Original article

Protection, systemic IFNγ, and antibody responses induced by an ISCOM-based vaccine against a recent equine influenza virus in its natural host

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Abstract – In the horse, conventional inactivated or subunit vaccines against equine influenza virus (EIV) induce a short-lived antibody-based immunity to infection. Alternative strategies of vaccination have been subsequently developed to mimic the long-term protection induced by natural infection with the virus. One of these approaches is the use of immune-stimulating complex (ISCOM)-based vaccines. ISCOM vaccines induce a strong antibody response and protection against influenza in horses, humans, and a mouse model. Cell-mediated immunity (CMI) has been demonstrated in humans and mice after ISCOM vaccination, but rarely investigated in the horse. The aim of this study was to evaluate EIV-specific immune responses after intra-muscular vaccination with an ISCOM-EIV vaccine (EQUIP F) containing both equine influenza H7N7 (A/eq/Newmarket/77) and H3N8 (A/eq/Borlänge/91 and A/eq/Kentucky/98) strains. The antibody response was measured by single radial haemolysis (SRH) assay using different H3N8 EIV strains. Stimulation of type-1 immunity was evaluated with a recently developed method that measures EIV-specific IFNγ synthesis by peripheral blood lymphocytes (PBL). The protective efficacy of this ISCOM-based vaccine against challenge infection with a recent equine influenza (H3N8; A/eq/South Africa/4/03) strain was also evaluated. Vaccinated ponies developed elevated levels of EIV-specific SRH antibody and increased percentage of EIV-specific IFNγ$^+$ PBL, whereas these responses were only detected after challenge infection in unvaccinated control ponies. Vaccinates showed minimal signs of disease and did not shed virus when challenged shortly after the second immunisation. In conclusion, evidence of type-1 immunity induced by an ISCOM-based vaccine is described for the first time in horses.

equine influenza virus / vaccine / ISCOM / equine IFN gamma / immunity

1. INTRODUCTION

Influenza is a respiratory disease in the horse induced by equine influenza virus (EIV) of the H7N7 and H3N8 subtypes. While the presence of H7N7 EIV has not been detected since 1991 [12], H3N8 EIV is still circulating and the cause of influenza outbreaks in horses[1] [17], despite widespread vaccination in certain populations. Conventional vaccines against EIV, containing whole inactivated virus or sub-unit antigens, stimulate an antibody-based immune response leading to an efficient, but short-lived and highly specific, immunity to infection. Strategies of vaccination developed in the last decades (reviewed in [6, 18]) aim to mimic the long-term protection induced by natural infection. One of these approaches is the use of immune stimulating complex

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[1] Newton R., Elton D., Bryant N., Rash A., Hammond T.A., Equine influenza in England, Letter, Vet. Rec. (2007) 160:851 (letter).

(ISCOM)-based vaccines, which have been used for several years in horses.

ISCOM particles are spontaneously formed, negatively charged, and stable cage-like structures resulting from the combination of antigens with cholesterol, phospholipids, and *Quillaja* saponins (Quil-A) [30]. ISCOM particles play the role of adjuvant, improving presentation, distribution, and release of the antigen when compared with the soluble protein alone. ISCOMs associate with intracellular lipid membranes and have been localised within the cytoplasm and vesicular compartment in antigen-presenting cells (APC) [2, 37]. Antigens are therefore processed by both endogenous and exogenous pathways and presented via major histocompatibility complex (MHC) class I and II molecules, respectively [13]. ISCOM adjuvant also induces the synthesis of pro-inflammatory, Th1 and Th2 cytokines (e.g. TNFα, IL-6, IFNγ and IL-4), which modulate T cell immune responses [29, 30]. Up-regulation of MHC class II and co-stimulatory molecules in APC has also been demonstrated in mice [13, 26].

ISCOM vaccines have been used to induce both humoral and cellular immune responses to numerous antigens in several species [28]. ISCOMs induce a strong and long lasting antibody response against influenza haemagglutinin (HA) and neuraminidase (NA) glycoproteins when compared with conventional adjuvants (e.g. Freund's complete adjuvant or aluminium hydroxide) [31]. In mice, ISCOM-influenza vaccines protect against experimental challenge infection with homologous or heterologous strains of influenza virus [27, 32]. In humans, administration of ISCOM-influenza (H1N1 or H3N2) vaccine has been shown to accelerate the antibody response and to stimulate T lymphocyte proliferation and cytotoxic T lymphocyte (CTL) activity [23]. In the horse, ISCOM-vaccines containing EIV antigens have been studied for two decades. These vaccines were shown to induce a high and long-lasting antibody response, when compared with conventional killed whole influenza virus vaccine, and protection against experimental infection with

homologous EIV strains[2] [4, 5, 15, 16, 33]. Stimulation of Th1 T lymphocytes and cellular immune response (type-1 immunity) after vaccination with ISCOM-based vaccines has been largely described in humans [23] and several animal models [3] but this has not been investigated in vaccinated horses due to a lack of specific methods available for this species. The measurement of virus-specific IFNγ synthesis has been recently developed to evaluate stimulation of type-1 immunity after experimental infection with equine respiratory viruses such as equine herpesvirus-1 (EHV-1) [19, 21] or EIV [20], or vaccination with vector-based vaccines specially designed to stimulate an antigen-specific CMI [22].

The aims of this study were to determine whether the current formulation of a commercialised EIV-ISCOM vaccine, containing equine influenza H7N7 strain A/eq/Newmarket/77 and H3N8 strains A/eq/Borlänge/91 and A/eq/Kentucky/98, would stimulate both humoral and type-1 immune responses, and protect against challenge infection with a recently circulating equine influenza strain, A/eq/South Africa/4/03 (H3N8), which is the subject of a vaccine strain update recommendation[3].

2. MATERIALS AND METHODS

2.1. Viruses

Different strains of EIV were used in this study (Tab. I). EIV strain A/eq/Sussex/89 was used for culture amplification of peripheral blood mononuclear cells (PBMC) and in vitro stimulation. EIV

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[2] Hannant D., Jessett D.M., O'Neill T., Sundquist B., Mumford J.A., Nasopharyngeal, tracheobronchial, and sytemic immune responses to vaccination and aerosol infection with equine-2 influenza A virus (H3N8), in: Equine Infectious Diseases V: Proceedings of the Fifth International Conference, Powell D.G. (Ed.), University Press of Kentucky: Lexington, KY, USA, 1987, pp. 66–73.

[3] OIE, Conclusion and recommendations from the Expert Surveillance Panel on Equine Influenza Vaccines, 2006, pp. 35–36: http://www.oie.int/eng/publicat/BULLETIN%20PDF/Bull%202006-2-ENG.pdf [consulted 12 November 2007].

Table I. Equine influenza virus (EIV) strains used in this study.

EIV strain	Subtype	H3N8 Sublineage	Use in the study
A/eq/Newmarket/77	H7N7	na	Vaccination
A/eq/Sussex/89	H3N8	European	IFNγ assay
A/eq/Borlänge/91	H3N8	European	Vaccination/SRH assay
A/eq/Kentucky/98	H3N8	American	Vaccination/SRH assay
A/eq/SouthAfrica/4/03	H3N8	American	Challenge/SRH assay

na = Not applicable. SRH = Single radial haemolysis.

strains A/eq/Borlänge/91, A/eq/Kentucky/98, and A/eq/South Africa/4/03 were used in the single radial haemolysis (SRH) assay. EIV strain A/eq/South Africa/4/03 was used for experimental infection of ponies. Viruses were all grown in embryonated hen's eggs. The virions were concentrated from allantoic fluid by centrifugation for 3 h at $54\,000 \times g$ and 4 °C in an LKB RP19 rotor (LKB, Bromma, Sweden) and further purified through a 15/30/60% (wt/vol) discontinuous sucrose cushion in phosphate-buffered saline (PBS) for 2 h at $72\,000 \times g$ and 4 °C (LKB 28SA rotor, LKB). The virions were harvested from the 30/60% sucrose cushions interface, re-suspended in PBS and pelleted by centrifugation for 2.5 h at $90\,000 \times g$ and 4 °C (LKB 28SA rotor, LKB). EIV titre was measured by haemagglutination assay. The HA titre was taken as the last virus dilution giving at least 50% haemagglutination of an equal volume (25 μL) of a 1% suspension of chicken erythrocytes and expressed as HA units/mL. Equine herpesvirus-1 strain Ab4/14 was used as a control for the IFNγ assay at specified 50% tissue culture infectious dose ($TCID_{50}$) defined on an equine embryonic lung (EEL) cell line.

2.2. Experimental animals

Ten Welsh mountain ponies (yearlings), seronegative and unprimed for EIV, were used. Only healthy ponies in good clinical condition as ascertained by a qualified veterinary surgeon were included in the study. All work involving experimental ponies was performed under a Home Office Project Licence and was approved by the Animal Health Trust's Ethical Review Committee. All ponies were fully recovered at the end of the study.

2.3. Vaccine, vaccination protocol, and challenge infection

The EQUIP™ F ISCOM vaccine (Schering Plough Animal Health, Uxbridge, UK) containing 10 μg A/eq/Newmarket/77 (H7N7), 20 μg A/eq/Borlänge/91 (H3N8), and 20 μg A/eq/Kentucky/98 (H3N8) was used at 2 mL per dose. The ponies were randomly assigned to two groups (A and B) with six ponies in group A and four in group B. Vaccinates (group A) received two doses of EQUIP™F ISCOM given six weeks apart (days 0 and 42). Animals in group B were not vaccinated. After each vaccination, ponies were examined during three days for the occurrence of local and/or systemic reactions. Temperatures > 38.8 °C were considered abnormal. No local reactions and no pyrexia were observed after the first or second vaccinations (data not shown). All ponies were challenged on day 56 (14 days after the second immunisation) by exposure to an aerosol generated from 20 mL of allantoic fluid diluted in PBS containing $10^{6.66}$ 50% egg infectious doses (EID_{50}) of influenza A/eq/South Africa/4/03 (representative strain of recently circulating equine influenza virus from the American lineage) using a nebuliser (model ULTRA-NEB® 2000; DeVillbiss Health Care, Somerset, PA, USA).

2.4. Clinical signs of disease and virus excretion

Clinical signs associated with equine influenza, including nasal discharge, coughing, and dyspnoea were recorded daily for each pony from day 56 to day 70. The signs were scored using a standardised protocol [7]. On the same days, rectal temperatures were measured. Temperatures > 38.8 °C were considered pyretic. The ponies used in these studies are generally not used to manipulations (e.g. bleeding, nasal swabs, etc.) and the resulting "excitation" often increases basal temperature. To avoid false pyrexia due to excitation, 38.9 °C is the reference pyretic temperature used at the Animal Health Trust. Nasal swabs were taken from each pony from the day after challenge (day 1 post challenge) through to day 10. Infectious virus was titrated in embryonated hens' eggs

and expressed as EID_{50} per mL of swab extract as described previously [15]. Ponies developing apparent clinical signs were treated against secondary bacterial infection with trimethoprim potentiated sulphonamide antibiotic (Trimediazine; Fort Dodge Animal Health, Southampton, UK) paste administered orally on days 5 to 10 (inclusive) post challenge infection. Each treated pony was given a dose appropriate to a 250 kg pony.

2.5. Serological methods

Blood samples for serological analysis were taken on days 0, 7, 14, 42, 49, 56, and 70 and assayed for antibody by SRH [38] with A/eq/Borlänge/91, A/eq/Kentucky/98, and A/eq/South Africa/4/03 as representative antigens. The titres of antibody were expressed as the area of haemolysis (mm^2). An increase of 35 mm^2 or 50% in the area of the zone of haemolysis was regarded as a significant rise in antibody and indicative of infection if measured between pre- (day 56) and post- (day 70) challenge.

2.6. Measure of IFNγ synthesising cells

Heparinised blood samples were taken on days 0, 56, and 70. PBMC were purified from heparinised blood by centrifugation on Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden). EIV-specific effectors were derived from fresh PBMC co-cultured with EIV-infected PBMCs to increase the frequency of EIV-specific T cells, as previously described [20]. Briefly, 5×10^7 PBMC were treated with trypsin (BDH, Poole, UK) at 0.125 µg/mL for 1 h at 37 °C prior to being infected with EIV as follows: 5×10^7 PBMC were distributed in ten wells of a 96-well U-bottomed plate (5×10^6 PBMC per well) and centrifuged (7 min, $175 \times g$). The supernatant was discarded and replaced with 50 µL amplification medium containing 1×10^4 HA units/mL of A/eq/Sussex/89 and trypsin at 0.125 µg/mL. Amplification medium consisted of AIMV® (50%; GIBCO™, Invitrogen Corp., Paisley, UK) and RPMI 1640 (45%) supplemented with 2 mM L-glutamine, 55 µM 2-mercaptoethanol, 0.05 mM NEAA, 0.5 mM sodium pyruvate, 50 µg/mL gentamycin, 5% foetal calf serum, and 5% heat-inactivated equine serum (all supplied by Sigma Ltd, Poole, UK). PBMC were incubated at 37 °C for 1 h. Infected PBMC were washed once (7 min, $175 \times g$) and

then cultivated in amplification medium, in the same flask as autologous uninfected PBMC (5×10^7 PBMC), in the presence of 5% heat treated horse serum (Sigma Ltd.). After seven days of culture, effector cells were purified by centrifugation on Fycoll-Hypaque and washed three times in PBS prior to stimulation. IFNγ synthesis was measured as previously described [19,22]. A total of 10^6 fresh PBMC were stimulated in vitro with A/eq/Sussex/89 (H3N8) at 10^3 HA unit/mL. Brefeldin A (BFA; BD GolgiPlug™, BD Biosciences, Oxford, UK) was added 4 h after the stimulation (1 µL/mL final concentration according to the manufacturer's recommendation). After overnight incubation, expression of CD5 or CD8 was determined using the mouse anti-equine CD5 antibody (clone HB19a, IgG2a isotype; VMRD, Pullman, USA) or the mouse anti-equine CD8α antibody (clone 73/6.9.1, IgG3 isotype; VMRD). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG2a (Serotec Ltd; Oxford, UK) or FITC-conjuguated goat anti-mouse IgG3 (Southern Biotechnology, Cambridge, UK) were used as secondary antibodies. Cells were fixed in 3% formaldehyde in PBS v/v prior to detection of equine IFNγ by intra-cellular cytokine staining (ICC) using a biotinylated antibody specific for equine IFNγ (clone CC302, Serotec Ltd) [19] and streptavidin-rhodamine phycoerythrin (RPE) (Serotec Ltd) in permeabilisation buffer (PBS with 0.5% bovine serum albumin and 0.20% saponin, Sigma Ltd). For single colour staining to detect intracellular IFNγ, unbiotinylated clone CC302 antibody and FITC-conjugated goat anti-mouse IgG antibody (Stratech Scientific Ltd, Newmarket, UK) were used. IFNγ detection was performed in duplicate or triplicate. The percentage of virus specific IFNγ synthesis was calculated according to the formula (% virus stimulated IFNγ synthesis)–(% medium stimulated IFNγ synthesis). T lymphocytes $CD8\alpha^{neg}$ or $CD8\alpha^{high}$ phenotypes were defined using none activated PBMC purified from an unvaccinated and uninfected pony.

2.7. Statistical analysis

Statistical analyses were performed with StatView (version 4.5) or the StatGraphics Plus® program for Windows (Statistical Graphics Corporation, Orlean, VA, USA). Student's t-test was used to test significance of SRH antibody response and IFNγ data between the vaccinated and unvaccinated

groups (Tuckey HSD or Boneferroni procedure with a confidence interval of 95%). The level of significance was set at p-value < 0.05. If variances between each group were not homogenous (p-value < 0.05), data were log transformed to allow t-test.

3. RESULTS

3.1. SRH antibody response

3.1.1. After vaccination

None of the control ponies (group B) developed an antibody response prior to challenge infection with EIV (Fig. 1 and Tab. II). All of the vaccinated ponies responded to vaccination and developed an SRH antibody response measured with A/eq/Kentucky/98 and A/eq/South Africa/4/03 antigens. One of the six vaccinates (#A2) did not possess SRH antibodies detectable with A/eq/Borlänge/91 antigens after the first vaccination. By day 49 (7 days after V2), all vaccinated ponies showed an antibody response to vaccination measurable with all three virus strains. The mean SRH results in the vaccine group at the time of challenge (14 days after the second vaccination) obtained with A/eq/South Africa/4/03 antigens ($137 \pm 24 \, \text{mm}^2$) were equivalent to those obtained with A/eq/Kentucky/98 or A/eq/Borlänge/91 antigens (133 ± 19 and $123 \pm 23 \, \text{mm}^2$, respectively). Two of the vaccinated ponies (#A2 and #A6) had relatively low SRH antibody levels on day 56, one pony (#A2) was below the 120–$150 \, \text{mm}^2$ cut-off thought to be required to be assured of protection against clinical signs of disease and virus shedding after challenge with a strain homologous or related to the vaccine strain [14, 16].

3.1.2. After challenge infection with EIV

All control ponies had seroconverted against all three strains tested at 14 days post challenge (Fig. 1 and Tab. II), the highest individual response recorded ($176.7 \, \text{mm}^2$) was against the challenge virus A/eq/South Africa/4/03 but the mean was highest to A/eq/Kentucky/98. Only one vaccinated pony
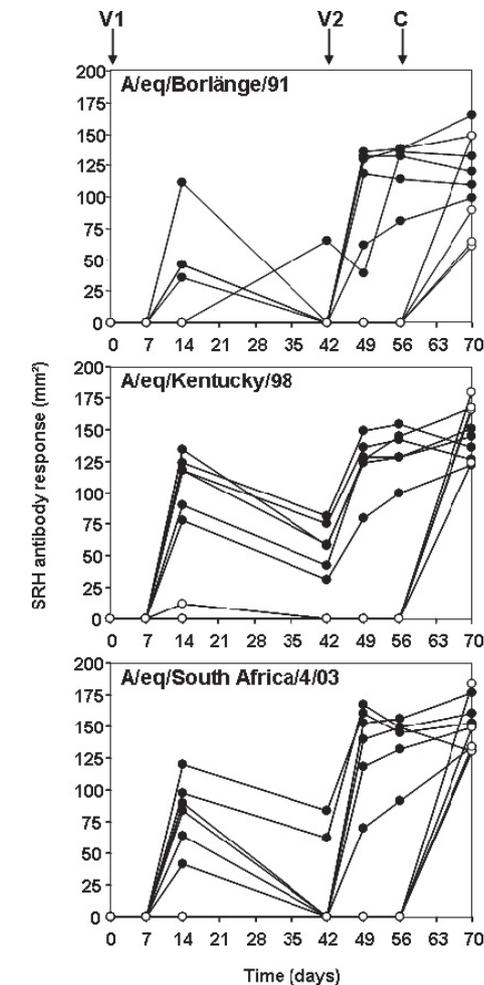
Figure 1. Kinetics of single radial haemolysis (SRH) antibody responses to A/eq/Borlänge/91, A/eq/Kentucky/98, and A/eq/South Africa/4/03 reference antigens in vaccinated ponies (closed circles) and control ponies (open circle). V1 = first vaccination; V2 = second vaccination; C = experimental challenge with A/eq/South Africa/4/03.

(#A2), which had the lowest pre-challenge antibody level, demonstrated a significant antibody increase ($>35 \, \text{mm}^2$) post challenge. This significant increase was only detected using the challenge strain A/eq/South Africa/4/03 as antigen.

Table II. Mean titres of single radial haemolysis antibody.

	Antigens	Day						
		V1	V1 + 7	V1 + 14	V2	V2 + 7	V2 + 14/C	C + 14
		0	7	14	42	49	56	70
Group A (vaccinates)	B/91	0	0	40 ± 41	11 ± 27	103 ± 41	123 ± 23	129 ± 25
(n = 6)	K/98	0	0	110 ± 22	58 ± 19	124 ± 24	133 ± 19	141 ± 17
	SA/4/03	0	0	82 ± 27	24 ± 38	135 ± 36	137 ± 24	150 ± 17
Group B (controls)	B/91	0	0	0	0	0	0	91 ± 41
(n = 4)	K/98	0	0	3 ± 6	0	0	0	159 ± 24
	SA/4/03	0	0	0	0	0	0	149 ± 25

B/91 = A/eq/Borlänge/91; K/98 = A/eq/Kentucky/98; SA/4/03 = A/eq/South Africa/4/03; V = vaccination; C = challenge infection.

3.2. EIV-specific IFNγ response

#### 3.2.1. Response to vaccination and experimental infection with EIV

PBMC were purified on the day of the first vaccination (V1), immediately prior to in vivo challenge infection with A/eq/South Africa/4/03 (i.e. 14 days after the second vaccination) and 14 days after challenge infection. PBMC were culture amplified with EIV A/eq/Sussex/89 for seven days prior to overnight stimulation with either EIV A/eq/Sussex/89 strain or EHV-1 and IFNγ ICC staining. As shown in Figure 2, the percentage of EIV-specific IFNγ+ PBL was low in both groups at V1 ($0.22\% \pm 0.3$ and $0.03\% \pm 0.2$ for groups A and B, respectively). Two weeks after the second vaccination, the percentage of EIV-specific IFNγ+ PBL strongly increased in vaccinates ($4.8\% \pm 3.9$) when compared with percentages measured in control ponies, which remained at a background level ($0.48\% \pm 0.5$). Two weeks after challenge infection with EIV, the percentage of EIV-specific IFNγ+ PBL increased in control ponies ($2.38\% \pm 1.6$) and remained high in vaccinates ($3.75\% \pm 1.2$). No increase of virus-specific IFNγ+ PBL was measured at any of the time points studied when PBMC were stimulated overnight with EHV-1, indicating that the response was specific to EIV.

3.2.2. Phenotype of EIV-specific IFNγ+ PBL

The phenotype of EIV-specific IFNγ+ PBL induced by vaccination with the EIV-ISCOM vaccine was studied two weeks after V2. As illustrated in Figure 2B, the majority of EIV-specific IFNγ+ PBL were CD5+ T lymphocytes ($95.2\% \pm 2$) and the percentage of EIV-specific CD5+ IFNγ+ PBL was significantly different between groups A (vaccinates) and B (control) (p-value = 0.01). Lymphocytes of the CD8α phenotype represented only $19.2\% \pm 8.9$ of the total EIV-specific IFNγ+ cell population (Fig. 2C). Four of the six vaccinates showed increased percentage of CD8α+ IFNγ+ PBL when compared with the group of control ponies. After challenge infection with A/eq/South Africa/4/03, the phenotype of EIV-specific IFNγ+ PBL was similar between groups. EIV-specific IFNγ+ PBL were CD5+ in the majority ($95.5\% \pm 4$ and $97.1\% \pm 4.5$ for groups A and B, respectively), with $28.5\% \pm 8.2$ and $23.8\% \pm 10.6$ of cells having a CD8α+ phenotype (groups A and B, respectively).

3.3. Expression of CD8α molecule by PBL

Interestingly, expression of the CD8α molecule in the overall CD8α+ cell population was affected by vaccination. As shown in Figure 3, the expression level of the CD8α
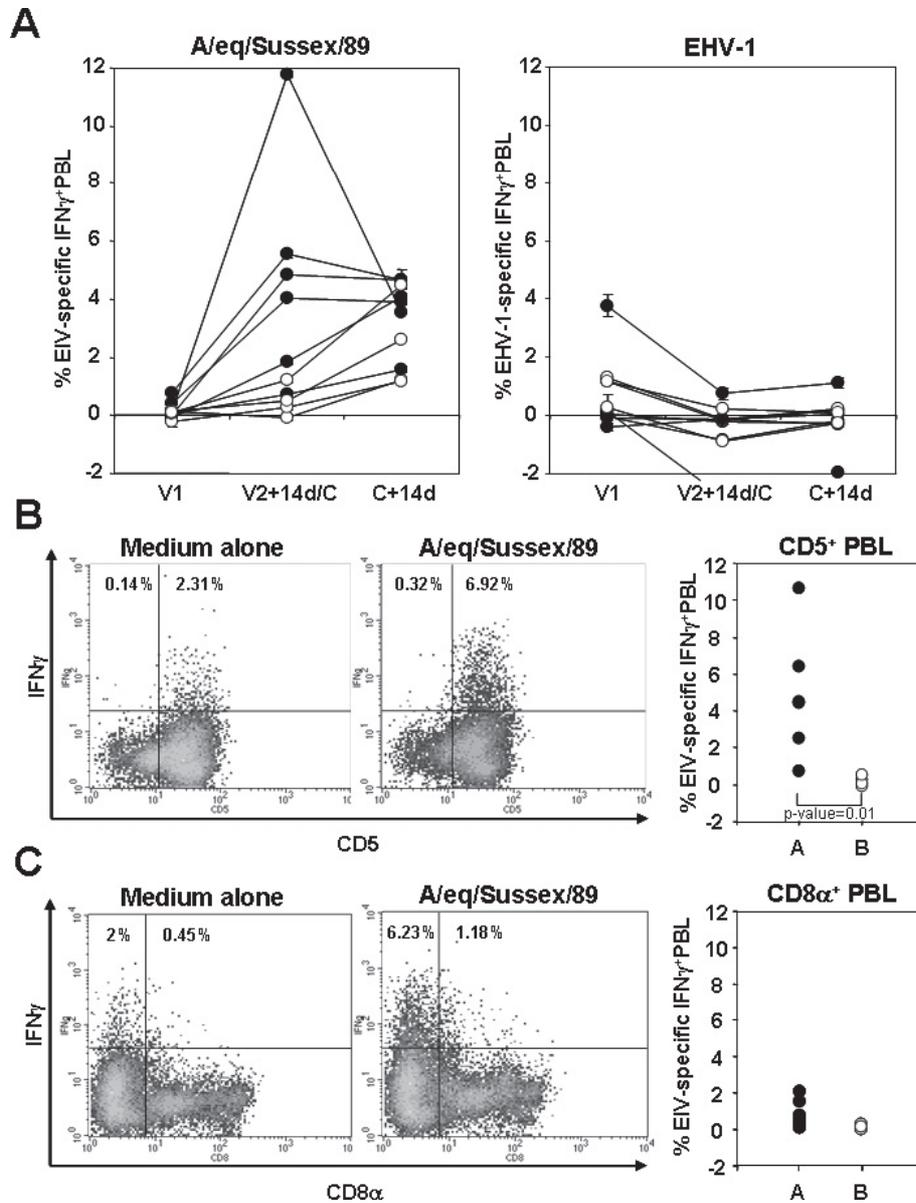
Figure 2. Equine influenza virus (EIV)-specific IFNγ response: (A) Kinetics of virus-specific IFNγ$^+$ PBL purified from control ponies (open circles) or vaccinates (closed circle), culture amplified and stimulated overnight with either A/eq/Sussex/89 or EHV-1 as a control of virus specificity. V1 = first vaccination; C = experimental challenge with A/eq/South Africa/4/03. CD5 (B) and CD8α (C) phenotype of EIV-specific IFNγ$^+$ PBL, 2 weeks after the second vaccination. Typical CD5- or CD8α-IFNγ double staining of PBL from pony #A2, culture amplified and stimulated overnight with medium alone or A/eq/Sussex/89 and comparison of the percentage of EIV-specific CD5$^+$ or CD8α$^+$ IFNγ$^+$ PBL in vaccinated (group A) and unvaccinated ponies (group B).
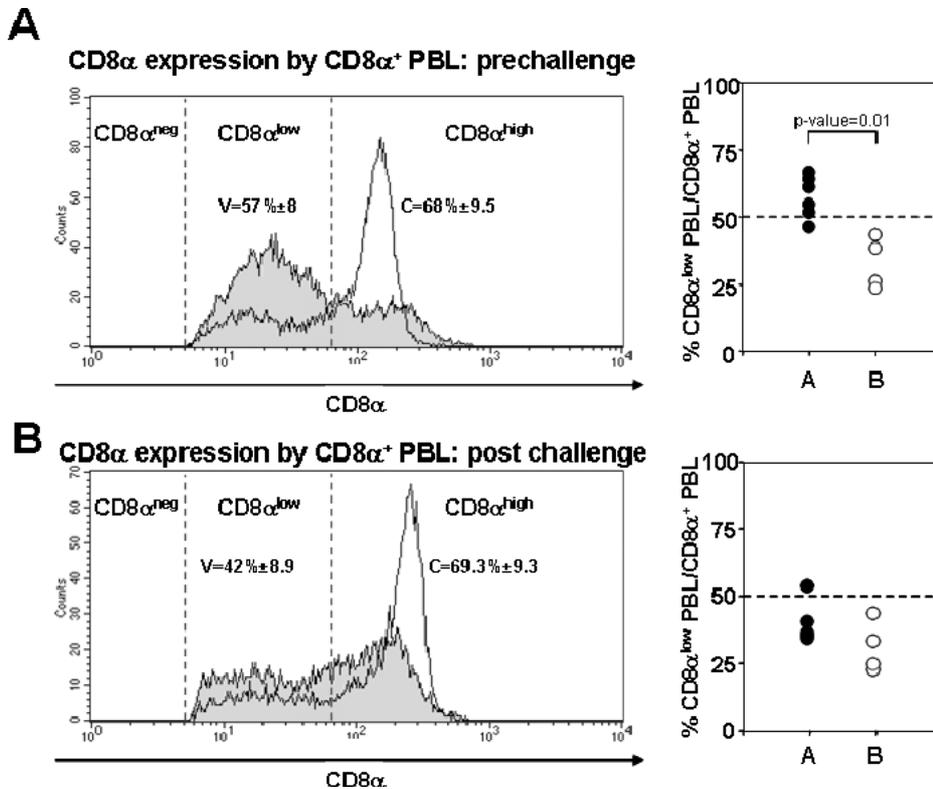
Figure 3. Expression of the CD8α molecule by PBL. Overlay histogram of CD8α molecule expression by CD8α⁺ PBL purified from ponies prior to (A) and after challenge infection with a/eq/South Africa/4/03 (B). Histograms are representative of controls (white; C) or vaccinates (grey; V). The mean proportion of CD8α^{low} or CD8α^{high} inside CD8α⁺ PBL is calculated for vaccinates and controls, respectively. The proportion of CD8α^{low} PBL inside the CD8α⁺ cell population is calculated for each ponies and compared between groups.

molecule was predominantly low (CD8α^{low}, 57% ± 8) in CD8α⁺ PBL purified from vaccinated ponies (group A), two weeks after the second administration of the EIV-ISCOM vaccine. In contrast, CD8α⁺ PBL purified from control ponies were mostly CD8α^{high} (68% ± 9.5), as is usually observed in ponies. This difference of CD8α molecule expression by PBL between groups was statistically significant (p-value = 0.01). Two weeks after experimental infection with EIV, the percentage of CD8α^{high} PBL remained unchanged in control ponies (69.3% ± 9.3). The CD8α phenotype shifted from low to high in the vaccinated group (58.2% ± 8.9 of CD8α^{high} PBL) when compared with the

pre-challenge infection level (42.9% ± 7.9 of CD8α^{high} PBL at V2 + 2 weeks).

3.4. Clinical signs of disease induced by EIV

3.4.1. Coughing and dyspnoea

None of the vaccinated ponies (group A) were observed to cough throughout the challenge period (Tab. III). All of the control ponies (group B) were observed to cough for a total of six to nine days during the first ten days post challenge (mean 7.2 days). Furthermore, laboured breathing was observed in all four of the control ponies between days 2 and 10 post challenge.

Table III. Clinical signs of disease and virus shedding after experimental infection with A/eq/South Africa/4/03.

	Signs	Day pre/post experimental challenge											
		C	1	2	3	4	5	6	7	8	9	10	Total
Group A	Cough	0	0	0	0	0	0	0	0	0	0	0	0
(vaccinates)	Dysponea	0	0	0	0	0	0	0	0	0	0	0	0
($n = 6$)	ND[1]	1	2	1	2	2	3	4	2	5	7	2	31
	BT[2]	0	0	0	0	0	0	0	0	0	0	0	0
	Total	1	2	1	2	2	3	4	2	5	7	2	31
	VS[3]	0	0	0	0	0	0	0	0	0	0	0	na
Group B	Cough	0	0	0	6	8	6	4	3	4	5	3	40
(controls)	Dysponea	0	0	1	1	3	4	2	2	2	1	1	17
($n = 4$)	ND[1]	3	4	5	4	9	14	8	3	5	6	3	64
	BT[2]	0	0	0	3	3	3	1	0	1	0	0	11
	Total	3	4	6	14	23	27	15	8	12	12	7	132
	VS[3]	0	0	0	0	1.6 ± 1.2	0.3 ± 0.5	0.5 ± 0.6	0	0	0	0	na

[1] Nasal discharge.
[2] Body temperature.
[3] Virus shedding.
na = Not applicable; C = challenge infection.

3.4.2. Nasal discharges

One pony in group A and two ponies in group B presented nasal discharges on the day prior to challenge infection (Tab. III). After infection, nasal discharges were noted in all four of the control ponies (group B) and all had extremely evident mucopurulent discharge at some point between days 4 and 6 post challenge. In Group A, five out of the six vaccinated ponies were observed to have serous nasal discharge on at least one day, but mucopurulent discharge was only observed in three ponies and for one day only.
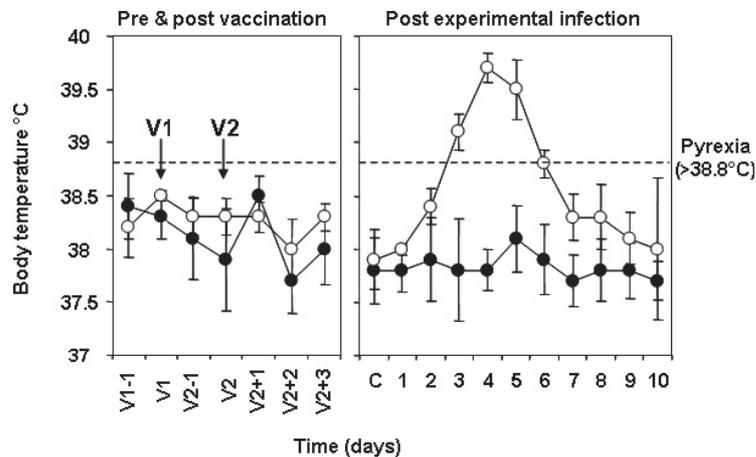
3.4.3. Temperature

Three of the four control ponies (group B) developed pyrexia (Fig. 4). All three were pyretic on three consecutive days (days 3 to 5). Two animals each had an additional day of pyrexia (one on day 6 and the other on day 8). The mean temperature was above 38.8 °C on days 3 to 5 for Group B. None of the ponies in Group A developed a pyretic temperature.

3.4.4. Total clinical scores

Total clinical scores for groups A and B were 31 and 132, respectively, with a mean of 5.2 and 33 (Tab. III). All four control ponies (group B) had high clinical scores post challenge and these were derived from pyrexia (with the exception of pony #B1), coughing, dyspnoea, and nasal discharges for all four ponies. The total clinical scores in group A were entirely derived from observed nasal discharges. No clinical signs were recorded during the 10-day post challenge period for one pony (#A1) in Group A.

3.5. Virus shedding after challenge infection with EIV

There was no detectable virus shedding in the vaccinated group from the day of challenge to ten days post challenge infection (Tab. III). All control ponies shed virus after challenge infection. Virus shedding was detected on day 4 in three of the four ponies. One pony was shown to be shedding virus on both days 4

Figure 4. Mean of rectal temperatures for control pony group (open circle) and vaccinated pony group (closed circle) taken pre- and post-vaccination (V1 and V2) and for ten consecutive days after experimental challenge with A/eq/South Africa/4/03 (C). Temperatures > 38.8 °C (dotted line) were considered pyretic.

and 5. Pony #B1 only started shedding virus at day 6. These levels of virus shedding were lower than anticipated; therefore virus titration was repeated on duplicate aliquots of nasal swab extracts that had not been previously thawed and were found to be in accordance with those reported here (data not shown).

4. DISCUSSION

ISCOM-based vaccines for EIV are part of a whole generation of vaccines commercialised during the last decade (reviewed in [18]), which are designed to present antigen via both MHC class I and II molecules and subsequently to stimulate an immune response not restricted to highly virus-specific antibodies. A broader immunity induced by vaccination, involving both humoral and cell-mediated immunity, is predicted to improve protection and recovery in the case of significant mismatch between the vaccine EIV strain and field isolates. Previous studies of EIV-ISCOM vaccination in ponies were generally limited to measurement of the antibody response[4] [15, 16, 33]. Concerning this particular commercialised EIV ISCOM

vaccine, protection was challenged with a strain of EIV isolated more than a decade ago, namely A/eq/Newmarket/1/93 [4, 5]. The present study has broadened the investigation of immune responses induced by vaccination by measuring EIV-specific $IFN\gamma^+$ PBL in peripheral blood, which is a new marker of type-1 immunity in the horse, in addition to the classical SRH assay for antibody levels. Vaccinated ponies were also challenged with A/eq/SouthAfrica/4/03 to assess cross-protection against this recent strain of EIV.

The EIV-ISCOM vaccine (EQUIP™ F; Schering Plough Animal Health) was well tolerated and there were no local reaction to its administration. As expected, high levels of SRH antibody specific to EIV strains contained in the vaccine (A/eq/Borlänge/91 and A/eq/Kentucky/98) were detected after two immunisations. SRH antibody cross-reacting with the challenge strain (A/eq/South Africa/ 4/03) was also measured after vaccination

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[4] Hannant D., Jessett D.M., O'Neill T., Livesay G.J., Mumford J.A., Cellular immune responses stimulated by inactivated virus vaccines and infection with equine influenza virus (H3N8), in: Equine Infectious Diseases VII, Proceedings of Seventh International Conference on Equine Infectious Diseases, Nakajima H., Plowright W. (Eds.), R & W Publications Ltd, Newmarket, UK, 1994, pp. 169–174.

with values around the threshold thought to protect against infection with EIV [14, 16]. In control ponies, SRH antibody levels were almost undetectable throughout the sampling period from the first vaccination to the day of challenge infection, suggesting that no field infection of equine influenza occurred during this period. After challenge infection with A/eq/SouthAfrica/4/03, all four unvaccinated ponies developed clinical signs of infection with protracted coughing being particularly noteworthy, although only three developed pyrexia. Nasal discharge was not regarded as a reliable sign of infection as some ponies entered the challenge phase of the study with nasal discharges evident. All four unvaccinated ponies shed virus on one or two days post infection, although the titres of virus shed were low, late in onset and of limited duration. The overall outcome of this challenge infection with A/eq/South Africa/4/03 can be considered as a mild disease. None of the vaccinated ponies coughed at any time during the post challenge observation period and none developed pyrexia nor did the vaccinated ponies shed virus after challenge infection. All four unvaccinated ponies seroconverted after challenge infection whereas only one of the six vaccinated ponies had a significant increase in SRH antibody using the A/eq/South Africa/4/03 strain after infection. Protection against challenge infection with A/eq/South Africa/4/03 was probably induced by the presence of high level of SRH antibody, leading to sterilising immunity and absence of either clinical signs of disease, virus shedding or even seroconversion in five of the six vaccinated ponies. The sixth pony, who had the lowest level of antibody prior to challenge infection, below levels predicted to protect, did seroconvert after infection but did not develop clinical signs of disease or shed virus.

Vaccines able to stimulate a second line of defence (i.e. CMI) will improve the outcome of disease and recovery after equine influenza if the first line of protection (i.e. neutralising antibody) is insufficient and fails to prevent infection. Until recently, the lack of reliable methods to measure EIV-specific CMI was an obstacle to investigate such responses in

horses and therefore also to fully evaluate vaccine efficiency. IFNγ is an important mediator of the anti-viral immune response and is involved in several activities such as immuno-modulation, lymphocyte recruitment and activation, and anti-pathogen activity [1]. IFNγ promotes CMI both by increasing the presentation of viral peptides by APC and promoting the development of T helper 1 (Th1) lymphocytes. IFNγ therefore is a key factor in the development of a type-1 response involving CTLs, which ultimately leads to the destruction of virus-infected cells and recovery from infection. EIV-specific IFNγ synthesis has recently been characterised and used as a marker of EIV-specific type-1 immunity by our group [20, 22]. Greater EIV-specific CTL activity has been associated with an increased percentage of EIV-specific IFNγ^+ PBL in ponies experimentally infected with A/eq/Sussex/89 [20]. This marker was applied to assess the immune response induced by the EIV-ISCOM vaccine. Previous studies in horses have not demonstrated any evidence of type-1 immunity stimulation after immunisation with ISCOM-based vaccines against EIV or other equine pathogens such as EHV-1. Indeed, ponies vaccinated with a subunit vaccine containing Quil A as adjuvant failed to induce an influenza-virus specific CTL activity[4]. However, the reduced signs of disease and virus shedding measured in a few ponies vaccinated with an EIV ISCOM-based vaccine and presenting low levels of SRH antibody ($< 50\,mm^2$) [16] were an indication of defence mecanisms that were not directly linked to a strong antibody response immediately prior to challenge infection. This hypothesis was supported in the current study as an increased frequency of EIV-specific IFNγ^+ PBL has been demonstrated for the first time in ponies immunised with an ISCOM-based vaccine when compared with controls. Potential stimulation of EIV-specific CTL by the EIV-ISCOM vaccine and the nature of epitopes targeted will need further investigation.

EIV-specific IFNγ^+ cells detected after immunisation were mostly CD5$^+$CD8α^- T lymphocytes after culture amplification and

re-stimulation. These IFNγ$^+$ cells are presumably CD4$^+$ T lymphocytes as expression of the equine CD4 antigen on extrathymic equine T lymphocytes is mutually exclusive with the CD8 antigen [9]. This result confirms the potent ability of ISCOMs to prime equine Th1 lymphocytes in vivo. A fraction of EIV-specific IFNγ$^+$ cells were CD5$^+$CD8α$^+$ T lymphocytes. In previous studies, both CD4$^+$ and CD8$^+$ T lymphocytes have been efficiently antigenically stimulated by murine dendritic cells treated with ISCOM-antigen [24, 25]. Interestingly, a large proportion of CD8α$^+$ T lymphocytes purified after immunisation and culture amplified one week in the presence of EIV displayed a low expression of the CD8α molecule at their surface (CD8^{low} phenotype). As a co-receptor of the T cell receptor (TCR), CD8 molecules are internalised and degraded when the TCR is triggered [35] giving rise to a transient CD8^{low} phenotype. The function of CD8^{low} T lymphocytes is controversial. In humans, circulating T cells expressing low CD8 have been shown to represent a subset of highly activated CD8 T lymphocyte effectors, synthesising IFNγ and displaying increased cytotoxicity [34]. This is in contrast to CD8^{low} T lymphocytes derived from naïve CD8$^+$ T cell cultivated in presence of IL-4, as previously described in mice [10, 11]. In these studies, CD8^{low} T lymphocytes have been shown to be a stable subpopulation with noncytolytic activity, able to synthesise both IL-4 and IFNγ after antigenic stimuli. Reduced cytotoxicity allowed IL-4-induced CD8^{low} cells to provide B cell help, increasing IgM and IgG1 antibody levels [11]. In the current study, the origin and function of the large population of CD8^{low} lymphocytes transiently detected in PBMC purified from vaccinated ponies are unclear. This population could be activated/exhausted lymphocytes resulting from proliferation during the amplification culture of EIV-specific CD8$^+$ lymphocytes induced by vaccination. However, the hypothesis of a noncytolytic sub-population of CD8$^+$ T lymphocytes cannot be excluded as the synthesis of IL-4 after ISCOM vaccination has been reported in several studies (reviewed in [30]). The recent development of an

antibody specific for equine IL-4 will enable further investigation of the CD8^{low} cell population [36]. Finally, another possibility is activation of lymphocytes in an antigen independent way through an unidentified bystander effect (e.g. CD8 downregulation has been shown in the absence of specific peptide-MHC complexes, such as after stimulation with superantigens or incubation with monovalent anti-CD3 antibodies [35]).

The EIV strain A/eq/Sussex/89 was the strain of reference for the development of the EIV-specific IFNγ assay [20, 22] due to high concentration obtained after culture in hens' eggs and purification. This EIV strain was used in the current study to stimulate PBMC in vitro. The importance of strain specificity to the EIV-specific IFNγ assay is unknown but currently under investigation. The cross-reactivity of A/eq/Sussex/89 and the vaccine strain A/eq/Borlänge/91 was expected from analysis of results obtained by the hemagglutination-inhibition assay. The similarity in reaction of these strains with monospecific ferret sera indicated that the glycoproteins are of the European sublineage and very closely related antigenically[5]. Glycoproteins from A/eq/Sussex/89 and the challenge strain A/eq/South Africa/4/03 are antigenically and genetically different. However, the EIV-antigens that stimulate CMI are expected to be located on molecules such as nucleoprotein (NP) or matrix (M), as described for human influenza [8]. Amino acids sequences of these molecules are well conserved between EIV strains (data not shown). Therefore, A/eq/Sussex/89 was expected to stimulate in vitro EIV-specific PBL induced by challenge infection with A/eq/South Africa/4/03 to some extent.

In conclusion, the current formulation of EQUIP™ F ISCOM (Schering Plough Animal Health) gave protection against clinical signs and virus shedding when ponies were challenged with A/eq/South Africa/4/03 two weeks after a second dose of vaccine. This protection is based on both an antibody response,

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[5] Lewis N., Animal Health Trust, personal communication.

characterised by cross-reactive SRH antibody, and stimulation of EIV-specific IFNγ^+ PBL after immunisation. To our knowledge, this demonstration of the stimulation of IFNγ synthesis by EIV-specific lymphocytes in vaccinated ponies is the first evidence of ISCOM-induced type-1 immunity in the horse.

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