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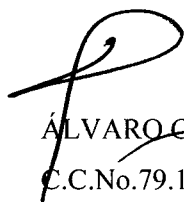
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Solicitud de Patente de Invención titulada: "Vacunas multivalentes contra la  
influenza aviar"

Solicitante: Wyeth

**SOLICITUD DE EXAMEN DE PATENTABILIDAD SEGÚN EL ARTÍCULO 44 DE LA DECISIÓN**  
**486. GACETA NO. 580**

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado según aparece al pie de mi firma, en mi condición de apoderado de la sociedad arriba mencionada, solicito que se examine la invención de la referencia según los requisitos de patentabilidad previstos en la Decisión 486 de la Comisión de la Comunidad Andina de Naciones. Adjunto recibo de caja por valor de \$420.000 para cubrir la tasa correspondiente según la Resolución 44372 del 26 de diciembre de 2007.

Atentamente,

  
ÁLVARO CORREA ORDÓÑEZ  
C.C.No.79.143.366 de Usaquén  
T.P.20.281

Anexo: Recibo de caja No. 08-19059 por valor de \$420.000

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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FECHA : MARZO 3 DE 2008

\*\*\*\*\* CONSIGNACION \*\*\*\*\*

| DEPOSITANTE      | TIPO PAGO    | BANCO           | CUENTA    | Nº. PAGO | FECHA PGO  | Vr. PAGO      |
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| BAKER & MCKENZIE | CONSIGNACION | BANCO DE BOGOTA | 062754387 | 2924733  | 03/03/2008 | 67,398,000.00 |

\*\*\*\*\* CONCEPTO \*\*\*\*\*

| CANT. | RENTISTADO  | CONCEPTO                              | TOTAL CONCEPTO |
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| 1     | 50005-01-01 | SOLICITUDES                           |                |
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**DIRECCIÓN DE NUEVAS CREACIONES**

RADICACIÓN EXPEDIENTE No. PCT 07-34174

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **580** DE

FECHA **28 DE SEPTIEMBRE DE 2007** EL TÉRMINO HÁBIL SEÑALADO POR

LA LEY EXPIRA EL **27 DE DICIEMBRE DE 2007**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

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## Efficacy of avridine and liposomes as adjuvants for avian influenza virus antigens in turkeys

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### SUMMARY

Four-week-old turkeys were vaccinated with H4N8, H5N2 and H7N3 inactivated avian influenza viruses (AIV) in one of the following adjuvants: avridine, positively charged liposomal avridine, liposomal avridine with covalent attachment to the AIV antigens and oil-emulsion; and without any adjuvant. Two vaccinations were given at a 4-week interval. Antibody concentrations in sera and in respiratory lavages were measured over a period of 8 weeks. The turkeys responded with high cumulative geometric mean HI titres to the H4N8, H5N2 and H7N3 components of the vaccines, while the cumulative geometric mean HI titres for the antigens without adjuvant were significantly ( $P < 0.05$ ) lower.

The most efficacious adjuvant, positively charged liposomal avridine, gave the highest local immune response as measured by total antibody specific to AIV in respiratory lavages. The difference in weight gains between vaccinated and non-vaccinated turkeys was significant ( $P < 0.05$ ). Virus isolations 2 weeks post-challenge decreased in vaccinated turkeys.

### INTRODUCTION

Avian influenza (AI) is a disease of considerable economic importance. Inactivated whole virus vaccines containing the currently prevalent virus strains have been used to immunize chickens and turkeys in the control of avian influenza (Eskelund, 1984). Several studies have demonstrated the ability of inactivated whole AI vaccines constructed as water-in-oil emulsions to protect chickens and turkeys against the disease (Homme & Easterday, 1970; Allan *et al.*, 1971; Gough *et al.*, 1975; Bahl & Pomeroy, 1977; Butterfield, 1978; Butterfield & Campbell, 1978; Bahl *et al.*, 1979; Brugh *et al.*, 1979).

One disadvantage of the mineral oil in oil-emulsion vaccines is that it is non-biodegradable and tends to leave residues in the tissues. The protective efficacy of inactivated vaccines is further limited by their low immunogenicity especially in unprimed hosts and their inability to induce cytolytic T cell (Tc) responses (Jones *et al.*, 1988). Thus, there is a need for improved AI vaccines. One way of improving AI vaccines is by incorporating other immunological adjuvants such as avridine and liposomes as components of inactivated vaccines.

Avridine is a small, synthetic lipoidal amine [*N,N*-dioctadecyl-*N,N*-bis(2-hydroxyethyl)-propanediamine], previously described as CP-20961, and has been shown to have

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interferon-inducing properties (Hoffman *et al.*, 1973). It also has potent adjuvant effects with a wide range of viral, bacterial, protozoan and other antigens (Anderson & Reynolds, 1979; Siddiqui *et al.*, 1981; Knudsen, 1982; Jensen, 1986). It is a macrophage-activating agent (Jensen, 1986), can enhance the bactericidal activity of neutrophils (Woodard *et al.*, 1983) and enhance lymphocyte cytotoxicity (Jensen, 1986). Avridine has been found to be most efficient as an adjuvant when incorporated into liposomes (Pierce & Sacci, 1984).

## MATERIALS AND METHODS

### Vaccine virus antigens

The three influenza viruses used in the preparation of the trivalent vaccines were H4N8 (A/Turkey/MN/1164/80); H5N2 (A/Turkey/MN/1700/82); and H7N3 (A/Turkey/Ontario/5254/81). Each subtype virus was propagated in 10-day-old embryonated chicken eggs at 37°C for 72 h. The harvested allantoic fluid (AF) was clarified by centrifugation at 2000 *g* for 30 min at 4°C. The viruses in the AF were inactivated with beta-propiolactone (Brugh *et al.*, 1979) and the absence of residual infection was confirmed by egg inoculation.

To maintain the integrity of the vaccine antigens, thimerosal (merthiolate, Eli Lilly and Co., Indianapolis, Indiana) (0.01% w/v) was added as preservative (Stone, 1987). Vaccine antigens from AF harvest of each virus subtype were concentrated through 50 ml of 30% (w/v) sucrose gradient in TN buffer (0.1 M Tris, 0.1 M NaCl; pH 7.2) by centrifugation for 6 h at 4°C in a Beckman JA-10 rotor. The pellets from the three virus subtypes were, respectively, resuspended in TN buffer.

The three inactivated concentrated virus antigens were assayed for their antigen content by the haemagglutination (HA) test.

### Experimental turkeys

Three-hundred-and-ninety 2-week-old female turkeys were obtained from a commercial source and housed in isolation units. Pre-vaccination sera were tested and found to be negative for antibody to AI virus when screened by the agar gel precipitin test.

### Preparation of vaccines

For all vaccines, the concentration of each of the antigens that made up the trivalent vaccines was adjusted before the incorporation of the adjuvants, such that all vaccines had equivalent antigenic mass of the three subtype antigens, as determined by their HA titres.

#### *Vaccine 1—vaccine without adjuvant*

The three vaccine antigens were mixed in equivalent amounts in TN buffer.

#### *Vaccine 2—avridine-adjuvanted vaccine*

The ethanol formulation of avridine was prepared as previously described (Knudsen *et al.*, 1986), but with some modifications. Briefly, a 20 mg/ml solution was prepared by dissolving 650 mg of avridine, kindly provided by Dr W. W. Hoffman (lot number 11, 635-68-3, Pfizer Central Inc; Groton, Connecticut) in 2.82 ml warm (37°C) absolute

ethanol, to which 0.65 ml of Tween 80 (ICI United States Inc., Wilmington, Delaware) was added. This was followed by 29.03 ml of 0.01 M phosphate buffered saline (PBS), pH 7.4. Each concentrated vaccine virus subtype antigen was added to the avidine formulation. The final vaccine contained 10 mg avidine per dose.

#### *Vaccine 3—positively charged avidine liposomal vaccine*

The liposomal avidine suspension was prepared by the ethanol injection method (Batzri & Korn, 1973; Anderson *et al.*, 1985; Knudsen *et al.*, 1986), and contained avidine and dimyristoyl-L-phosphatidylcholine (DMPC) (Sigma Chemical Company, St. Louis, Missouri), in a molar ratio of 2:1. Briefly, avidine (15 mg/ml) and DMPC (7.5 mg/ml) were dissolved in warm (37°C) absolute ethanol. One volume of this solution was injected into 15 volumes of 0.15 M NaCl containing 0.005 M sodium phosphate buffer, pH 7.4. Stearylamine (Sigma Chemical Company) was added (molar ratio of DMPC:stearylamine was 5:1); to create the positive charge (Latif & Bachhawat, 1984; Schwendener *et al.*, 1984). This preparation was then concentrated to 40 mg/ml of avidine using a YM 100 ultrafiltration membrane with molecular weight cut-off point of 100 KDa (Amicon Div; W. R. Grace and Co, Danvers, Massachusetts). Rapid stirring and low pressure were needed during the concentration process to avoid the formation of larger and more heterogeneous liposomes. After the addition of the different inactivated vaccine antigens to the preparation, the final product contained 10 mg avidine per vaccine dose. The liposomal avidine suspension contained liposomes of the small unilamellar type.

#### *Vaccine 4—vaccine containing antigens covalently linked to the surface of avidine-containing liposomes*

The covalent coupling of antigens to liposomes was achieved by using glutaraldehyde (Torchilin *et al.*, 1978; Latif & Bachhawat, 1978). Briefly, liposomes were prepared and contained DMPC and cholesterol in a molar ratio of 1.4:1 with cholesterol being required for liposomal stability. One millilitre of liposome suspension containing 30 mg lipid per ml was mixed with 12 mg of antigens (4 mg of each of the three antigens). Glutaraldehyde to a final concentration of 15 mM, was slowly added to the mixture of liposome and antigens. The resulting mixture was allowed to react under mild stirring condition for 8 min at 4°C. Excess antigens and glutaraldehyde were removed by repeated washing in PBS buffer by ultracentrifugation. The HA content of the antigens associated with the vesicle was determined after disrupting the liposomes by 1% Triton X-100.

#### *Vaccine 5—oil-emulsion vaccine*

One part of the pooled concentrated antigens was emulsified in 4 parts of mineral oil (sontex 55 NF, Penrenco Morco, Dickinson, Texas) containing 10% Arlacel 80 (sorbitan mono-oleate, ICI United States, Inc., Wilmington, Delaware) and 1% Tween 80 (poly-sorbate 80 ICI United States, Inc.). This ensured that the oil-emulsion vaccine contained the same amounts of antigens as the other vaccines (1-4). The final concentration for each of the trivalent vaccines (HA units per vaccine dose) was 4096 each for H4N8, H5N2 and H7N3.

### Experimental design

At 4 weeks of age, turkeys were divided randomly into six groups, each group comprising 65 turkeys. Five groups were vaccinated subcutaneously in the mid-dorsal neck region with a single 0.5 ml dose of vaccine. Revaccination was done at 8 weeks of age. Group 6 served as non-vaccinated controls.

### Collection of respiratory lavages

Respiratory lavages were collected at 0, 3, 6 and 8 weeks post vaccination. Five Turkeys per group were killed for each collection. At 0, 3, 6 and 8 weeks post-vaccination 40 ml, 60 ml, 80 ml and 100 ml of PBS, respectively, were infused through the larynx into the trachea using a dispenser (Dispensette, Brinkham, West Germany). The tracheal opening was closed by haemostatic forceps, allowing the infused buffer to remain in the respiratory passage for a period of 10 min. The respiratory lavages (about 30 to 60 ml per bird) were collected into clean centrifuge tubes, clarified at low speed and then concentrated through an Amicon Diaflow membrane filter YM 30 (30 KDa cut-off). Each sample was concentrated to 1/10th of its original volume and stored frozen at  $-20^{\circ}\text{C}$  until tested for antibody content.

### ELISA for antibodies in respiratory lavages

The total content of immunoglobulins specific for AIV antigens was measured using the ELISA. A broad-spectrum AIV subtype antigen, H9N2 (A/Turkey/California/6889/80) as previously described (Fatunmbi *et al.*, 1989), was used for the indirect ELISA. The concentrated respiratory lavages were diluted 1:100. An antigen concentration of 0.8  $\mu\text{g/ml}$  was used while the conjugate (goat anti-turkey IgG (H and L) alkaline phosphatase (Kirkegaard and Perry, Gaithersbury, Maryland) was diluted 1:1600. Absorbance readings obtained reflected the total immunoglobulins specific to AIV antigens in tested samples.

### Challenge viruses

The challenge viruses, viz, H4N8 (A/Turkey/MN/1164/80), H5N2 (A/Turkey/MN/1700/82) and H7N3 (A/Turkey/Ontario/5254/81); were of low infectivity (Karanakaran, 1980). To increase their infectivity, they were activated by trypsin treatment (Klenk *et al.*, 1975) and at the time of challenge, had infective titres of  $10^{6.1}\text{EID}_{50}$ ,  $10^{5.1}\text{EID}_{50}$  and  $10^{5.2}\text{EID}_{50}$ , respectively.

### Challenge

Each group of turkeys was subdivided into three with 15 turkeys per subgroup. The challenge study comprised three main groups of 90 birds in each group. Each group of 90 birds was challenged with a different virus subtype. Each turkey was challenged intraocularly with 1 ml of infective AF containing the challenge virus at doses which were previously determined in turkeys of different ages to be suitable for vaccine evaluation. Following intraocular virus challenge, the turkeys were closely observed daily for clinical signs of disease including coughing, sneezing, swollen sinuses, depression, anorexia and diarrhoea.

### Virus isolation

On days 4, 7 and 14 post-challenge, tracheal and cloacal swabs were collected from each bird and placed in separate tubes containing 2 ml of brain-heart infusion broth containing penicillin (10,000 U/ml), streptomycin (2 mg/ml), gentamicin (1 mg/ml), kanamycin (65 µg/ml) and amphotericin (20 µg/ml). Ten-day-old embryonated chicken eggs were inoculated with 0.2 ml of the brain-heart infusion broth containing the swab samples, after clarification using low speed centrifugation. Two embryos were used per swab sample. AF was harvested from inoculated eggs 96 h post-inoculation and checked for HA activity. Identity of the HA agent recovered was confirmed by haemagglutination-inhibition (HI) test with homologous and normal sera.

### Serology

Sera were collected biweekly, from the same 15 turkeys in each group and stored at -20°C until tested. HI test was performed (Beard, 1980) on each serum sample using H4N8, H5N2 and H7N3 as antigens. The HI titres were expressed as the reciprocal of the dilution at which there was complete inhibition of HA. A geometric mean titre (GMT) for each group of sera was determined (Brugh, 1978).

### Challenge assessment

Infection and body weight gains were the criteria used to determine response to challenge. Virus isolation from trachea and cloaca, and weight gain were indices of infection.

### Statistical analysis

The HI GMT in each vaccine group, the data on local immune response and percent weight gains were evaluated using a computer program called STATISTIX 3.1 (Analytical Software, St. Paul, MN). The data for the various vaccine groups were analyzed by analysis of variance and the group means were compared by Tukey's honest significant difference at 5% probability level (Snedecor & Cochran, 1989).

## RESULTS

### Humoral response to vaccines

Table 1 shows the HI responses of vaccinated turkeys using different adjuvanted vaccines.

#### Antibodies to H4N8 component of vaccines

The positively charged liposomal avridine vaccine produced the highest HI responses (with a cumulative mean titre of 87) and these were the most significant ( $P < 0.05$ ) of all vaccinated turkeys.

#### Antibodies to H5N2 component of vaccines

The most significant ( $P < 0.05$ ) HI responses were recorded by the positively charged liposomal avridine vaccine with a cumulative mean titre of 100.

Table 1. HI GMT,\* 2, 4, 6 and 8 weeks post vaccination<sup>y</sup> of turkeys vaccinated with different adjuvanted vaccines

| Group          | H4N8 |                   |     |    | H5N2               |                    |                   |     | H7N3 |                   |     |     |
|----------------|------|-------------------|-----|----|--------------------|--------------------|-------------------|-----|------|-------------------|-----|-----|
|                | 2    | 4                 | 6   | 8  | 2                  | 4                  | 6                 | 8   | 2    | 4                 | 6   | 8   |
| 1 <sup>z</sup> | 9    | 8                 | 20  | 7  | 10                 | 9                  | 21                | 11  | 13   | 10                | 28  | 14  |
|                |      | (11) <sup>x</sup> |     |    |                    | (13) <sup>f</sup>  |                   |     |      | (16) <sup>f</sup> |     |     |
| 2              | 42   | 37                | 84  | 42 | 49                 | 42                 | 91                | 84  | 52   | 45                | 97  | 72  |
|                |      | (51) <sup>c</sup> |     |    | (67) <sup>b</sup>  |                    | (71) <sup>b</sup> |     |      |                   |     |     |
| 3              | 74   | 69                | 119 | 84 | 74                 | 69                 | 137               | 119 | 84   | 74                | 147 | 137 |
|                |      | (87) <sup>a</sup> |     |    | (100) <sup>a</sup> | (111) <sup>a</sup> |                   |     |      |                   |     |     |
| 4              | 45   | 34                | 91  | 39 | 60                 | 37                 | 97                | 74  | 56   | 42                | 104 | 97  |
|                |      | (52) <sup>c</sup> |     |    | (65) <sup>b</sup>  |                    | (75) <sup>b</sup> |     |      |                   |     |     |
| 5              | 14   | 34                | 42  | 23 | 28                 | 18                 | 56                | 42  | 34   | 21                | 69  | 37  |
|                |      | (28) <sup>e</sup> |     |    |                    | (36) <sup>d</sup>  |                   |     |      | (40) <sup>d</sup> |     |     |
| 6              | 0    | 0                 | 0   | 0  | 0                  | 0                  | 0                 | 0   | 0    | 0                 | 0   | 0   |
|                |      | (0) <sup>b</sup>  |     |    |                    | (0) <sup>b</sup>   |                   |     |      | (0) <sup>b</sup>  |     |     |

\* Means having different superscripts are significantly different ( $P < 0.05$ ).  
<sup>x</sup> Haemagglutination-inhibition geometric mean titre.  
<sup>y</sup> Revaccination done at 4 weeks post-primary vaccination. Figures in parenthesis are 8-week cumulative means (HI GMT).  
<sup>z</sup> Group 1 received antigens only. Group 2 received avridine-adjuvanted vaccine. Group 3 received positively charged avridine liposomal vaccine. Group 4 received vaccine containing antigens covalently linked to the surface of avridine-containing liposomes. Group 5 received oil-emulsion vaccine. Group 6 did not receive any vaccine.

Antibodies to H7N3 component of vaccines

The positively charged liposomal avridine vaccine had the highest and the most significant ( $P < 0.05$ ) HI titre with a cumulative mean of 111.

The avridine-adjuvanted vaccine and the vaccine with antigens covalently linked to avridine-containing liposomes did not differ significantly ( $P > 0.05$ ) from each other in antibody responses.

Local immune response

The total specific immunoglobulin activity in the respiratory lavages (Table 2) was highest in group 3 that received the positively charged liposomal avridine vaccine, with a cumulative absorbance mean of 167.8, followed by group 2 which was given avridine-adjuvanted vaccine. These values were significantly ( $P < 0.05$ ) different from any of the other groups. Total specific immunoglobulin values in groups 1, 4 and 5 were significantly different from that of the unvaccinated controls (group 6).

Body weight gain

The percentage weight gains two weeks post-challenge are presented in Table 3. A significant ( $P < 0.05$ ) difference was found between vaccinated and unvaccinated birds in percentage body weight gain.

Clinical signs

No clinical signs were observed in vaccinated groups. More than 50% of the unvaccinated controls were depressed and two of them had swollen sinuses.

Table 2. Total AIV-specific antibody\* in respiratory lavages following vaccination

| Group          | Weeks post primary vaccination |       |       |       | Cumulative mean <sup>y</sup> |
|----------------|--------------------------------|-------|-------|-------|------------------------------|
|                | 0                              | 3     | 6     | 9     |                              |
| 1 <sup>z</sup> | 16.4                           | 51.2  | 66.8  | 52.7  | 52.7 <sup>c</sup>            |
| 2              | 16.4                           | 119.4 | 156.4 | 194.6 | 121.7 <sup>a</sup>           |
| 3              | 16.4                           | 142.8 | 184   | 328   | 167.8 <sup>a</sup>           |
| 4              | 16.4                           | 76.4  | 86    | 151.6 | 82.6 <sup>b</sup>            |
| 5              | 16.4                           | 60    | 67.2  | 94.4  | 59.5 <sup>b</sup>            |
| 6              | 16.4                           | 19    | 17.2  | 20.2  | 18.2 <sup>d</sup>            |

\* As determined by ELISA using goat anti-turkey IgG (H and L).  
\* Values expressed are the absorbance readings at 405 nm. Absorbance readings are multiplied by a factor of 10<sup>3</sup>.  
<sup>y</sup> Means with different superscripts are significantly different (*P* < 0.05).  
<sup>z</sup> Vaccines received by these groups are defined in Table 1.

Table 3. Mean body weight gain between pre- and post-challenge, expressed as percentage body weight gain of turkey groups challenged with H4N8, H5N2 and H7N3, respectively.

| Group          | Mean body weight gain (g) |      |      | Percentage body weight gain <sup>y</sup> |                    |                    |
|----------------|---------------------------|------|------|------------------------------------------|--------------------|--------------------|
|                | A                         | B    | C    | A                                        | B                  | C                  |
| 1 <sup>z</sup> | 1167                      | 1047 | 1047 | 16.36 <sup>c</sup>                       | 14.87 <sup>c</sup> | 16.26 <sup>c</sup> |
| 2              | 1180                      | 1287 | 1347 | 17.03 <sup>c</sup>                       | 18.86 <sup>b</sup> | 18.93 <sup>b</sup> |
| 3              | 1567                      | 1687 | 1793 | 23.07 <sup>a</sup>                       | 23.65 <sup>a</sup> | 24.33 <sup>a</sup> |
| 4              | 1220                      | 1106 | 1480 | 18.90 <sup>b</sup>                       | 19.07 <sup>a</sup> | 19.73 <sup>a</sup> |
| 5              | 1027                      | 1020 | 1180 | 16.72 <sup>c</sup>                       | 16.72 <sup>c</sup> | 16.34 <sup>c</sup> |
| 6              | 1010                      | 1010 | 1010 | 14.17 <sup>d</sup>                       | 14.17 <sup>d</sup> | 14.17 <sup>d</sup> |

<sup>a</sup> Turkeys challenged with H4N8 virus.  
<sup>b</sup> Turkeys challenged with H5N2 virus.  
<sup>c</sup> Turkeys challenged with H7N3 virus.  
<sup>y</sup> Percentage body weight gains with different superscript letters are significantly different (*P* < 0.05).  
<sup>z</sup> Vaccines received by these groups are defined in Table 1.

Virus isolation

Table 4 shows the virus recovery from the tracheal and cloacal swabs at 4, 7 and 14 days post-challenge. High virus detection rates were generally observed at day 4 post-challenge and declined at subsequent isolation attempts. Birds vaccinated with the positively charged liposomal avridine vaccine had the lowest virus detection with 30%, 20% and 30% rates of detection, from the trachea, when challenged with either H4N8, H5N2 or H7N3 viruses, respectively, on day 4 post-challenge. There were 10%, 0% and 20% rates of detection, respectively, from cloacal swabs collected from birds at day 4 post-challenge. These detection rates declined to 10%, 0% and 10%, respectively, from the trachea at 7 days; no viruses were isolated from tracheal or cloacal swabs at day 14 post-challenge. However, there was 100% rate of virus detection from both the tracheal and cloacal swabs collected from unvaccinated birds, infected either with H4N8, H5N2 or H7N3 at days 4, 7 and 14 post-infection.

Table 4. Virus detection at 4, 7 and 14 days post-challenge exposure

| Group          | Days post-challenge | Challenge virus |                |                |                |                |                |
|----------------|---------------------|-----------------|----------------|----------------|----------------|----------------|----------------|
|                |                     | H4N8            |                | H5N2           |                | H7N3           |                |
|                |                     | T <sup>a</sup>  | C <sup>a</sup> | T <sup>a</sup> | C <sup>b</sup> | T <sup>a</sup> | C <sup>b</sup> |
| 1 <sup>z</sup> | 4                   | 6               | 5              | 7              | 2              | 6              | 1              |
|                | 7                   | 5               | 4              | 6              | 4              | 5              | 4              |
|                | 14                  | 3               | 2              | 3              | 2              | 3              | 2              |
| 2              | 4                   | 5               | 2              | 5              | 2              | 6              | 2              |
|                | 7                   | 3               | 2              | 1              | 1              | 3              | 2              |
|                | 14                  | 0               | 1              | 1              | 0              | 1              | 1              |
| 3              | 4                   | 3               | 1              | 2              | 0              | 3              | 2              |
|                | 7                   | 1               | 1              | 0              | 1              | 1              | 1              |
|                | 14                  | 0               | 0              | 0              | 0              | 0              | 0              |
| 4              | 4                   | 6               | 3              | 7              | 5              | 6              | 5              |
|                | 7                   | 4               | 3              | 4              | 3              | 4              | 4              |
|                | 14                  | 1               | 2              | 1              | 2              | 1              | 2              |
| 5              | 4                   | 5               | 3              | 5              | 2              | 7              | 3              |
|                | 7                   | 3               | 3              | 3              | 3              | 5              | 3              |
|                | 14                  | 1               | 1              | 1              | 1              | 1              | 1              |
| Control        | 4                   | 10              | 10             | 10             | 10             | 10             | 10             |
|                | 7                   | 10              | 10             | 10             | 10             | 10             | 10             |
|                | 14                  | 10              | 10             | 10             | 10             | 10             | 10             |

<sup>a</sup> Number of tracheal swabs positive/10 swabs examined.<sup>b</sup> Number of cloacal swabs positive/10 swabs examined.<sup>z</sup> Vaccines received by these groups are defined in Table 1.

## DISCUSSION

The results of this study indicate that avridine has adjuvant activity for both humoral and local immune response when incorporated in trivalent AIV antigens. Turkeys given positively charged liposomal avridine vaccine gave the highest HI and local responses, and percentage weight gain, and lowest virus recovery rates after challenge; thus, showing it to be the best vaccine.

Parenterally inoculated antigens contained in mineral oil adjuvant generally stimulate higher and more persistent antibody titres than equivalent amounts of antigen inoculated without adjuvant (Becher, 1957; Zanella, 1969; Phillips, 1973; Stone *et al.*, 1978). Our results with AIV vaccines support this observation. Furthermore, the trivalent AIV antigens contained in the other adjuvants used in this study elicited higher and more persistent systemic and local immune responses than the oil-emulsion vaccine. Three antigenic subtypes of AIV were combined to prepare polyvalent vaccines without any apparent loss of immunogenicity. This is of considerable practical importance in areas where AI is enzootic and where there are multiple subtypes of virus responsible for the outbreaks, among which little or no cross protection is induced (Easterday & Beard, 1984). This is consistent with the observation of Brugh *et al.* (1979).

In an early report (Karunakunan *et al.*, 1987) observed protection in vaccinated turkeys with low and insignificant HI titres, although this protection was not absolute since virus was still recovered from 11% of the tracheal swabs. An earlier observation (Fatunmbi *et al.*, 1992a) that revaccination was required to reduce the rate of virus

recovery from the tracheal and cloacal swabs post-challenge was confirmed in the present study. Revaccination increases stimulation of the immune system and thus enhances the degree of protection offered against infection and/or clinical disease.

The importance of secretory immunity in the defence against respiratory viruses in man has been recognized (Smith *et al.*, 1966; Ogra *et al.*, 1968). The local immune system is optimally primed by the local administration of the antigens (Ogra *et al.*, 1971; Klipstein & Engert, 1980) and a live replicating antigen is superior to an inactivated preparation (Ogra & Karzon, 1969; Keren *et al.*, 1982). However, inactivated vaccines when combined with adjuvants can still elicit local immunity (Wright *et al.*, 1983). Antibody activity against Newcastle disease in chickens was associated mainly with IgA (85%) and partly with IgG (15%) (Parry & Aitken, 1977), whereas IgG was the only immunoglobulin found in the lower respiratory tract (Zakay-Rones *et al.*, 1972). IgA has also been found in turkey saliva, lacrimal secretions and tracheal washings (Dohms *et al.*, 1978), but not in the lower respiratory tract. There is no available information in the literature on the local immune response of turkeys to AI vaccines. Positively charged liposomal avridine vaccine produced the highest and most significant AIV-specific local immunoglobulins and this was responsible for the lowest rate of virus isolation from the trachea and cloaca in this group. No attempts were made to determine the classes of the local specific immunoglobulin antibodies against AIV due to non-availability of anti-turkey IgA. Hence, the ELISA readings should be interpreted as the total immunoglobulins specific to AIV antigens in tested samples.

The lipoidal amine avridine has been shown to be an effective adjuvant for viral vaccines in chicken (Rweyemamu *et al.*, 1986). Avridine was originally characterized as a potent interferon inducer and antiviral agent (Hoffman *et al.*, 1973). Later studies have indicated that avridine can enhance antibody responses (Anderson & Reynolds, 1979; Niblack *et al.*, 1979; Molitor *et al.*, 1984) and stimulate mucosal immunity especially when combined with liposomes (Pierce & Sacci, 1984; Anderson *et al.*, 1985; Knudsen *et al.*, 1986).

Based on our results, positively charged liposomal avridine vaccine was more efficacious than avridine alone. In fact, from a preliminary study, this positively charged liposomal avridine vaccine was superior to the negatively charged or neutral liposomal avridine vaccine (Fatunmbi *et al.*, 1992b). The vaccine with antigens covalently linked to avridine containing liposomes was less efficacious than the positively charged liposomal avridine vaccine. This is in contrast to other reports (Latif & Bachhawat, 1987). The efficacy of the avridine-adjuvanted vaccine did not appear to be different from that of avridine-containing liposomes covalently linked to the antigens. Both vaccines gave very similar results. Avridine, especially when given in liposomes, was an efficient adjuvant for AI vaccine and was capable of sustaining levels of immunity in turkeys which were comparable and superior to immunity induced by oil-emulsion vaccine.

The mechanisms of adjuvant actions of avridine are not fully understood, but proposed hypotheses include actions as a cofactor with lymphokines, such as the interleukins, or induction of interleukin-1 or similar autocoids with immunostimulatory properties (Hoffman, 1984; Jensen, 1986). Avridine appears to act through a mechanism similar to Freund's complete adjuvant (Anderson & Reynolds, 1979) in that it enhances cellular traffic through antigen depots and potentiates the expansion of immunoreactive clones.

Despite the induction of high levels of serum antibodies by the different avridine-adjuvanted vaccines in our study, some virus isolations from infected birds were made as early as 4 days post-challenge. This is an indication that for complete protection from virus infection of the respiratory system, an appropriate vaccine for AI should be

designed to stimulate local antibody production to prevent upper respiratory infection. Since avridine has been reported to stimulate mucosal immunity (Pierce & Sacci, 1984; Anderson *et al.*, 1985) liposomal avridine-containing AI vaccines should be evaluated using an aerosol (intraoculonasal) route of inoculation.

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