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Feeding Eggs from Hens Immunized with Specific KLH-Conjugated HIV Peptide Candidate Vaccines to Chicks Induces Specific Anti-HIV gp120 and gp41 Antibodies that Neutralize the Original HIV Antigens

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Isolation of antibodies from the egg yolk of chickens is of particular interest as a source of specific antibodies for oral administration to prevent infections and use them as immunodiagnostic reagents. This study assessed the hypothesis that immunization with human immunodeficiency virus 1(HIV-1) viral peptides could induce a potent immune response that could be evaluated in chicken eggs. **Methods:** Nine healthy brown Leghorn layer hens (3 per HIV immunogen), aged 7 months, were immunized intramuscularly at multiple sites on the breast with specific KLH-conjugated HIV peptide candidate vaccines. The anti-HIV antibody response was measured using ELISA. Chicks were fed HIV hyper-immune eggs and their blood was collected for testing for anti-anti-idiotypic antibodies that neutralize the original HIV antigen using an inhibition ELISA. **Results:** The immunogenicity results showed that HIV peptide vaccines effectively produced strong anti-HIV immune responses in immunized brown Leghorn layer hens. The binding inhibition assay showed that 13.9%–20.3% inhibition of the binding of avian anti-HIV gp 41 (fragment 579-601) or anti-HIV-gp120 antibodies (Ab-1) to immobilized HIVgp41, HIVgp120 peptide by anti-HIV gp 41 (fragment 579-601) or anti-HIV gp120 (fragments 308-331 or 421-438) antibodies present in serum sample replicates of chicks tested, suggesting that the chicks anti-HIV gp 41 (fragment 579-601) or anti-HIV-gp120 antibodies (fragments 308-331 or 421-438) were anti-anti-idiotypic antibodies. This inhibition was not observed in the sera of chicks that were not fed with the hyperimmune eggs. **Conclusion:** Feeding chicks with hyperimmune eggs could potentially stimulate the production of anti-anti-idiotypic antibodies that can neutralize the original HIV antigen (gp120 or gp41). This could be an avenue for immunotherapy to improve the fight against HIV infections. However, more studies and clinical trials are required to demonstrate similar human immune responses.

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INTRODUCTION

Anti-idiotypes that possess an internal image of antigens can produce effective humoral immune responses toward microbes [1]. Immunization with anti-idiotypic vaccines may prevent established infections [2]. Human immunodeficiency virus infects CD4+ T cells by attaching HIV gp120 to CD4 receptors on T cells [1]. In this study, we used HIV peptides as immunogens to create specific anti-HIV antibodies in layer chickens. Then, feeding chicks with anti-HIV hyper-immune eggs, and evaluate the effect of this oral immunization *in vitro*.

Several ELISAs were used in this study to assess the immunogenicity of HIV peptides in vivo. Chicken are used as laboratory animals because it is well known that chicken IgY has therapeutic capabilities [3].

It has been documented that oral administration of IgY has been used in immunotherapy of a variety of infections, including *Escherichia coli*, *Salmonella spp*., human rotaviruses, *Staphylococcus aureus*, and coronaviruses [4]. To the best of our knowledge, the capacity of IgY from birds to confer active

immunity has not been reported in the literature, even though it is well known that IgY technology has demonstrated the suitability of IgY from birds to treat infections or diagnostically [5]. In this study, we demonstrate that IgY is a versatile protein that can be used to prevent experimentally HIV infections.

MATERIALS AND METHODS

Ethics Statement

Ethical approval was granted by the Campus Ethics Committee of the University of the West Indies, Mona Campus. The laboratory work described was conducted under EU Directive 2010/63/EU for experiments.

Immunogenicity Studies of Experimental HIV Vaccine Candidates in Chickens

All grade reagents used in this study were commercially available from Sigma-Aldrich, (USA). The study was repeated 3 times and similar results were obtained. The HIV immunogens used in these experiments were keyhole limpet hemocyanin (KLH) conjugated to the following HIV peptides:

HIV gp 41 (fragment 579-601): Arg-lle-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly lle-Trp-Gly Cys-Ser-Gly Lys [6].

HIV gp120 (308-331): Asn-Asn-Thr-Arg-Lys-Ser-lle-Arg-lle-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-lle- Gly-Lys-lle-Gly [7].

HIV gp120 (421-438): Lys-Gln-Phe-lle-Asn-Met-Trp-Gln-Glu-Val-Gly-Lys-Ala-Met-Tyr-Ala-Pro-Pro [8].

Dimerization of HIV Peptides and Preparation of HIV Immunogens

The C-terminal cysteine was added to the amino acid sequences of HIV peptides (fragment 579-601 of HIV gp 41, and fragments 308-331 and 421-438 of HIV gp120). These peptide fragments were dimerized via cysteine oxidation in dimethyl sulfoxide [9].

Chicken Immunization

Nine healthy brown Leghorn layer hens (3/ HIV immunogen), aged seven months, were immunized intramuscularly (IM) at multiple sites on the breast with a specific KLH-conjugated above-mentioned HIV peptide vaccine. Chickens were vaccinated on day 0, with 0.5 mg/mL of the immunogens in 0.5 ml of complete Freund's adjuvant (Sigma-Aldrich), and on days 14, 28, and 45 after the first immunization, hens received booster doses of 0.25 mg/mL of the immunogen in 0.5 mL incomplete Freund's adjuvant. Eggs were collected daily before and after the immunization. Additionally, 9 healthy brown Leghorn chickens were fed hyper-immune eggs for 45 days (3 birds for each above-mentioned candidate vaccine. Likewise, 3 healthy brown Leghorn chickens were fed non-hyper-immune eggs for 45 days, and their response to each peptide was measured. The procedure was blinded so the scientists did not intervene in the feeding of the birds nor knew none of the groups.

Feeding of Chicks

Feeding of chicks with HIV hyper-immune eggs with a titer of 1:15000 was done to 15 chicks aged zero and divided into three groups fed for one month. The first group of 5 chicks was supplied with hyper-immune eggs against fragment 579-601 of the HIV gp 41 (fragment 579-601) peptide and corn. Likewise, the second group of five chicks was fed a hyper-immune egg against segments 308-331 of the HIV gp120 peptide and corn. The third group of five chicks was supplied with a hyper-immune egg against fragments 421-438 of the HIV gp120 peptide and corn. An additional ten chicks were divided into two groups: a group of five chicks that received non-hyperimmune HIV eggs and corn, and the last group of five chicks was fed with only corn. At the end of the feeding schedule, blood samples were obtained from all the chicks and investigated using an inhibition ELISA. The water-soluble fraction (WSF) contains an elevated IgY concentration which is separated from the lipid content by the partial application of the Polson methodology [10], which uses only chloroform separation.

ELISA for Anti-HIV Peptide Antibodies

The 96-well polystyrene microplates (U-shaped bottom, Sigma-Aldrich) were coated with 100 ng of fragment 579-601 of HIV gp 41, fragments 308-331, or fragment 421-438 from HIV gp120 in coating buffer for 4 h at 37°C. Each microplate was washed four times with 10% PBS-Tween 20, and the blocking solution (3% non-fat milk in PBS) was added to each well (51 µL). The microplates were incubated for 1.30 h at RT. The microplates were washed as previously described. Fifty microliters of WSF diluted 1:50 with the sample diluent was added to the wells. Each microplate was then incubated for 1 h at RT and washed four times as above. Then, 50 µL of horseradish peroxidase-labeled anti-IgY conjugate (Sigma-Aldrich) diluted 1:30,000 was poured into each well. The microtiter plates were incubated again for 1 h at RT and washed four times. A volume of 50 µL tetramethylbenzidine (TMB, Sigma-Aldrich) was added. After further incubation for 16 min in the dark, the reaction was stopped with a solution of 3M HCl, and each microplate was read in a microplate reader at 450 nm. The cut-off points of ELISAs for the detection of the anti-HIV peptide (579-601), anti-HIV peptide (308-331), and anti-HIV peptide (421-438) were 0.42, 0.40, and 0.44, respectively.

Positive and negative controls were homemade. Four positive controls were used in each assay, prepared from egg yolk samples with the highest titers of specific anti-HIV peptide antibodies, and their OD values were between 1.20 and 1.50 at 450 nm. Four negative controls were used in each assay. They were prepared from the egg yolks of non-immunized birds, and they showed OD values of 0.170-0.20 at 450 nm. Three replicates of each WSF sample per bird, collected on day 60, were assayed for the presence of anti-HIV peptide antibodies using the above ELISA, standardized using commercially available reagents and materials (Sigma-Aldrich Co, USA).

Inhibition Immunoassays

Ninety-six-well polystyrene microplates (U-shaped bottom, Sigma-Aldrich) were coated with 50 µL/well of 1 ng/µL solution of fragments 308-331 or 421-438 from HIV gp120 peptides or fragment 579-601 of HIV gp41 (Sigma-Aldrich) in carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich) for 4 h at 37°C. The microplates were then washed four times with PBS Tween-20 and blocked with 3% non-fat milk in PBS, 25 µL/well, 1 h, RT). Triplicates of serial doubling dilutions of chick sera diluted in PBS 3% non-fat milk (pH 7.4) were added to the microplate, which was then incubated for 90 min at 37°C. The microplates were washed four times with 150 µL of PBS-Tween 20, and 25 µL of a WSF with anti-HIV gp120 (fragments 308-331 or 421-438; Sigma-

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Aldrich Co, USA) antibody titer of 1:5000 or anti-HIV gp41 (fragments 579-601; Sigma-Aldrich Co, USA) with an antibody titer of 1:1000 were added to each well. Following incubation for 90 min at 37°C, the microplates were washed 4X and 25 µL of a 1:30,000 dilution of rabbit anti-chicken IgY-HRP was added to each well. The microplates were then incubated at 37°C for 1 h before final washing (PBS-Tween 20, 4 times), and 25 µL of TMB was added before incubation in the dark for 15 min. The reaction was stopped using 3M H2SO4. Microplates were read at a wavelength of 450 nm using a microplate reader. The percentage inhibition (%I) was calculated using the following formula. The inter-assay coefficients of variation were then calculated.

$$\%I = 100 - \frac{\text{XOD of sample-XOD of blanks}}{\text{XOD 100\% HIVgp120 or HIVgp41 peptide binding to anti-gp120 or anti-gp41- XOD of blanks}} \times 100$$

RESULTS

Effectively Production of strong Anti-HIV Immune Responses in Hens

Table 1 shows the water-soluble fractions (WSF) of egg yolks collected at zero and 60-days post-immunization were assayed for the presence of anti-HIV peptide antibodies using ELISA (6 samples in total were assayed which were evaluated 3 times). These immunogenicity results showed that HIV peptide vaccines effectively produced strong anti-HIV immune responses in immunized brown Leghorn layer hens. There was a statistically significant difference ($P < 0.01$) that explains the notable degree of difference (related to the anti-HIV antibody levels in the egg yolks) between pre-immunized and post-immunized birds in the 3 experimental vaccines. These immunogenicity results showed that HIV peptide vaccines effectively produced a strong anti-HIV immune response in the immunized brown Leghorn layer hens.

Table 1. Results of immunogenicity studies of experimental HIV vaccine in brown Leghorn layer hens.

Candidate Vaccines	XOD (SD), day 0 (Pre-immunized birds)	XOD (SD), 45 days post-Immunized Birds	*P*-value
HIV gp 41 (fragment 579-601) 3 birds	0.170 (0.021)	0.885 (0.044)	<0.001
HIV gp120 (fragment 308-331) 3 birds	0.156 (0.015)	0.910 (0.023)	<0.001
HIV gp120 (fragment 421-438) 3 birds	0.188 (0.01)	0.865 (0.037)	<0.001

Table 2. Inhibition experiments. Mean of binding inhibition percentage (X%I), and Standard deviation (SD).

Candidate vaccines	X%I (SD) of non-fed chicks with hyper-immune eggs and corn	X%I (SD) of fed chicks with hyper-immune eggs and corn (15-days post-immunization)	*P*-value
HIV gp 41 (fragment 579-601)	3.13 (0.37)	18.05 (2.40)	0.007
HIV gp120 (fragment 308-331)	2.41 (0.63)	19.62 (3.13)	0.009
HIV gp120 (fragment 421-438)	3.19 (0.51)	13.92 (3.96)	0.041

Table 2 shows the results of the inhibition of fragments of HIV gp120 (fragments 308-331 or 421-438) or HIV gp41 (fragment 579-601) and anti-HIV gp120 or anti-HIV gp41 reactions by anti-anti-idiotypic HIV gp120 (fragments 308-331 or 421-438) or HIV-gp41 antibodies, respectively. This inhibition was statistically significant ($P < 0.05$).

The binding inhibition assay showed that 13.9%–20.3% inhibition of the binding of avian anti-HIV gp 41 (fragment 579-601) or anti-HIV-gp120 antibodies (Ab-1) to immobilized HIVgp41, HIVgp120 peptide by anti-HIV gp 41 (fragment 579-601) or anti-HIV gp120 (fragments 308-331 or 421-438) antibodies present in serum sample replicates of chicks tested, suggesting that the chicks anti-HIV gp 41 (fragment 579-601) or anti-HIV-gp120 antibodies (fragments 308-331 or 421-438) were anti-anti-idiotypic antibodies. This inhibition was not observed in the sera of chicks that were not fed with hyperimmune eggs. This confirms the hypothesis that feeding chicks with hyperimmune eggs stimulates the production of anti-anti-idiotypic antibodies that neutralize the original HIV antigen (gp120 or gp41). Table 3 shows that chicks fed with corn and water failed to inhibit the binding or reactivity of fragments of HIV gp120 (fragments 308-331 or 421-438) or HIV gp41 (fragment 579-601) and anti-HIV gp120 (fragments 308-331 or 421-438) or anti-HIV gp41 (fragment 579-601) by anti-anti-idiotypic HIV gp120 (fragments 308-331 or 421-438) or HIV-gp41 antibodies, respectively; and again the chicks fed with hyper-immune eggs inhibited successfully the reactivity, suggesting that the anti-anti-HIV antibody against each fragment of gp120 or gp41 was able to recognized the original antigen and therefore reacting and protecting against it.

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Table 3. Inhibition of fragments of HIV gp120 or HIV gp41 and anti-HIV gp120 or anti-HIV gp41 reaction by anti-anti-idiotypic HIV gp120 or HIV-gp41 antibodies, respectively.

Candidate vaccines	X%I (SD) of non-fed chicks with hyper-immune eggs (given corn and water)	X%I (SD) of fed chicks with hyper-immune eggs and corn (30-days post-immunization)	*p*-value
HIV gp 41 (fragment 579-601) 3 birds	3.13 (0.37)	20.08 (3.23)	0.011
HIV gp120 (fragment 308-331) 3 birds	2.41 (0.63)	18.55 (2.47)	0.005
HIV gp120 (fragment 421-438) 3 birds	3.19 (0.51)	16.79 (3.85)	0.024

DISCUSSION

Eggs from the pre-immunized and post-immunized healthy brown Leghorn layer hens immunized IM at multiple sites on the breast with three specific KLH-conjugated HIV peptide vaccines on days 0, 15, 30, and 45 were collected and the WSF of each egg was evaluated by ELISA. The coefficients of variation (inter-assays) were less than 10%. This suggests that the assays were reproducible. We could not compare our HIV tests with a commercially prepared assay because such a study was not available at the time of this study. We showed that feeding chicks with anti-idiotypic HIV gp41 (fragment 579-601) or gp120 peptide antibodies induced anti-peptide antibodies (Ab-3), which also recognized a recombinant HIV gp41 (fragment 579-601) or HIV gp120 (fragments 308-331 or 421-438), and also inhibited its interaction with anti-HIV-gp41 or anti-HIV-gp120 antibodies (Ab1). This study provides evidence that is supported by a previous study in which intramuscular immunization of BALB/c mice with anti-idiotypic HIV gp160 peptide antibodies induced anti-peptide antibodies (Ab-3), which also recognized a recombinant HIV gp160 preparation, and inhibited its interaction with anti-HIV-gp160 antibodies (Ab1) [11]. 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 the literature [12]. Our results are also supported by a study on the development of an idiotype-anti-idiotype network of antibodies to bovine serum albumin (BSA) in eggs from chickens immunized with BSA [13]. 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, neutralizing HIV infectivity in vitro [14]. Our study showed a statistically significant percentage inhibition. Oral immunizations had a p-value < 0.05, which indicated the presence of anti-anti-idiotypic antibodies (Ab3) against HIV gp120 (fragments 308-331 or 421-438) and HIV gp 41 (fragment 579-601) fragments, which inhibited the formation of complexes between HIV gp120 (fragments 308-331 or 421-438) peptide or HIV gp 41 (fragment 579-601) and anti-HIVgp120 or anti-HIV gp 41 (fragment 579-601) idiotypic antibodies, respectively.

Boudet *et al*. (1992) reported that one of the well-characterized motifs mapped to a loop within the third hypervariable region (V3) of the exterior envelope glycoprotein gp120 at amino acid positions 308–331 and is referred to as the principal neutralizing determinant (PND). The sequence of this V3 loop raises the immunogenicity and diversity of the antibody response to PND. We show here that this neutralization-related motif is highly immunogenic in HIV-positive subjects and experimentally immunized primates and rodents subjected to various anti-HIV immunization regimens [15]. Our study showed that this peptide could generate a robust immune response in chickens, consistent with previous research by Boudet et al. The immunogenicity study was statistically significant (p <0.05), suggesting that it could be a good immunogen for a candidate vaccine.

Bell *et al*. (1992) reported the use of a series of overlapping synthetic peptides derived from a conserved region of the envelope gp41 (aa 572-613). They identified an immunodominant 12-mer peptide sequence, gp41(8) (aa 593-604), which consistently elicited both T cell blastogenic and antibody responses in asymptomatic HIV-seropositive individuals but not in ARC and AIDS patients [16]. Linear regression analysis showed that in asymptomatic persons, there was a strong positive correlation (P less than 0.0005) between the absolute CD8+ T cell and the magnitude of blastogenic responses to gp41(8) (aa 593-604) [16]. These experiments suggest that the involved peptides have a high immunogenicity capacity, as suggested by our study. Therefore, the fragment HIV gp 41 (fragment 579-601) (572-613) is a promising vaccine candidate, as shown in our study in immunogenicity studies and binding inhibition assays.

The first (B138) is linear and spans the envelope residues 421-438; the second (1005/45) encompasses amino acids 418-445 and is cyclized by a disulfide bond joining its terminal cysteines. Both species have been shown to inhibit syncytia formation in a conventional bioassay, with B138 being the most efficient. Both peptides elicit high titers of anti-peptide antibodies in immunized mice, rabbits, and goats, with titers exceeding 1:10 in many cases [8]. Our study does not show that anti-idiotypic antibodies inhibit syncytia formation, because this was not our aim. We are not aware of the literature on other immunogenicity studies performed with the HIV gp120 peptide fragments 308-331 or 421-438.

In conclusion, we hypothesized that feeding chicks with hyperimmune eggs would stimulate the production of anti-anti-idiotypic antibodies that neutralize the original HIV antigen (gp120 or gp41). Therefore, it may be an avenue for immunotherapy to improve the fight against HIV infections. However, more studies and clinical trials are required to demonstrate similar human immune responses as observed in our experimental birds.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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