti-idiotypic antibody [3].

## Material and Methods

### HIV peptide

The immunogen used in this experiment was the keyhole limpet hemocynin (KLH) conjugated to a synthetic peptide, fragment 254-274 of HIV-gp120, that has amino acidic sequence as follows: Cys-Thr-His-Gly-lle-Arg-Pro-Val-Val-Ser-Thr-Gln-Leu-Leu-Leu-Asn-Gly-Ser-Leu-Ala-Glu. This peptide is the second conserved domain of gp120, and is important for HIV infectivity and antibody neutralization [5].

### Preparation of HIV immunogens

The fragment of HIVgp120 was conjugated to KLH by the glutaraldehyde method as follows: 1 mg hemocynin was dissolved in 2 ml 0.1 M borate buffer, pH 10, in a 15-ml glass tube by gentle stirring, 1 µmol of synthetic peptide and 0.2 ml 0.3% glutaraldehyde solution were added slowly with stirring at room temperature (RT). It was allowed to stand for 2 hr at RT until a yellow coloration was observed. To block excess glutaraldehyde, 0.25 ml of 1 M glycine was added and the mixture left for 30 min at RT. The conjugate was then dialyzed against 1L 0.1M borate buffer, pH 8.3 overnight at 4°C, then against 1L of the same buffer for 8 hr at 4°C. The dialysate was stored at 4°C.

### Chicken immunization with KLH-gp120 fragment (254-274) conjugate

Two healthy layer chickens (brown Leghorn), aged approximately 6 months, were injected intramuscularly at multiple sites on the breast with the peptide-KLH conjugate. The chickens were immunized on day 0, with 0.2 µmol/ml of the peptide-KLH conjugate in 0.5 ml complete Freund's adjuvant (Sigma-Aldrich Co, St. Louis Missouri), and on days

***Corresponding author:** Dr. Patrick Eberechi Akpaka, Department of Para-Clinical Sciences, The University of the West Indies, St. Augustine, Trinidad & Tobago, Tel: +868-736-0440; Fax: +868-663-3797; Email: peakpaka@yahoo.co.uk, patrick.akpaka@sta.uwi.edu

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15, 60, and 90 with 0.2 µmol/ml of the immunogen in 0.5 ml incomplete Freund's adjuvant (Sigma-Aldrich Co, St. Louis Missouri). The eggs were collected post-immunization.

The watery soluble fraction (WSF) was separated using the Polson method [6].

### Feeding of cats with hyper-immune eggs

The anti-gp120 positive eggs were fed to five (5) adult cats, 2-3 years old (1 male and 4 female). Each cat received on average, of 2 eggs diluted in 5 volumes of soya milk weekly for 10 weeks. Of the 5 cats used in the study, 3 were fed hyper-immune eggs (anti-gp120), while the remaining 2 cats were fed eggs from non-immunized chickens. And these two cats that were fed with eggs from non immunized chickens were used as controls. Blood samples (2 ml) were collected from each cat, after completion of the feeding and were tested for anti-HIV antibodies by an indirect HIV ELISA.

### ELISA for detection of anti-HIV antibodies in eggs

The 96 well polystyrene microplates (U-shaped bottom) were coated with 50 ng of the synthetic HIVgp-120 peptide in coating buffer overnight at 4°C. The microplates were washed 4 times (PBS-Tween-20) and blocked with 3% non-fat milk in PBS, 25 µl/well, 1h, RT. The microplates were washed 4 times (PBS-Tween-20) and triplicates of 25 µl of WSF of the egg yolk in PBS non-fat milk were added. After incubation for 90 min at RT the microplates were washed 4 times and 25 µl of rabbit anti-chicken IgY-HRP diluted 1:30,000 was added and the micro plates were then incubated for 1h at RT, washed four times. Tetramethylbenzidine (TMB) solution (50 µl) was used. After a further incubation of 15 min in the dark, the reaction was stopped and read in a micro plate reader at 450 nm. Geometric mean antibody titer was calculated using Perkins method as previously reported [7].

### Indirect ELISA for detection of anti-HIV antibodies in cats

The ELISA assay described above (with modifications) was used to determine the presence of anti-HIV antibodies in cat sera. The modifications were that triplicates of 1/16 dilutions of cat serum in PBS non-fat milk were added. After incubation for 90 min at RT the microplates were washed 4X (PBS-Tween 20) and 25 µl of protein LA-HRP diluted 1:1000 (Sigma) was added and the assay follows as the previous ELISA. Protein LA-HRP (Horseradish peroxidase labelled recombinant Protein LA) is an immunoglobulin binding protein conjugated with HRP (Horseradish peroxidase) for immunodetection. It has the same effect as protein-A-HRP (Horseradish peroxidase labelled purified protein A). So this conjugate was used instead of anti-cat-HRP in this particular ELISA.

### Inhibition immunoassay of the HIVgp120-anti-HIVgp120 reaction by anti-anti-HIVgp120 antibodies

Ninety six well polystyrene microplates (U-shaped bottom, Sigma-Aldrich Co, St Louis Missouri) were coated with 50 µl/well of 1 ng/µl solution of gp-120 peptide (Sigma) in carbonate-bicarbonate buffer pH 9.6 (Sigma) for 4 hours at 37°C. The micro plates were then washed 4 times with PBS Tween-20 and blocked (3% non-fat milk in PBS, 25 µl/well, 1h, RT). Triplicates of serial doubling dilutions of cat serum diluted in PBS-3% non-fat milk, pH 7.4 were added to the micro plate which then was incubated for 90 min at 37°C. The micro plates were washed 4 times with 150 µl/well of PBS-Tween 20 and 25 µl of a WSF containing anti-HIV-gp120 antibodies (titer = 1:1024) was added to each well. After adding WSF and following incubation (90 min, 37°C) the microplate were washed 4 times and 25 µl of a 1:30,000 dilution of rabbit anti-chicken IgY-HRP (Sigma-Aldrich Co) was added to each well. The micro plate was then incubated at 37°C for 1 h, before final washing (PBS-Tween 20, 4 times) and 25 µl of TMB added before incubation in the dark for 15 min. The reaction was stopped with 3M $H_2SO_4$. The micro plates were read at 450 nm in a micro plate reader.

**Ethical approval:** The protocol for this study was approved by the Ethics Committee of the Faculty of Medical Sciences, The University of the West Indies, Mona Campus, Jamaica.

**Statistical analysis**: The chi-squared test and Fisher's exact test (Epi Info 3.5.3 software, CDC, Atlanta, GA, USA) were used as appropriate to compare the percentage of inhibition of the HIV-gp120 and anti-HIV-gp120 reaction by anti-anti-idiotypic HIV-gp120 antibodies. A P value <0.05 was considered significant.

## Results

### Indirect enzyme linked immunosorbent assay for detection of anti-HIV-gp120 antibodies in egg yolk and anti-anti-HIV-gp120 in cat serum

The geometric mean antibody titer was 1024 and shows a high post-immunization specific antibody titer in all egg samples tested (Table 1). The indirect ELISA for the detection of anti-HIV gp120 peptide showed that all of the samples of egg yolk that were tested contained anti-HIV-gp120 antibodies. Antibodies were produced from week 2 post-immunization and continued through week 12 (Table 2).

The 3 cats that were fed anti-HIV-gp120 positive eggs produced antibodies that reacted with the synthetic HIV-gp120 peptide in the indirect ELISA (Table 3). The readings obtained using sera from cats fed eggs from non-immunized chickens were below the cut-off point.

### Inhibition of the HIV-gp120 and anti-HIV-gp120 reaction by anti-anti-idiotypic HIV-gp120 antibodies

The binding inhibition assay showed 10% -16.2% inhibition of the binding of avian anti-HIV-gp120 antibodies (Ab-1) to immobilised HIVgp120 peptide by anti-HIV-gp120 antibodies (Ab-3) present in triplicates serum samples of cats tested, suggesting that the feline anti-

| No of egg samples | Antibody titer | $Log_{10}$ |
|---|---|---|
| 3 | 512 | 8.1279 |
| 3 | 1024 | 9.0309 |
| 3 | 2048 | 9.9339 |
| 9 | | 27.0927 |

$Log_{10}$ Geometric mean titer = 27.0927/9 = 3.0103. Geometric mean titer=1024. The geometric mean antibody titer was calculated according to Perkins method [17].

**Table 1**: **Geometric mean antibody titer detection of anti-HIV antibodies in eggs**

| Post Immunization (Weeks) | Mean OD (±SD) at 450 nm |
|---|---|
| 0 | 0.17 (±0.018) |
| 2 | 0.82 (±0.03) |
| 8 | 0.80 (±0.046) |
| 12 | 0.86 (±0.007) |

OD = Optical Density; SD = Standard Deviation
The cut-off point 0.445 demonstrates the development of anti-HIV antibodies from 2 weeks post immunization.

**Table 2**: **Indirect ELISA detection of anti-HIV gp120 antibodies in chicken's egg yolk**

| Animals | Mean OD (± SD) at 450 nm | Results |
|---|---|---|
| Cat 3 | 0.43 (± 0.001) | Positive |
| Cat 4 | 0.51 (± 0.02) | Positive |
| Cat 5 | 0.38 (± 0.02) | Positive |
| Cat 1 (control, non fed) | 0.25 (± 0.03) | Negative |
| Cat 2 (control, non fed) | 0.27 (± 0.02) | Negative |

OD = optical density; SD = standard deviation
The cut-off point = 0.35, was calculated as a mean of the negative controls + (2 x SD). Mean values were calculated from the absorbance values of triplicate samples.

**Table 3:** Results of detection of anti-anti-gp120 in the Cats' serum by ELISA

| Fed animals | Mean of % inhibition in fed animals ( ±SD ) | Results |
|---|---|---|
| Cat 1 | 13.0 ±0.3 | Positive |
| Cat 2 (fed) | 16.2 ± 0.7 | Positive |
| Cat 3 (fed) | 10.0 ± 0.5 | Positive |
| Cat 4 (non fed) | 1.37 ± 0.07 | Negative |
| Cat 5 (non fed) | 1.63 ± 0.04 | Negative |

The results of this experiment suggest that the feline anti-HIV-gp120 antibodies were anti-anti-idiotypic antibodies, able to inhibit the binding of HIV-gp120 peptide to anti-HIV-gp120 antibodies. The results showed statistically significant difference between the means of % inhibition of fed and non-fed animals, p = 0.015.

**Table 4**: Inhibition of the HIV-gp120 peptide and anti-HIV-gp120 Ab reaction by anti-anti-idiotypic HIV-gp120 antibodies

HIV-gp120 antibodies were anti-anti-idiotypic antibodies (Table 4). This inhibition was not observed with sera of the cat that were used as controls (non-fed animals). The percentage of inhibition of the HIV-gp120 and anti-HIV-gp120 reaction by anti-anti-idiotypic HIV-gp120 antibodies were compared and it was noted to be significantly different (p = 0.003).

## Discussion

The experiments described in this study may represent the first report of a chicken and egg system for production of anti-HIV-gp120 antibodies. Eggs from chickens immunized with a specific immunogen might be considered as a special type of oral anti-idiotypic vaccines. This was shown by the development of anti-anti-HIV gp120 antibodies in cats fed anti-HIVgp120 positive eggs. The presence of these antibodies could prove useful in exploring the development of an oral active immuninization to fight HIV in asymptomatic carriers and patients with AIDS. The authors are not aware of documented studies describing the use of anti-HIV positive eggs as oral anti-idiotypic vaccine. The use of anti-idiotypic vaccines in HIV/AIDS, using a monoclonal antibody (mAb 13B8.2) directed against the CDR3-homologous CD4/D1 region has previously been reported in literature [8]. It has also been reported that HIV-1 gp160-specific secretory IgA was detected in the saliva of all rabbits orally immunized with HIV-immunosomes, these antibodies neutralized HIV infectivity *in vitro* [9].

*In vivo* administration of eggs containing anti-HIV-gp120 antibodies induced anti-anti-idiotypic antibodies, which recognized the epitope defined by peptide 254-274 of the HIV gp120. This indicated the presence of idiotypic antibodies, Ab1 and anti-idiotypic antibodies (Ab2) in eggs of chickens immunized with the HIV-gp120 peptide-KLH conjugate (which was able to stimulate the production of Ab3, when orally administered). In literature, study by other workers reported the development of an idiotype-anti-idiotype network of antibodies to bovine serum albumin (BSA) in eggs from chickens immunized with BSA [10]. Our study showed percentages of inhibition that were statistically significant, that were an indication of the presence of anti-anti-idiotypic antibodies (Ab3) against the HIV-gp120 fragment, which inhibited the formation of complexes between HIV-gp120 peptide and anti-HIVgp120 idiotypic antibodies. It was reported that intramuscular immunization of BALB/c mice with anti-idiotypic HIV gp160 peptide antibodies induced anti-anti-peptide antibodies (Ab-3), which also recognized a recombinant HIV gp160 preparation, and also inhibited its interaction with anti-HIV-gp160 antibodies (Ab1) [11].

The fact that antibodies belonging to the IgA and IgM isotypes are found in egg whites might enhance the immunogenic capability of the whole egg. In addition, immunization by oral routes is more 'user friendly'. Antibodies given by mouth are subject to digestion but there is some evidence that antibody protein rich solutions such as egg yolks are more resistant to enzymatic digestion [12,13]. The effectiveness of a therapeutic HIV vaccine could be established in a clinical trial in humans. Parameters of the humoral immunity (e.g. titre of neutralizing anti-HIV antibodies) and cellular immunity (e.g. induction of specific anti-HIV CD8+ T cells) should be evaluated. The immunogen used to immunize chickens, gp120 peptide conjugated KLH, was an example of peptide vaccine [14,15], which induced the presence of antibodies against the original peptide; fragment 254-274 of the HIV gp120 in chicken eggs. The potential HIV vaccine proposed in this work ("egg vaccine") has the advantage of being both an oral and an anti-idiotypic vaccine. Oral vaccines in general have the capacity to induce mucosal immunity, which involve strong humoral and cellular reactions [4] and therefore it could be a better candidate for a HIV therapeutic vaccine. Future work should prove the development of cellular immunity in recipients of anti-HIV positive eggs. In addition, the egg can be considered a complete nutrient as it provides several proteins with anti-microbial properties such as lysozyme [16], and antioxidants such as phosvitin [17]. These molecules along with antibodies would enhance the anti-HIV immunity in HIV+ carriers, improving their quality of life. It is expected that the egg, which is a safe product does not have the potential side effects produced by the ant-retroviral therapy employed in HIV infections.

## Conclusion

Clearly, the results of the preliminary experiments strongly suggest that the chicken and egg system is a potential and novel approach for the development of anti-idiotypic vaccines that could prove useful in the treatment of microbial infections including HIV infections.

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