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Innate immune responses following emergency vaccination against foot-and-mouth disease virus in pigs

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Abstract

Inactivated "emergency" foot-and-mouth disease virus (FMDV) vaccine of high potency will induce early protection against the disease, implying a critical role for innate immune defences. At 3 and 6 days post-vaccination (dpv), there was no evidence of vaccine-induced specific anti-FMDV antibodies (Abs), nor enhanced uptake and destruction of opsonised virus by macrophages. Sera from vaccinates and control animals showed similar capacity to neutralise the virus, and were not different from the pre-vaccination sera. There were also no distinguishable changes in the distribution of the different peripheral blood leucocyte (PBL) subpopulations. Nor was any vaccine-induced increase in production of acute phase proteins noted. In contrast, chemotaxis assays identified an increase in PBL migratory activity which was vaccine-related. Furthermore, sera from 3 days post-vaccination contained elevated chemotactic potential. These results demonstrate that enhanced chemotaxis of cells of the innate immune defences, could play an important role during the early protection induced by emergency FMDV vaccines.

Keywords: Foot-and-mouth disease virus; Emergency vaccination; Innate immunity

1. Introduction

Foot-and-mouth disease virus (FMDV) is part of the *Picornaviridae*, and sole member of the genus Aphthovirus (reviewed in [1]). Serologically, FMDV can be classified into seven antigenically distinct serotypes, O, A, C, South African Territories (SAT) 1, SAT2, SAT3 and Asia 1. Immunologically, there is no cross protection between serotypes. FMDV causes an economically important highly contagious vesicular disease of cloven-hoofed animals, notably with cattle, sheep and pigs. Infection with FMDV is commonly via the respiratory tract following inhalation of airborne virus, the major site of primary replication being the mucosal epithelia of the nasopharynx. In susceptible animals, the virus has an incubation period of 2–8 days, with high mortality in young animals. Ruminants can become carriers of the virus, presenting a potential reservoir of infection, while pigs are particularly important in virus dissemination of the disease, due to the large quantities of infectious virus excreted per day [2]. Thus, a rapid spread of the virus within a susceptible population can even occur before clinical signs become apparent.

Since 1991, a non-vaccination policy has been enforced in Europe for the control of foot-and-mouth disease (FMD). In the case of an epidemic, slaughter of animals on and around a farm diagnosed as FMD-positive, together with strict control of animal movement, are the primary actions to be taken against the spread of the disease. However, the well-publicised outbreak of the Pan Asia type O FMDV in the UK [3] clearly demonstrated that these actions are only successful when the correct resources and contingency plans are in place. The approach taken in The Netherlands during 2001 of additionally using vaccine in such an emergency showed the importance of this approach when the means to execute successfully a "stamping-out" programme are constrained.

The most important property of an emergency FMDV vaccine of high potency is the rapid induction of early protective immunity. Traditionally, immunity against FMD is documented as being primarily mediated by neutralising anti-FMDV antibodies (Abs) (reviewed in [4]). Eventual destruction of the virus requires the phagocytosis of antibody-opsonised virus by macrophages [5]. However, trials using high potency vaccines based on inactivated

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whole virus vaccine in cattle [6], pigs [7] and sheep [8] have demonstrated that animals can be protected as early as 4 days post-vaccination (dpv), in the absence of significant levels of anti-FMDV Abs. This would imply that events other than specific Ab were involved. Considering the rapidity with which the protection develops, it seems plausible that innate immunity would be an important element in these early stages of protection.

Research emphasis to date has tended to focus on humoral and specific immunity against FMDV. Consequently, there is a lack of knowledge concerning the innate arm of immune defences against FMDV, which may be equally important for protection. By comparing the immunological activity contained in serum and peripheral blood leucocyte (PBL) populations during the first 6 days post-vaccination, we sought to identify alterations in innate immune responses induced by FMDV vaccine. The aim was to identify those aspects of innate immunity which could be important with respect to protection early post-vaccination.

2. Materials and methods

2.1. Animals

Seventeen Swiss White Landrance pigs between the ages of 4 months and 2 years old were employed in these studies. These were housed under specific pathogen-free conditions at our institute.

2.2. Vaccine preparation

A single dose of the double oil emulsion "emergency" FMDV vaccine formulation was prepared as described [7]. This is the vaccine which can protect pigs within 4–5 days post-vaccination against indirect aerosol challenge infection. Essentially, this vaccine contains inactivated type C_1 Oberbayern FMDV antigen (antigen payload of 2.9 μg per dose), formulated in a water-in-oil-in-water emulsion adjuvant using Montanide ISA 206 (Seppic, Paris), prepared in the International FMD Vaccine Bank (Institute for Animal Health, Pirbright, UK).

2.3. Vaccination trials

In the vaccination trials, each pig was injected intramuscularly with a dose of one of the following: the formulated vaccine, phosphate buffered saline (PBS) as negative control, adjuvant alone, or inactivated C_1 Oberbayern FMDV antigen in PBS. Blood samples were collected from the vena cava 7 days prior to vaccination (pre-bleed), as well as 3 and 6 days post-vaccination.

Trial 1. Nine pigs, split into groups of three were either administered with the vaccine formulation (541, 542, 543), adjuvant (537, 538, 539) or PBS (534, 535, 536).

Trial 2. Five pigs were inoculated with the vaccine formulation (603, 605, 615, 514, 515) and three with PBS (518, 519, 520).

2.4. Virus preparation

The subtype C_1 Oberbayern isolate of FMDV was grown in baby hamster kidney (BHK-21) cell monolayers as described [9]. Briefly, BHK-21 cells in serum-free Glasgow Modified Eagle Medium (GMEM, Gibco) supplemented with 2 mM L-glutamine and 7.5% (w/v) bicarbonate, were infected at a multiplicity of infection (MOI) of 0.001 $TCID_{50}$ per cell, and incubated for 24 h at 37 °C, 6% (v/v) CO_2, until cytopathic effect (CPE) was observed by light microscopy. Cells were harvested, sonicated 2 × 10 s, and clarified at 3000 × *g* for 30 min at 4 °C. Virus-containing supernatant was stored at −70 °C, and the virus titre calculated from thawed virus stock by titration on BHK-21 cells.

2.5. Sandwich ELISA for the detection of anti-FMDV antibodies

This ELISA was based on the method described [10], with modifications. Briefly, Maxisorb 96-well ELISA plates (Nunc, GibcoBRL, Life Technologies, Basel, Switzerland) were coated with 100 μl per well of rabbit anti-FMDV serotype C_1 Oberbayern antibodies (diluted 1:10,000 in 0.05 M carbonate–bicarbonate coating buffer, pH 9.6) and incubated overnight at 4 °C. The plates were washed five times with PBS containing 1% (v/v) Tween-20 and 1% (w/v) bovine serum albumin (BSA). FMDV C_1 Oberbayern, pre-diluted in PBS containing 1% (v/v) Tween-20 and 1% (w/v) BSA, was added to each well (100 μl) and incubated for 2 h at 37 °C. The concentration of the virus used was such that the amount captured would enable maximum binding (saturation) by a reference anti-FMDV guinea-pig serum. Test sera and positive serum taken from a hyperimmune pig were pre-diluted 1:2 and 1:10 in PBS/Tween20/1% BSA/1% (v/v) normal rabbit serum. The plates were washed as above, followed by the addition of sera in triplicate (100 μl per well), and incubated for 1, 5, 10 or 30 min at 37 °C before washing away unbound serum proteins. As a negative control (background), test sera were added to wells that did not contain the antigen. Bound antibodies were detected with 100 μl per well of rabbit anti-swine immunoglobulin conjugated with horseradish peroxidase (DAKO Diagnostics, Zug, Switzerland) (1:1000 dilution in PBS/Tween 20/1% BSA), incubated for 1 h at 37 °C. The plates were washed five times, and the reaction developed with 2,2′-azino-di- (3-ethylbenzthiazoline-6-sulfonate) in 0.01% hydrogen peroxide ($ABTS/H_2O_2$) substrate, followed by reading at 405 nm in an ELISA reader. The measurements were carried out by reading the absorbance of the reactions in the wells when that of the positive control serum approximated to 1.00 OD unit.

The percentage relative reactivity was calculated as follows, by averaging the results from replicate wells:

$$\frac{\text{average (test serum)} - \text{average (background)}}{\text{average (positive control)}} \times 100$$

2.6. Serum neutralisation

BHK-21 cell monolayers were grown in 96-well microtitre plates (Costar, Cambridge) in GMEM supplemented with 2 mM L-glutamine, 7.5% (w/v) bicarbonate and 2% (v/v) foetal calf serum (FCS). Serum samples were diluted 1:2 in the above medium, and incubated with 100 $TCID_{50}$ in 100 μl of C_1 Oberbayern FMDV for 1, 5, 10 and 30 min in a separate plate. The medium was removed from the BHK-21 cells, and the virus/serum suspension then transferred on to the BHK-21 cell monolayers (100 μl per well), followed by incubation for 2 h at 39 °C. Appropriate virus, serum and cell controls were included. The plate was then washed three times with warm PBS, fresh GMEM containing 0.5% (v/v) FCS was added, and incubation continued for a further 46 h at 39 °C until CPE was observed under the light microscope in the wells containing virus without serum. The cells were then fixed and stained with 90% (v/v) ethanol containing 0.5% (w/v) crystal violet for 10 min at room temperature, followed by washing with water. Crystal violet staining of the cells was measured at 590 nm in an ELISA reader, after dissolving with acid/ethanol. The percentage relative cell survival was calculated as follows, after averaging the values for replicate wells:

$$\frac{\text{average (test serum)} - \text{average (negative control)}}{\text{average (positive control)}} \times 100$$

2.7. Opsonisation of FMDV

Peripheral blood mononuclear cells (PBMC) were obtained from citrated vena cava blood of a non-vaccinated, untreated donor pig, using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation, and monocyte-derived macrophages obtained as described [11]. PBMC were seeded at 4×10^6 cells/ml in Dulbecco's Modified Eagles Medium (DMEM, Gibco), supplemented with 10% (v/v) pooled heparinized porcine plasma and 2 mM L-glutamine. After 24 h incubation at 39 °C, non-adherent cells were removed, and the adherent cells cultured in DMEM supplemented with 30% (v/v) pooled heparinized porcine plasma for 3–5 days at 37 °C to generate macrophages [12].

A pre-determined amount of virus (10^6 $TCID_{50}$/ml) was then incubated with an equal volume of test serum for 30 min at 39 °C prior to being added to the macrophage cultures. This titre of virus was found to be optimum for the subsequent detection of virus infectivity associated with and destruction by the macrophages. At 10, 30 and 60 min post-infection, macrophages were removed, washed and then lysed to measure the amount of cell-associated virus. After the 1 h adsorption period at 39 °C, residual unbound virus was removed by washing the macrophage cultures three times with warm PBS (containing divalent cations). Incubation was then continued for a further 1, 3, 5 and 23 h in fresh medium. After incubation, the medium was removed, the cells harvested in cold PBS^A-0.03% (w/v) EDTA and centrifuged at 350 × *g* for 15 min, 4 °C. Supernatant was removed and the cell pellet resuspended in 1 ml of DMEM, before freeze-thawing the cells three times in liquid nitrogen to release any cell-associated virus into the supernatant. The frozen-thawed cell suspension was centrifuged at 3000 × *g* for 30 min, 4 °C, and the clarified supernatant was then serially titrated on BHK-21 monolayers, in GMEM containing 1% (v/v) FCS. CPE was recorded after 2 days incubation at 37 °C, and the titre of infectious virus calculated using the method of Reed and Muench [13]. As a control, virus alone was employed in place of opsonised virus.

2.8. Acute phase protein assays

Porcine α_1-acid glycoprotein (α_1-AG) and haptoglobin (Hp) were measured with a single radial immunodiffusion kit (Cardiotech Services, Louisville, Kentucky, USA). As explained in the manufacturer's instructions, 5 μl of standard A (1000 μg/ml) and standard B (250 μg/ml) were dispensed in separate wells. Serum test samples were dispensed in an identical manner, except that samples to be measured for Hp were diluted five-fold with PBS. The plate was covered, placed in a humidified box and incubated for 48 h at 39 °C. Precipitation ring diameters were measured, and the Hp or α_1-AG concentrations determined from the standard curve. In the case of the Hp measurements, the results were multiplied by the dilution factor of five.

Porcine C-reactive protein (CRP) and serum amyloid A (SAA) were measured using solid phase sandwich ELISA kits (Tridelta Development Ltd., P.O. Box 14, Greystones Co., Wicklow, Ireland). As explained in the manufacturer's instructions for the measurement of CRP, 100 μl of serum test sample (pre-diluted 1:100 in diluent buffer) or standard were added per well of the 96-well ELISA plate provided, and incubated for 15 min at 37 °C. The wells were washed four times with washing buffer to remove unbound sample before adding 100 μl of anti-porcine CRP conjugate per well, and incubating for a further 15 min at 37 °C.

For the measurement of SAA, 50 μl of biotinylated anti-SAA followed by 50 μl of standard or serum test sample (pre-diluted 1:500 in diluent buffer) were added per well of the 96-well ELISA plate provided. The plate was incubated for 1 h at 37 °C and then washed four times with wash buffer. After washing, 100 μl of strepavidin-peroxidase were dispensed to each well, and incubated at room temperature in the dark for 30 min.

For both ELISA assays the plates were finally washed before adding 3′,3′,5′,5′-tetramethylbenzidine (TMB) substrate (100 μl) with incubation for 15 min at RT in the dark. After incubation, 100 μl of stop solution was added per well. The

plates were read with a microplate reader (VERSAmax™, Molecular Devices Corp., Sunnyvale, CA, USA) at 450 nm absorbance wavelength. Sample concentrations were determined by reading from the standard curve and multiplying by the appropriate dilution factor.

2.9. Preparation of peripheral blood leucocytes (PBL), plasma and serum

Citrated blood was collected from each pig for the isolation of peripheral blood leucocytes (PBL). Initially, the blood was centrifuged at 1000 × *g* for 25 min, 2 min brake, 4 °C. The plasma layer was removed, aliquoted and stored at −20 °C. Cold distilled water was then added to the cell pellet to lyse the erythrocytes, mixed for 30 s and tonicity restored by the addition of 10 × concentration of cold PBS. The cells were centrifuged at 800 × *g* for 20 min, 2 min brake, 4 °C, and the supernatant removed. This procedure was then repeated to remove any remaining erythrocytes. PBL were then washed twice with cold Ca^{2+}–Mg^{2+}-free phosphate buffered saline (PBS-A) supplemented with 0.03% (w/v) EDTA, and centrifuged at 350 × *g* for 15 min, 4 °C.

Serum was collected from non-citrated blood by incubating the blood at 37 °C, then at 4 °C overnight, followed by centrifuging at 3000 × *g* for 10 min, 4 °C and storing the serum at −20 °C.

2.10. Chemotaxis assay

PBL migration was measured in a 96-well chemotaxis chamber (ChemoTx, Neuro Probe, Gaithersburg, MD) as described [14]. Briefly, the PBL preparation from each pig was resuspended at a final cell concentration of 5×10^6/ml in DMEM, supplemented with 10% (v/v) heat-inactivated FCS. Calcein AM (Molecular Probes, Eugene, OR), a fluorescent cell marker, was added to the cell suspension at a concentration of 5 μg/ml and incubated for 30 min at 37 °C. After incubation, the PBL were washed twice in cold PBS^A-0.03% (w/v) EDTA and resuspended at 5×10^6 cells/ml in DMEM, supplemented with 1% (v/v) heat-inactivated FCS.

As chemoattractants, the following were employed: autologous plasma (derived from the same animal and experiment as PBL), pooled plasma obtained from 20 donor animals, zymosan-activated serum (ZAS), recombinant porcine interleukin-8 (IL-8) (Biosource, Camarillo, California, USA), DMEM (as a control for random migration). ZAS was prepared by resuspending Zymosan A (2 mg/ml) in FCS and incubating at 37 °C for 60 min followed by centrifugation at 2000 × *g* for 10 min [15]. All except IL-8 were diluted in PBS to give a concentration of 100, 75, 50, 25 and 10% v/v. IL-8 was diluted in PBS to give a concentration of 500, 100, 10, 1 and 0.1 ng/ml. The wells in the chamber were filled with 32 μl of diluted chemoattractant or PBS as a negative control. In order to prepare a standard curve of fluorescence verses cell number, 25 μl of 5.0, 4.0, 3.0, 2.0, 1.0, 0.75, 0.5, or 0.25×10^6 fluorescent-labelled PBL/ml were dispensed directly into the wells. A polycarbonate cell permeable membrane (5 μm pore size) was placed on top of the wells, and 25 μl of autologous PBL pipetted on to the filter sites. The plate was incubated for 2 h at 37 °C. Non-migrating cells on the top of the filter were gently wiped away with a tissue and the filter carefully removed. The fluorescence-labelled PBL that had migrated into the wells were read by a spectrofluorimeter (SPECTRAmax™ GEMINI, Molecular Devices Corp., Sunnydale, CA, USA) (excitation 485 nm, emission 530 nm), and the number of cells migrated determined from the standard curve.

The above method was also used to measure the migration of PBL taken from a pig that was not used in the vaccination trials. This animal was a non-vaccinated, untreated, uninoculated pig. The PBL from this animal would not be displaying any effects due to inoculation or vaccination. By employing PBL from this pig, the "background" migratory activity of cells in the absence of any inoculation were determined. In addition, these PBL permitted the measurement of the chemotactic activity present in the plasma from vaccinated animals.

2.11. Absolute cell counts and flow cytometry (FCM)

After PBL isolation, total cell counts were obtained by mixing the cell suspension at a 1 in 10 dilution with Turk's solution for 30 s at room temperature. Turk's solution will lyse any contaminating erythrocytes. This mixture was dispensed into a haemocytometer chamber, and the total PBL counted under a light microscope, multiplying by the diluting factor.

PBL at a cell concentration of 1×10^6 cells/100 μl were labelled with monoclonal antibodies (MAbs) SWC1 (11/8/1, IgG2b isotype), SWC3 (74-22-15, IgG1 isotype) (both kindly donated by A. Saalmüller, BFAV, Tübingen, Germany) and SWC8 (MIL-3, IgM isotype) (kindly donated by K. Haverson, University of Bristol, UK). This labelling distinguishes monocytes ($SWC1^+$, $SWC3^+$, $SWC8^-$), neutrophils ($SWC1^+$, $SWC3^+$, $SWC8^+$), eosinophils ($SWC1^-$, $SWC3^+$, $SWC8^+$), B lymphocytes ($SWC1^-$, $SWC3^-$, $SWC8^+$), T lymphocytes ($SWC1^+$, $SWC3^-$) [16–18]. After washing the cells with CellWASH (Becton Dickinson), appropriate isotype-specific conjugates (goat $F(ab')_2$ anti-mouse Igs; Southern Biotechnology Associates, Birmingham, AL) were added. All MAb incubations were for 20 min at 4 °C; conjugates were for 15 min at 4 °C. The FACScan Flow Cytometer and Cellquest programme (Becton Dickinson AG, Basle, Switzerland) were used to acquire and analyse 20,000 events per sample. Appropriate gates, using a combination of the fluorescence channels Fl-1, FL-2, or FL-3, along with forward scatter/side scatter (FSC/SSC) profiles were employed to identify and measure the different cell subpopulations. Absolute counts of each subpopulation were calculated by multiplying the total PBL count by total percentage of each cell population gated.

2.12. Cell surface chemokine and integrin receptor expression

PBL were seeded at 4×10^6 cells/ml in teflon containers holding 7 ml of DMEM, supplemented with 30% (v/v) citrated plasma taken from control or FMDV vaccinated animals. After 4 h or 24 h incubation at 39 °C, the cells were centrifuged for 15 min, 350 × *g*, at 4 °C and resuspended at 1×10^6 cells/100 μl in CellWASH (Becton Dickinson). The cells were labelled with the following MAb: mouse anti-porcine wCD11R1 (MIL-4, IgG1, Serotec Ltd., Oxford, UK.) [19], mouse anti-human CD49d (HP2/1, IgG1, Immunotech, Marseille, France), mouse anti-human CXCR-1 (IL-8 RA) (clone 42705.111, IgG2a, R & D Systems, Minneapolis, USA), mouse anti-human CCR-2 (clone 53504.111, IgG2b, R & D Systems), mouse anti-human CCR-5 (clone 45549.111, IgG2b, R&D Systems), mouse anti-human CXCR-4 (clone 44708.111, IgG2a, R&D Systems), SWC1 and SWC8 as previously described, and mouse anti-human mannose receptor, conjugated with *R*-phycoerthyrin (PE) (clone 3.29B1.10, IgG1, Immunotech, Marseille, France). Fluorochrome labelling and FCM was performed as described above.

3. Results

3.1. Serum anti-FMDV antibodies

It has been clearly demonstrated that the vaccine employed in these studies will protect pigs against indirect FMDV aerosol challenge infection ([7]; Barnett et al., personal communication). Considering that specific anti-FMDV antibodies (Abs) are known to play an important role in long-term protection against FMD (reviewed in [4]), it was important to measure the level of anti-FMDV Abs that had been produced early post-vaccination. Therefore, sera from the animals used in the present experiments were titrated in the neutralisation test and ELISA.

It was not until 7–10 days post-vaccination that titres of specific anti-FMDV Abs were clearly distinguishable in the diluted sera from the vaccinates, but not in sera from the control or adjuvant inoculated animals (data not shown). At 3 and 6 days post-vaccination, serum diluted as little as 1 in 10 gave <10% neutralisation of virus infectivity, and there was no difference between vaccinates and controls (data not shown). In contrast, at a 1 in 2 dilution, FMDV infectivity for the BHK-21 indicator cells could be neutralised by the sera (Fig. 1). Nevertheless, this was found both before vaccination and with sera from the control and adjuvant-inoculated animals.

Despite these results, it was considered possible that a vaccine effect may have been manifested in the kinetics of neutralisation, as reported by Scicluna et al. [20]. Following only 1 min of incubation with the virus, sera (diluted 1:2) taken at pre-bleed, 3 and 6 days post-vaccination were capable of inhibiting virus-induced CPE in at least 50% of the BHK-21 monolayer (Fig. 1a). With 5 and 10 min incubation of serum with the virus before adding to the BHK-21 cells, the neutralisation of virus infectivity ranged from a 60–90%. These observations were obtained again with all serum samples (Fig. 1a–c). Therefore, this virus neutralisation effect could not be related to the formulated vaccine.

3.2. Uptake and destruction of opsonised FMDV by macrophages

McCullough et al. ([4,5]), demonstrated that an important role for macrophages is to phagocytose and destroy opsonised FMDV. Consequently, the interplay between macrophages and FMDV opsonised with the sera used in Fig. 1. was analysed. It was noted that destruction of infectious virus which had interacted with the macrophages became discernible within 2–4 h (Fig. 2). By 24 h, infectious

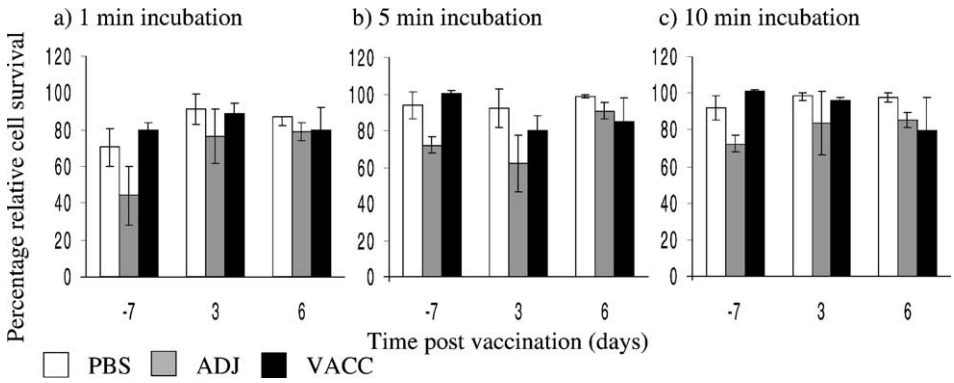

Fig. 1. Neutralisation of FMDV with serum from animals before and after vaccination. Serum was collected at the indicated times post-vaccination from animals which received PBS (white bars), adjuvant (grey bars) or vaccine (black bars). These sera were incubated with 100 $TCID_{50}$/100 μl of FMDV for (a) 1 min, (b) 5 min and (c) 10 min. The serum/virus mixtures were then dispensed on to a BHK-21 cell monolayer, and incubated for a further 48 h. Any cells, which had not been lysed by the virus after this incubation, were stained with crystal violet, and measured with an ELISA reader. Each bar represents the average of three animals ± S.D. from the first trial. The second trial gave similar results.

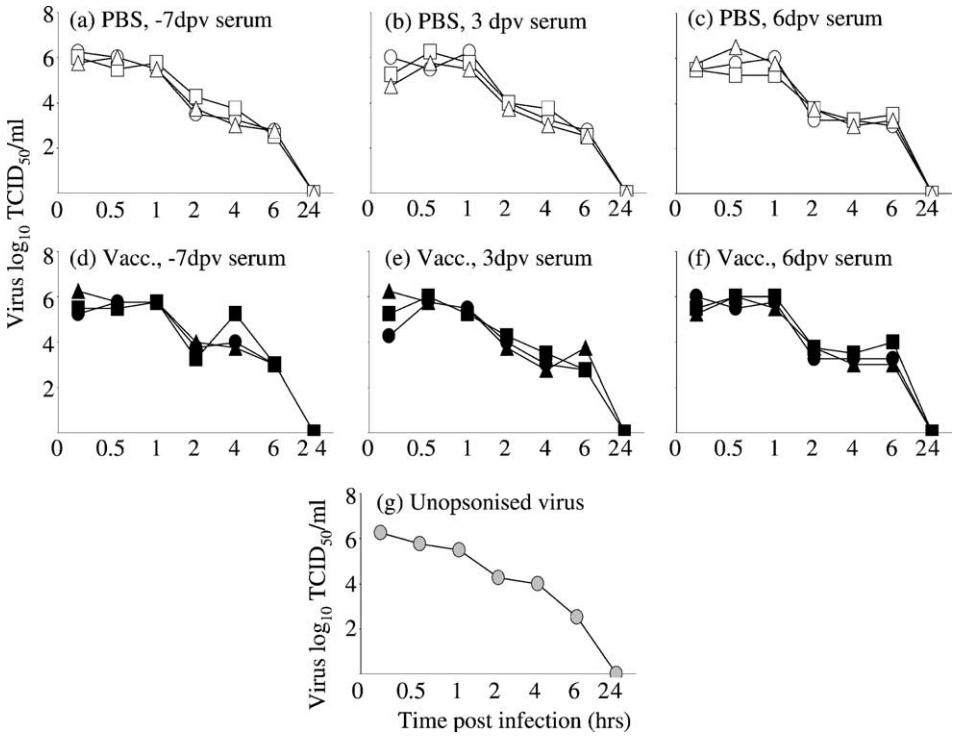

Fig. 2. Titres of macrophage-associated infectious FMDV following opsonisation. FMDV (10^6 $TCID_{50}$/ml) was incubated with an equal amount of serum taken from PBS-treated animals (a–c) and from vaccinated animals (d–f), at pre-bleed (−7 dpv) (a, d), 3 dpv (b, e) and 6 dpv (c, f) (dpv: days post-vaccination). Serum/virus mixture was incubated with blood monocyte-derived macrophages and incubated for the indicated times. The macrophages were then removed and centrifuged, frozen-thawed three times in liquid nitrogen, further clarified and the supernatant titrated on BHK-21 indicator cells. The uptake and destruction of non-opsonised virus is shown by way of comparison (g). The results are shown for samples from the first trial; the second trial gave similar results.

virus could no longer be detected. This uptake and destruction of the virus was not dependent on vaccine treatment, nor the time at which the serum was taken. Furthermore, there was also no difference when opsonised virus (Fig. 2a–f) was compared with unopsonised virus (Fig. 2g). These results imply that the vaccine did not have any effect on the capacity of the serum to enhance opsonisation and destruction of the virus by macrophages.

3.3. Acute phase proteins

Attention now focused more on how the activity of the phagocytes themselves would have been modified by the vaccination. A well described component of a localised inflammatory response is the production of inflammatory cytokines such as interleukin-1 (IL-1) [21–23], tumor necrosis factor-α (TNF-α) [24–26] and IL-6 [27–29] from activated macrophages. These cytokines, if produced in sufficient quantities, can have a systemic inflammatory effect. One element of this is the stimulation of hepatocytes to synthesise acute phase proteins (APP) [30]. A relatively high level of APP was noted in some animals at the time of pre-bleed (Fig. 3). After vaccination, there was no increase in any of the APP measured. In fact, some of these proteins fell to undetectable levels, but in both vaccinated as well as control animals (Fig. 3b and c). Interestingly, the level of alpha$_1$ acid glycoprotein (α_1-AG) remained relatively stable in the sera from the vaccinated animals (Fig. 3a, filled symbols). This contrasted with the control animals (Fig. 3a, open symbols), with which the α_1-AG increased two to four-fold during the 6 days post-vaccination. Taken together, these results would imply that the vaccine was not inducing a systemic inflammatory response.

3.4. Absolute PBL subpopulation numbers before and after vaccination

Another important element of phagocyte and antigen presenting cell activity during the immune response is migration into the tissues. Changes in the numbers within the different PBL subpopulations could give a gross indication of vaccine-induced migration of leucocytes into tissue sites. It would also demonstrate the influence of the blood sampling, which would be irrelevant to the analyses on vaccine effects. Overall, compared with PBS treatment, the vaccine showed no differential influence on the PBL subpopulation distribution (data not shown). Additional studies in pigs with the same vaccine formulation, as well as one containing O_1 Lausanne antigen, gave similar results (not shown).

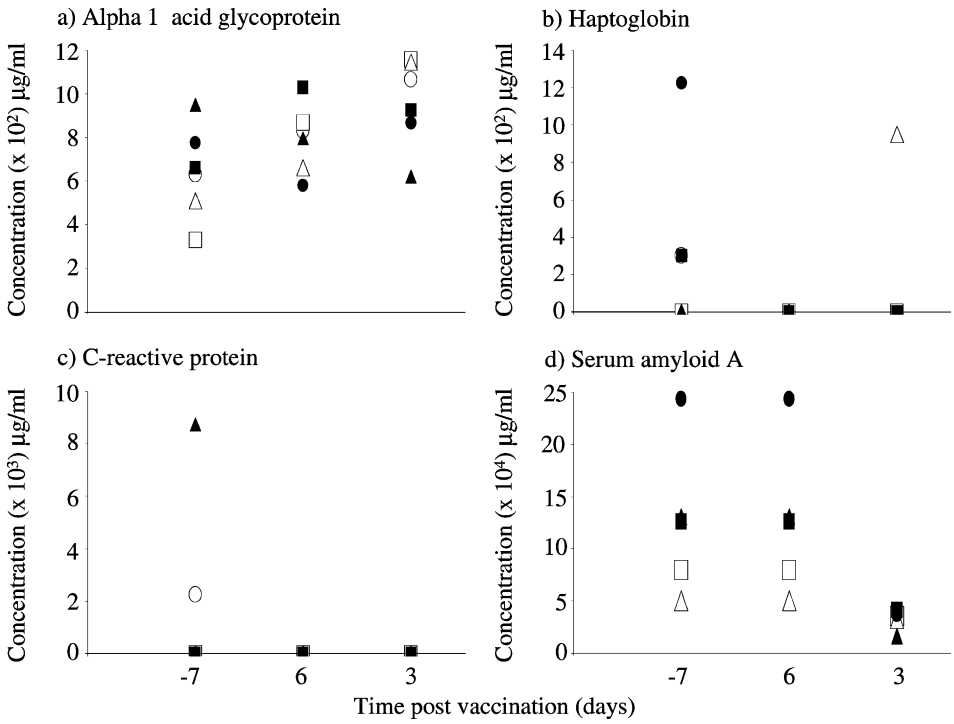

Fig. 3. Acute phase protein concentrations in serum before and after vaccination. At the times indicated post-vaccination (−7: the pre-bleed), serum from animals treated with PBS (open symbols) or vaccine (filled symbols) were measured either by single radial immunodiffusion for (a) α1 acid glycoprotein and (b) haptoglobin, or by ELISA for (c) C-reactive protein and (d) serum amyloid A, as described in Section 2. The same symbol in each graph and at each time point represents the same animal.

3.5. *Migration of PBL towards optimum concentrations of chemoattractants*

Further analyses on the leukocytes focused on the migration event itself. Study of leukocyte migration and chemotaxis is more sensitive than the simple measurement of PBL population dynamics. Both random migration (using DMEM in place of a chemoattractant) as well as migration towards optimum doses of potential chemotactic stimuli were studied. The pre-bleed samples showed no difference between the vaccinates and the "PBS" or "adjuvant" groups. With time post-vaccination, there was an increase in both the random migration (using DMEM) and the chemotaxis of PBL from all animals (data not shown). With some but not all vaccinates, elevated migration (greater than that with all PBS controls) was seen at 3 days post-vaccination. It was at 6 days post-vaccination that this migration was most notable with all vaccinates (Fig. 4, black symbols) compared with the other animals (Fig. 4, white and grey symbols).

This was clearly an effect of the vaccine, but not of the adjuvant alone, as seen with the PBL from the adjuvant-inoculated animals (Fig. 4, grey symbols). Interestingly, the highest level of migration was obtained with plasma and with ZAS. IL-8 was a poor chemoattractant for the cells, giving results which were not better than the random migration towards DMEM. Nevertheless it was also interesting that more PBL from the vaccinates than from the other two groups displayed random migration. These

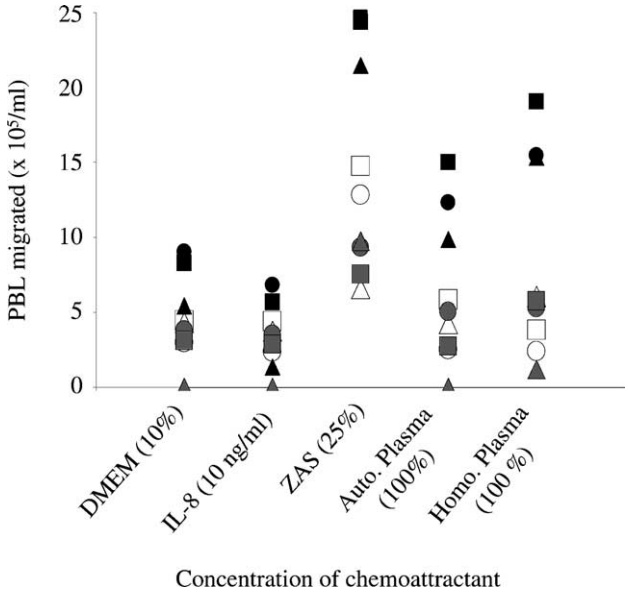

Fig. 4. PBL migration towards optimum chemoattractant concentrations. Fluorescent-labelled PBL from animals treated with PBS (white symbols), adjuvant (grey symbols) or vaccine (black symbols) were incubated for 2 h in the presence of different chemoattractants, in a chemotaxis chamber, as described in Section 2. DMEM was employed to measure random migration (non-chemokine). The number of PBL that migrated into the chemoattractant were read by a spectrofluorimeter.

results were also observed in the second vaccine trial using five vaccinates and three PBS controls (data not shown).

It would appear that the PBL from the vaccinates had an enhanced migratory activity, most clearly discernable at 6 days following the vaccination. These migratory cells were responsive to the chemotactic activity of ZAS and plasma, even plasma from untreated pigs ("homologous plasma"), but not to IL-8.

3.6. Comparison of plasma-dependent migration of PBL from vaccinated and control animals

PBL from vaccinates and PBS controls were tested for migration towards different concentrations of homologous pooled plasma as shown in Fig. 5a. The PBL from adjuvant inoculated animals are not shown, due to the fact that they responded in a similar manner to those from the PBS controls. This homologous plasma was pooled from 30 pigs which had not been inoculated. The same plasma was used for all PBL samples, to determine the relative chemotactic activity that the PBL from the vaccinates and PBS controls had possessed in vivo. At 6 days post-vaccination, PBL from the PBS controls (Fig. 5a, open symbols) showed reduced chemotactic activity at high concentration of plasma compared with the lower concentrations. This contrasted with the cells from the vaccinates (Fig. 5a, filled symbols), which maintained their chemotactic responsiveness regardless of the plasma concentration. This response of control PBL at high homologous plasma concentrations was actually reduced to the level of the random migration ($<10 \times 10^5$ cells/ml migrating-values shown within the rectangle of Fig. 5a, and marked "*R*"). The PBL from the vaccinates maintained a level of migration in the presence of homologous plasma which was higher than that of random migration.

3.7. Influence of autologous plasma on PBL migration

The PBL migration shown in Fig. 5a demonstrated that cells from the vaccinates had a different response to those from the controls. This raised the question of whether the vaccinate PBL had been altered or the PBL had been under the influence of chemotactic factors in vivo. Consequently, the PBL migration was measured in response to their autologous plasma (Fig. 5b). In the presence of autologous plasma (Fig. 5b), the higher than random level of migration was again seen with the vaccinated PBL. Furthermore, the chemotactic activity of the vaccinated PBL was higher towards autologous plasma (Fig. 5b, black shapes) than towards the homologous pooled plasma (Fig. 5a, black shapes). The activity of the control PBL was at best only slightly elevated above random migration.

3.8. Chemotactic activity of plasma from vaccinated and PBS control animals

The observation that autologous plasma further increased the PBL chemotactic activity, in a dose-dependent manner (Fig. 5b), suggested that plasma factors had also been modulated by the vaccinations. Consequently, the chemotactic potential of the autologous plasma was tested using PBL from a single untreated and non-vaccinated animal.

The plasma at pre-bleed showed variable levels of chemotactic activity, and there was no difference between plasma from vaccinates and PBS controls (data not shown). In contrast, plasma taken at 3 days showed a clear enhanced chemotactic potential when taken from the vaccinates (Fig. 6a, filled symbols). This chemotactic activity was potent, remaining high even when the plasma was diluted to a low concentration. By 6 days, the distinction of vaccinates from controls was no longer evident, except for one vaccinated animal (Fig. 6b). These results were confirmed in the second vaccine trial using five vaccinates and three controls (data not shown).

3.9. Plasma induced modulation of integrin and chemokine receptor expression on PBL

In order to 'sense' chemoattractants and respond by moving to sites of inflammation, cells have to regulate their chemokine receptors and integrins. Plasma from the

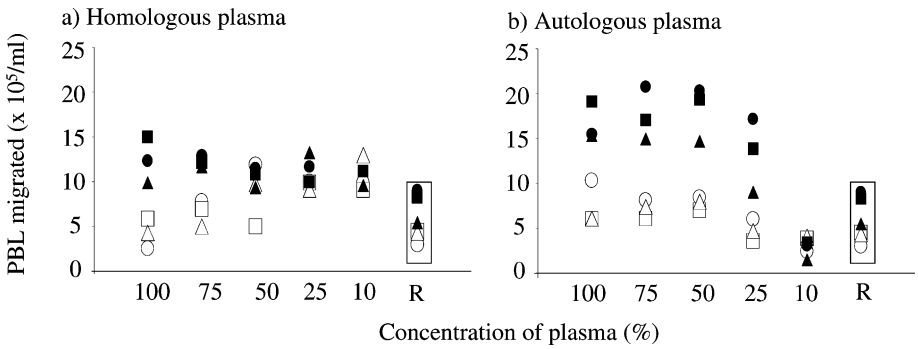

Fig. 5. Migration of PBL from PBS-treated (open symbols) and vaccinated (filled symbols) animals. Fluorescent-labelled PBL from 6 days post-vaccination or post-PBS inoculation were incubated with varying concentrations (*x*-axis) of either (a) homologous pooled plasma or (b) autologous plasma taken at the same time as the PBL. The homologous plasma was a pool from 30 animals, which were neither vaccinated nor inoculated. The PBL levels in the rectangular boxes marked "*R*" on the *x*-axis, represent the level of random migration in the presence of 10% (v/v) DMEM.

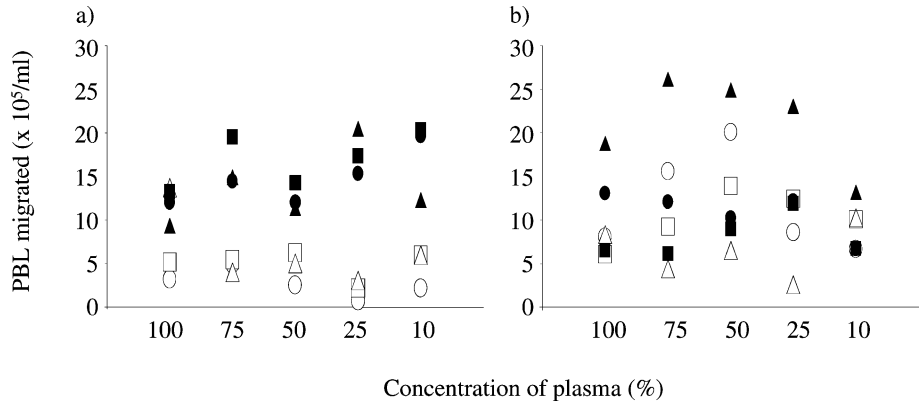

Fig. 6. Migration of PBL from a non-vaccinated, untreated donor animal towards plasma taken at (a) 3 or (b) 6 days from PBS-treated (open symbols) or vaccinated (filled symbols) animals. After a 2 h incubation, the amount of PBL that had migrated into the plasma was read by a spectrofluorimeter.

vaccinated animals and PBS controls were therefore compared for their ability to modulate such receptors on PBL. The PBL were incubated with the plasma samples for 4 and 24 h, but for reasons of clarity only analyses from plasma incubated with the PBL for 4 h are shown (Fig. 7).

There was no influence of the vaccination on plasma-dependent modulation of CD11R1 expression (data not shown). For CD49d expression variation was noted between the days of sampling and between the animals. Nevertheless, no CD49d modulation could be attributed to the vaccination (data not shown).

When plasma from vaccinates at 3 days post-vaccination was employed, a modulation of CCR1 expression was noted compared to the "−7 day" pre-bleed samples (Fig. 7a, filled symbols). The plasma from the PBS controls at 3 days post-vaccination did not have the same effect (Fig. 7a, open symbols). When plasma from 6 days post-vaccination was employed, there was no difference between that from vaccinates and that from PBS controls in terms of the influence on CCR1 expression. The level of expression of CCR5 (Fig. 7b) and CXCR1 (Fig. 7c) remained more constant, regardless of which plasma was employed with the donor

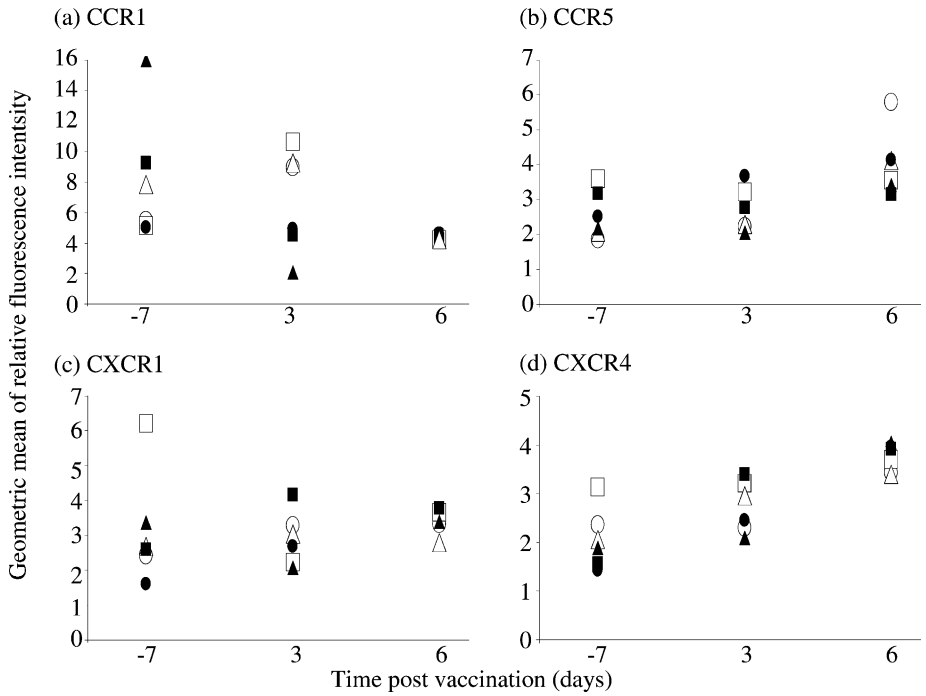

Fig. 7. Plasma induced modulation of (a) CCR1, (b) CCR5, (c) CXCR1, and (d) CXCR4 expression on PBL. Plasma collected at the times indicated post-vaccination from animals which received PBS (534, 535, 536; white circle, square and triangle, respectively) or vaccine (541, 542, 543; black circle, square and triangle, respectively) were incubated with PBL for 4 h. The PBL were then labelled with anti-CCR1, CCR5, CXCR1 or CXCR4 Mab, and expression measured by flow cytometry. Results are expressed as the geometric mean of fluorescence intensity less that of the conjugate control.

PBL. Expression of CXCR4 increased two-fold on donor PBL incubated with plasma from 6 days post-vaccination, in comparison with cells incubated in the "pre-bleed" plasma (Fig. 7d). However, there were no significant differences between plasma from vaccinates and controls with respect to modulation of CXCR4 expression.

3.10. Influence of plasma on differentiation markers

Mannose receptor expression was used as an indicator of differentiated monocytic cells, such as macrophages and dendritic cells. There was no apparent influence of the plasma, whether from vaccinated or PBS control animals, on mannose receptor expression (data not shown). The cell phenotype marker SWC1 which labels monocytes and *T* lymphocytes, but is lost upon monocyte differentiation to macrophages [12] was modulated by the plasma samples. However, this was variable and dependent on the sample, and no pattern particular to plasma from vaccinates was observed (data not shown). Similar observations were noted for SWC3 and SWC8 expression (data not shown).

4. Discussion

There has been renewed interest in innate immunity due to the recent work on dendritic cells (reviewed in [31]) pattern recognition receptors (Toll receptors, reviewed in [32]), antimicrobial peptides (defensins, reviewed in [33]) and chemokines/chemokine receptors (reviewed in [34]). In the context of vaccination, innate immunity would be triggered by the acute local inflammatory response at the site of injection, recruiting monocytes, macrophages and neutrophils [35]. Their activation would lead to inflammatory cytokine production, phagocytosis and enhancement of both innate and specific immune responses. However, the role of innate immunity is rarely studied in vaccination trials. Yet the indications are that innate immunity would play an important role in the initial protection induced by emergency vaccination against FMDV. The emergency FMDV vaccine is exceptional in that it can induce early protection in the absence of detectable specific immunity [6–8].

Although neutralising antibodies (Abs) are required for long-term protection against FMDV (reviewed in [1,4]), no specific anti-FMDV neutralising antibodies were detected at the early time points (3–6 days) post-vaccination in pigs when protection has been noted [7]. However, dilution of serum, as conventionally employed to measure neutralisation, may fail to detect natural Ab [10]. Using a 1 in 2 dilution of serum, neutralisation of FMDV infectivity in vitro was obtained, but there were no differences between the sera from vaccinates and PBS controls, nor between pre-bleed and post-vaccination samples.

Virus neutralisation does not always relate to the phagocytosis important for protection in vivo [5,36]. Macrophages will internalise FMDV opsonised with specific Ab within 2 h, and 50% of the virus will be destroyed by 4 h (Rigden et al., in press). Even in the absence of specific antibodies, >90% virus infectivity can be destroyed by macrophages within 6 h. Sera from the vaccinates or the controls early (3–6 days) post-vaccination were unable to enhance this uptake and destruction of the virus.

Due to these observations that serum opsonins alone could not be implicated in the early immunological events, attention turned to vaccine-induced modulation of leucocytes. Changes in blood leucocyte populations can indicate acute systemic inflammatory reactions [37]. Vaccination with the emergency FMDV vaccine did not modify body temperature, nor were clear changes in blood leucocyte population distribution identifiable. Furthermore, there was no elevation of acute phase protein production following vaccination. These results demonstrated that the vaccine did not promote a systemic inflammatory response.

One would expect vaccination to induce a local inflammatory response. A consequence would be increased chemotactic activity in the blood, and mobilisation of phagocytic cells from the bone marrow. Leucocyte chemotaxis was indeed modulated by the vaccine. This was not a simple effect of the adjuvant, but was dependent on the vaccine. These migratory leucocytes were seen to be dominated by granulocytes, followed by monocytes (data not shown), typical of innate defence responses [38].

Chemotaxis of leukocytes from the blood to sites of inflammation is regulated at least in part by the production of chemokines, associated with the modulation of chemokine receptor and integrin expression on different cell populations (reviewed in [38]). Although the present work was not able to identify the factors responsible for activating the leucocytes, it is clear that prominent chemotactic activity was present in the plasma within the first 3 days post-vaccination. By 6 days post-vaccination, the effect was most pronounced on the leucocytes. Such kinetics would relate to the generation of chemotactic factors from histiocytes in the site of vaccine deposition, followed by recruitment of blood leucocytes to that site. The reduced chemotactic activity of the plasma at 6 days compared with 3 days would relate to the leucocytes now utilizing the plasma chemokines. Although the individual chemokines in the plasma could not be identified, analysis of the cell receptors involved could shed some light in this area. Upregulation of β_1 integrins and the associated cross-linking of β2 integrins on neutrophils is an important mechanism in their extravasion from blood into tissues [39]. Our own results identified plasma-induced modulation of CD49d but not CD11R1. It is possible that the modulation of β_2 integrins may have occurred prior to 3 days post-vaccination. Nevertheless, the CD49d modulation could not be related solely to the vaccination. Similarly, the chemokine receptors CCR5, CXCR1 and CXCR4 were not modulated by the plasma. In contrast, modulation of CCR1 expression may have occurred at day 3 post-vaccination. CCR1 is an important receptor for recruitment of cells to inflammatory sites, being modulated as the cells migrate and

mature [40,41]. Downregulation of the receptor would reflect chemokine-induced capping and internalisation, but it is also known that agonists can desensitise CCR1 [40]. Nevertheless, the anti-CCR1 Ab employed was anti-human, and the specificity and its reactivity with porcine CCR1 is unconfirmed.

It can be concluded that initiation of cell migration and increased chemotaxis of leucocytes, probably to the site of injection, by the emergency FMDV vaccine are key factors in developing early protection. Through this activity, phagocytic function would also be enhanced, both in terms of the individual cell recruited and the increased number of migratory leucocytes. Under such conditions, it is likely that other elements, which alone could not be linked to the immunological events of early protection, such as the natural opsonins and the natural neutralising activity of serum, could become important. It is envisaged that oronasal infection by FMDV would involve epithelial cells of the oropharyngeal mucosa. As a consequence of cell death and tissue damage, inflammatory cells including monocytes, neutrophils, dendritic cells and NK cells would be attracted. The increased migratory capacity of leucocytes early post-vaccination would therefore be crucial in determining the outcome of the infection. Furthermore, dendritic cells migrating to the site of infection would play a pivotal role in promoting the development of the specific defences [1,4]. Clearly, emergency FMDV vaccines inducing early protection enhance leucocyte chemotactic activity. Identification of the elements in the vaccine formulation responsible for the enhanced leucocyte migration would be the next step, and facilitate a better understanding of the mechanisms involved with respect to the potency of the current emergency vaccine against FMD.

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