The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response

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African swine fever virus ASFV/NH/P68 is a naturally occurring, non-haemadsorbing and non-fatal isolate. Longitudinal clinical and immunological studies on 31 pigs inoculated oronasally or intramuscularly with this isolate defined two discrete groups of animals: those developing ASF chronic type lesions and those remaining asymptomatic. Animals developing lesions had viraemia and fever late after infection, NK activity levels close to that of control animals and high levels of anti-ASFV specific antibodies together with a marked hypergammaglobulinaemia involving IgG1, IgG2, IgM and IgA immunoglobulin isotypes. Pigs remaining asymptomatic after infection, on the other hand, did not have viraemia or fever after day 14 post-infection and had elevated NK cell activity, but normal plasma Ig concentrations and relatively low specific anti-virus antibody concentrations throughout the duration of the experiments. Importantly, the latter group of pigs virus were resistant to subsequent challenge with the highly virulent ASFV/L60 isolate and survived with no major changes in any of the parameters examined and referred to above. Finally, lymphoproliferative responses to the mitogens concanavalin A, phytohaemagglutinin and pokeweed mitogen were not depressed in either of the two clinically defined groups of pigs. Thus further studies with this infection model may provide new insights on mechanisms of protective immunity to ASFV.

Introduction

African swine fever (ASF) is caused by a large DNA virus classified as the only member of the recently named Asfarviridae family (Dixon et al., 2000). The disease is nowadays confined to sub-Saharan countries of Africa and to the island of Sardinia, but sporadic outbreaks have occurred in recent years in Europe. The disease represents a threat to the pig industry due to the expanding mobility of people and the potential global trade of pork products. Because there is no suitable vaccine, control of ASF is through rigorous sanitary measures following laboratory diagnosis. Although different African swine fever virus (ASFV) isolates share common biological features, their pathogenesis may range from rapidly fatal to subclinical, chronic or no overt symptoms of disease (reviewed by Plowright et al., 1994). This range of ASFV isolates showing different virulence is matched by an equally wide range of immune responses (reviewed by Martins & Leita4o, 1994), and suggests a complex array of virus–host interactions depending, at least partially, on the virulence of the aetiological agent (Pini & Wagenaar, 1974; Vigário et al., 1974; Thomson et al., 1979; Pan & Hess, 1984).

In this work, we have focused on an interesting ASFV isolate, the non-fatal, non-haemadsorbing ASFV/NH/P68 (NHV), isolated from a chronically infected pig and used to...
Table 1. Summary of the clinical and immunological studies on pigs infected with ASFV/NH/P68 and challenged with ASFV/L60

The pigs listed (S#) were infected with $5 \times 10^6$ CPE$_{50}$ of non-lethal NHV or highly pathogenic L60 strains of ASFV as indicated, and monitored for fever, viraemia and clinical manifestations of ASF. Those with clinical disease were killed. The remaining asymptomatic animals were challenged with $5 \times 10^6$ CPE$_{50}$ of L60 i.m. Additional immunological studies included anti-ASFV antibodies (anti-ASFV Abs), NK cell activity (NK), total plasma Ig isotype distribution (Ig) and proliferative lymphocyte responses to mitogens and viral antigens (lymphoprolif.). Two normal pigs were included in each experiment to provide a control reference for the studies.

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FBE
obtain anti-ASFV sera for diagnostic purposes (Vigário et al., 1974). Our observations extend previous studies on cellular immune responses in experimentally infected pigs (Martins et al., 1993; Leitão et al., 1998, 2000), and provide the basis for a useful and relevant infection model for studies on the mechanisms of protective immunity; in particular, the fact that exposure to NHV induces significantly high levels of NK cell activity and protects against subsequent infection with the highly virulent ASFV/L60 (L60). Thus, pigs inoculated with NHV and subsequently challenged with L60 were studied for the development of clinical signs, occurrence of viraemia and the development of cellular and serological immunity.

**Methods**

- **ASFV isolates.** Two natural occurring ASF isolates were used: the non-haemadsorbing ASFV/NH/PH6 (NHV), initially designated NHA2 (Vigário et al., 1974), and the highly virulent ASFV/L60 (L60) (Manso Ribeiro & Azevedo, 1961). Virus stocks were grown in blood-derived macrophage cultures for no more than six passages and titrated by observation of cytopathic effect (CPE) at end-point dilutions in macrophage cultures as previously reported (Martins et al., 1988).

- **Animals and animal inoculation.** Large White × Landrace cross-bred pigs, weighing 25–45 kg, were used for experimental inoculations. Pigs from the Lisbon slaughterhouse were used as blood donors to prepare macrophage cultures for virus propagation. Pigs were inoculated with 5 × 10^6 CPE_{50} NHV either by oronasal (o.n.) or intramuscular (i.m.) routes and monitored daily for body temperature and development of clinical signs. In each experiment there was a control group of two non-inoculated pigs. Blood samples were taken at different days post-inoculation (p.i.) from the anterior vena cava and collected in heparinized syringes (20 IU/ml blood). The clinical course and immunological parameters studied are summarized in Table 1. Animals developing clinical signs of ASF (ASF chronic type lesions) were euthanized by i.m. inoculation with 5 × 10^6 CPE_{50} of the highly virulent L60 isolate.

- **Blood-derived macrophage cultures.** Supernatants obtained after incubation of heparinized blood samples with 10% (v/v) of a 5% (w/v) dextran T 500 solution in Hanks’ balanced saline solution (HBSS) at 37°C, 15 min, were collected, diluted to twice the volume in culture medium (RPMI 1640 supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 20 mM HEPES), and incubated (37°C, 72 h) in tissue culture flasks. The non-adherent cells were removed by washing with HBSS leaving the adherent cells for virus propagation. Adherent cells were also harvested by treatment with 0.8 mM EDTA in PBS (8 g NaCl, 0.2 g KCl, 1.44 g NaHPO4, 0.24 g KH2PO4 per litre, pH 7.4) and used for virus titration (Martins et al., 1988) and for determining the levels of viraemia in inoculated animals.

- **Collection of peripheral blood mononuclear cells (PBMC) and plasma.** Heparinized blood samples collected from the anterior vena cava were layered on a Ficoll–Hypaque gradient (density = 1.077 g/cm³, Seromed) and centrifuged (room temperature, 400 g, 30 min). Cells from the interface were collected and washed with HBSS three times by centrifugation (4°C, 200 g, 10 min). The final pellet was resuspended in culture medium supplemented with 10% heat inactivated foetal calf serum (FCS). Cell viability, determined by trypan blue dye exclusion, was always higher than 95%. Plasma samples were clarified by centrifugation (4°C, 1000 g, 30 min), stored at −20°C and used to evaluate specific titres of anti-ASFV antibodies and total immunoglobulin (Ig) concentrations.

- **Estimation of viraemia in experimentally inoculated pigs.** Quadruplicate cultures of macrophages in 96-well microplates were inoculated with 20 µl blood per well and incubated (37°C, 7 days in 5% CO2 and > 80% humidity). Individual cultures were resuspended, transferred to cytospins slides, fixed with acetone (−20°C, 5 min) and screened with FITC-conjugated swine anti-ASFV serum. Positive samples were titrated as described above.

- **Lymphoproliferative responses to mitogens [phytohaemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogen (PWM)], and to ASFV (NHV and L60).** Lymphoproliferative responses to mitogens and ASFV were studied using PBMC in triplicate macrophage cultures (200 µl; 2.5 × 10^6 cells/ml in culture medium supplemented with 0.01 mM 2-mercaptoethanol and 10% FCS) and stimulating with PHA (50 and 100 µg/ml; Wellcome PHA HAI15), ConA (0.25 and 0.5 µg/ml; Sigma type IV C-2010) and PWM (0.5 and 1% v/v; Gibco 670-5360) and ASFV isolates NHV and L60 (m.o.i. 0.01). Cells without mitogens or virus were used as controls. Microplates were inoculated (37°C, 72 h in 5% CO2 and > 80% humidity) and then [3H]thymidine (1 µCi, 5 Ci/mmol) incorporation was allowed to proceed for 4 h. Cultures were harvested onto nitrocellulose filters, which were counted in scintillation fluid (Optiphase HiSafe 3, LKB 1200-437) using a scintillation counter. Incorporation of [3H]thymidine was measured as c.p.m. with coefficient of variation within triplicate cultures always less than 10%. The data from longitudinal samples were calculated for individual pigs as difference ratios at each time-point sampled, by dividing the observed c.p.m. in stimulated cultures by the observed c.p.m. of similarly stimulated PBMC cultures of the same pig at day p.i. 0. The final data were then expressed as the average ratio in each experimental infected group (infected with NHV symptomatic; infected with NHV healthy; infected with NHV healthy then challenged with L60) on a given day after commencement of the experiment minus the average ratio in the control uninfected group studied over the same time-scale. Thus values of zero indicate that infection with ASFV neither induces nor augments the subsequent response of PBMC to stimulation in vitro. The variation between ratios obtained on a given day within a given group of animals was always less than 20% and so, for clarity of presentation, this information is omitted from the graphical representation of the results. Levels of proliferation in the absence of antigen or mitogen were similarly low in all the samples and mitogen stimulation gave stimulation indices ranging from 30 to 100 while virus stimulation gave stimulation indices up to 10.

- **Measurement of anti-ASFV antibodies.** Plasma samples were tested by indirect ELISA using a crude preparation of ASFV proteins as antigen. The protein extract was obtained from ASFV-infected Vero cells by treatment with hypotonic buffer solution (67 mM sucrose, 5 mM Tris–HCl pH 8, 1% Nonidet P40) followed by centrifugation (4°C, 800 g, 20 min). The supernatant was adjusted to contain 50 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM Tris–HCl pH 8, and then centrifuged through a 20–60% sucrose gradient. The antigen collected at the 20–60% sucrose interface was suspended in 50 mM 2-mercaptoethanol, 2 mM EDTA, 0.5 M NaCl, 0.5% Nonidet P40, centrifuged (4°C, 100 000 g, 10 min), and resuspended in carbonate–bicarbonate buffer (1.59 g Na2CO3, 2.93 g NaHCO3 per litre, pH 9.6) at a concentration empirically determined for each antigen preparation for sensitizing ELISA 96-well microplates (Dynatech M129B) overnight at 4°C.

Serial twofold dilutions of plasma samples in PBS were added to the antigen-coated microplate wells and, after 1 h at 37°C, bound antibody was detected using protein A–peroxidase conjugate, and revealed with
α-phenylenediamine in the presence of hydrogen peroxide. The reaction was stopped with 1 M H₂SO₄, and the absorbance at 492 nm was read using a spectrophotometer. Sample titres were calculated by comparison with a reference anti-ASFV swine serum previously titrated by indirect immunofluorescence.

Measurement of total IgG1, IgG2, IgM and IgA concentrations by ELISA. Serial twofold dilutions of plasma samples and reference serum were dispensed in ELISA microplates and incubated overnight at 4 °C. After coating, plates were incubated (37 °C, 2–3 h) with PBS containing 0.5% (w/v) Tween 20. Differential detection of swine Ig classes was achieved by separate development with mouse monoclonal antibodies (MABs) (diluted 1/1000 in PBS) to swine-IgG1 (Serotec MCA635), -IgG2 (Serotec MCA636), -IgM (Serotec MCA637) and -IgA (Serotec MCA638). For negative controls, PBS replaced the anti-immunoglobulin MABs. After incubation (37 °C, 2 h), peroxidase-conjugated rabbit anti-mouse antibody (Dako P0260) was added (1/2000 dilution in PBS) and incubation was continued (37 °C, 1 h). After washing the substrate ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] solution (Boehringer Mannheim) was added, and after 40 minutes the reaction was stopped by addition of 2.5% (w/v) sodium fluoride, 5% (w/v) SDS. The plates were read (λ = 405 nm) in a spectrophotometer as above. The linear portion of the absorbance versus dilution plot was used to determine concentrations, after subtracting the non-specific background linear regression from the sample linear regression.

Concentrations of IgM and IgA were calculated with reference to a standard serum (kindly supplied by Dilip Patel, Department of Animal Husbandry, School of Veterinary Medicine, University of Bristol, UK) with concentrations of IgM and IgA of 2.9 g/l and 3.2 g/l respectively. Because a reference serum with known IgG1 and IgG2 concentrations was not available, relative concentrations of these two subclasses in the plasma samples were calculated arbitrarily, taking the absorbance for IgG1 plus the absorbance for IgG2 in the reference serum as 100 units, and using the following formula:

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\frac{(A_{\text{sample}} - A_{\text{sample background}})}{(A_{\text{st IgG1}} - A_{\text{st background}})} \times 100
\]

where ‘st’ stands for standard and ‘background’ refers to the background observed in wells tested without anti-immunoglobulin MAB.

NK activity assays. Target cells (K562, ATCC CCL 243) were harvested at exponential growth phase by centrifugation (4 °C, 2000 g, 10 min) and 10⁵ cells were labelled by incubation (1 h, 37 °C) with 100 μCi of Na₂⁵¹CrO₄ isotonic solution (Amersham). The cells were washed three times in cold HBSS, and finally suspended in culture medium at 10⁵ cells/ml. Microcytotoxicity assays were performed in triplicate 200 µl cultures in round-bottom 96-well tissue culture microplates, adding aliquots of effector cells and target cells at different volumes, to obtain different effector to target (E:T) ratios (10:1, 25:1, 50:1 and 100:1). As controls, target cells added with medium or with 10% Triton-X100 solution were included to measure spontaneous and total ⁵¹Cr release, respectively. Microplates were incubated (37 °C, 18 h, > 80% humidity and 5% CO₂), supernatants collected and sample radioactivity was counted in a γ-radiation counter. The percentage of specific lysis was calculated from the mean of the three replicate wells using the following formula: specific lysis = (mean c.p.m. wells with effector cells − mean c.p.m. spontaneous release)/mean c.p.m. spontaneous release) × 100. Spontaneous radioisotope release by target cells was always below 30% of total released radioactivity.

Statistical analysis. Statistical analysis of the results was performed using multifactor analysis of variance taking the time (day p.i.) as covariate. Differences between counts were considered significant at \( P < 0.05 \). Standard errors were below 20% of the mean, and thus for clarity are omitted from the graphs.

Results

Clinical status following infection with the NHV isolate

A summary of the individual experiments and their clinical and immunological studies is presented in Table 1. The data on all the pigs infected with ASFV strain NHV are combined in order to demonstrate the correlation between clinical status (asymptomatic versus chronic symptoms), fever and viraemia. All of the pigs inoculated with the highly virulent ASFV strain L60 (groups 5 and 6) developed acute ASF. In contrast, none of the pigs infected with the NHV isolate developed acute ASF. Indeed, many animals remained asymptomatic after infection. Some, on the other hand, exhibited slightly raised body temperatures, necrotic skin areas and joint swelling, corresponding to lesions previously described in chronic type ASFV infection (Petisca, 1965). In contrast, none of the pigs infected with the NHV isolate developed chronic ASF. In fact, many animals remained asymptomatic after infection. Indeed, many animals remained asymptomatic after infection. Some, on the other hand, exhibited slightly raised body temperatures, necrotic skin areas and joint swelling, corresponding to lesions previously described in chronic type ASFV infection (Petisca, 1965). In contrast, none of the pigs infected with the NHV isolate developed chronic ASF. In fact, many animals remained asymptomatic after infection.

Lymphoproliferative responses to mitogens

Lymphoproliferative responses of PBMC to mitogens after virus-inoculation, virus was detected, albeit at low titre (< 10⁶), in 36 samples. The presence of virus was not necessarily correlated with fever. Thus only 16 positive samples were collected when the animals’ body temperatures were above 40 °C. Viraemia was not detected in 9 out of 12 pigs inoculated o.n. and in 3 out of 19 pigs inoculated i.m. Two of these animals (one inoculated by each route) developed chronic ASF. Early viraemia in pigs not showing lesions was mainly detected in pigs inoculated i.m. (Table 1).

Of 240 blood samples collected at different times post-virus-inoculation, virus was detected, albeit at low titre (< 10⁶), in 36 samples. The presence of virus was not necessarily correlated with fever. Thus only 16 positive samples were collected when the animals’ body temperatures were above 40 °C. Viraemia was not detected in 9 out of 12 pigs inoculated o.n. and in 3 out of 19 pigs inoculated i.m. Two of these animals (one inoculated by each route) developed chronic ASF. Early viraemia in pigs not showing lesions was mainly detected in pigs inoculated i.m. (Table 1).
animals inoculated by the o.n. route only one developed chronic type lesions, whereas four of the seven inoculated by the i.m. route developed chronic type lesions. Lymphoproliferative responses to ConA, PHA or PWM were not depressed in any of the 15 inoculated animals, a consistent observation, independent of either the inoculation route or the presence or absence of chronic lesions. Indeed, in one of the two experiments (Fig. 1), the proliferative responses to mitogens of lymphocytes taken from days 7 to 28 p.i. were higher in the inoculated animals than in the control animals, with statistical significance ($P < 0.05$) for responses to ConA (0.5 µg/ml) and PHA (50 µg/ml). Moreover, lymphoproliferative responses to NHV and L60 virus antigens were also demonstrable with PBMC collected from day 7 p.i. onwards (Fig. 1).

Correlation of NK activity with resistance to NHV

A comparison of NK levels at days 0 and 7 p.i., in two experiments (groups 2 and 4, Table 1), revealed an increase in the NK levels at day 7 p.i. in inoculated animals which was clearly more pronounced and highly significant ($P < 0.001$) in those animals remaining asymptomatic until the end of the experiment (Table 2). In uninfected control pigs, on the other hand, NK activity at a time corresponding to day 7 p.i. was unchanged (one of three animals) or decreased (two of three animals). Thus, failure to develop clinical symptoms upon infection with NHV appears to be correlated with a significant development of NK cell activity.

In the first experiment on four pigs inoculated o.n. with NHV (group 2, Table 1), the increase in NK activity peaked at day 7 p.i., and returned to initial levels at day 14 p.i. in two asymptomatic pigs. However, the other two pigs, which developed chronic type lesions, showed a less distinct increase in NK activity at day 7 p.i. In a subsequent experiment (group 4, Table 1) (Fig. 2a, b) seven pigs were inoculated (three by the i.m. route and four by the o.n. route) and blood samples were collected at days $-2, 0, 2, 4$ and $7$ p.i. and then at $7$ day intervals. On day 2 p.i. there were decreased levels of NK activity in all the animals. From days 2 to 4 p.i., however, and in contrast to control animals, there was an increase in NK activity in the infected animals. Moreover, from days 4 to 7 p.i. in the five pigs that remained asymptomatic, this increase in NK activity was even more pronounced (Fig. 2a). Importantly, NK levels in the inoculated pigs which remained asymptomatic were significantly ($P < 0.001$) higher between days 7 and 28 p.i. when compared to controls, whether infected o.n. or i.m.
The two pigs that developed ASF chronic type lesions, on the other hand, maintained largely constant NK activity profiles over the experiment (Fig. 2b). An increase in NK activity in the controls was observed at day 14 p.i., perhaps an indication of stress (Fig. 2c). To assess the possible dependence of this phenomenon on stress associated with animal handling, the same two control animals were bled on days 0, 2, 4, 7, 14 and 21 at the end of the experiment (i.e. 5 weeks after the initiation of the experiment). The resulting PBMC samples were assayed for their NK activity. Once again, a very similar profile of NK activity was observed, with increased levels at day 14 p.i. but no change on day 7 p.i. (Fig. 2d). This observation emphasizes the significance of the increased NK activity levels observed at day 7 p.i. in the infected asymptomatic animals, and also contrasts with the absence of increased NK activity at day 14 p.i. in the two animals that developed ASF chronic type lesions.

**Anti-ASFV specific antibodies and Ig concentrations**

Anti-ASFV specific antibodies were detected by indirect ELISA from days 7 to 14 p.i. in all pigs inoculated by the i.m. route, and from days 10 to 18 p.i. in pigs inoculated o.n. Interestingly, anti-ASFV antibody titres were always low (400 to 3200 at days 29 to 39 p.i.) in pigs not developing lesions while in animals developing lesions very high values (in most of the cases between 6400 and 25600) were observed (Fig. 3).

Given the very elevated levels of specific anti-ASFV total Ig, we then asked whether this reflected the overall immune state of the animal, not simply as total serum Ig, but in terms of individual Ig isotypes. In short, we observed a significant increase in all serum isotypes tested in the group inoculated with NHV and developing lesions.

For the study of total Ig concentrations in plasma samples, 15 NHV-inoculated pigs were divided into two groups for independent experiments (groups 3 and 4, Table 1). In group 3,
Fig. 4. Mean values for IgG1 and IgG2 plasma concentrations in five pigs developing (■) and 10 pigs not developing (▲) ASF chronic type lesions after inoculation with NHV, as well as in four non-inoculated pigs (○) (groups 3 and 4, Table 1). The variation between determinations at each time-point was always less than 20% and thus for clarity this information is omitted from the figure. Graphics at top represent 95% confidence intervals for the mean considering concentrations observed from days 15 to 39 p.i. Mean values for the ratio of IgG2/IgG1 at each time-point are presented.

Fig. 5. Mean values for IgM and IgA plasma concentrations in five pigs developing (■) and 10 pigs not developing (▲) ASF chronic type lesions after inoculation with NHV, as well as in four non-inoculated pigs (○) (groups 3 and 4, Table 1). The variation between determinations at each time-point was always less than 20% and thus for clarity this information is omitted from the figure. Graphics at top represents 95% confidence intervals for the mean considering IgM concentrations observed from days 15 to 39 p.i.

Two groups of four pigs were infected i.m. or o.n. In group 4, four and three pigs were infected o.n. and i.m. respectively. Two control pigs were used in each experiment. There were no major oscillations in the levels of plasma IgG1 and IgG2 concentrations in the control animals during the time-course of the experiments. Asymptomatic inoculated animals (n = 10) had IgG1 and IgG2 levels similar to control animals (Fig. 4). In contrast, inoculated animals with lesions (n = 5) progressively developed increased levels of both IgG1 and IgG2. Thus, at day 39 p.i. these levels were nearly three times higher than those observed at day 0. The ratio of IgG2/IgG1 for each group of animals was nearly constant throughout the experiments (Fig. 4), although a slight increase in IgG2/IgG1 ratio can be identified in animals remaining asymptomatic after inoculation. At the same time, the IgG2/IgG1 ratio was consistently higher in the animals developing lesions.

Similar increases in plasma IgM and IgA were also observed in the animals developing ASF chronic type lesions (Fig. 5).
The IgM and IgA profiles for control and inoculated animals not showing lesions were similar and without the clearly increased levels observed in the inoculated animals suffering lesions.

Challenge inoculation with L60

All 19 pigs which remained asymptomatic when inoculated with the NHV isolate were resistant to i.m. challenge with the highly virulent L60 isolate and survived without clinical signs other than a mild 2 to 5 day fever episode in 15 animals. Indeed, there was no pyrexia at all in the remaining four animals. Titres of anti-ASFV antibodies in challenged animals remained essentially unchanged in all except one asymptomatic pig that showed a late viraemia just before inoculation with the L60 virus. In this case the anti-ASFV specific titre 5 days after L60 inoculation reached very high levels (51200). The three pigs that were monitored for 60 days after challenge with the L60 virus developed neither lesions nor fever.

Five pigs infected with NH virus and then challenged with L60 (group 4, Table 1) had normal lymphoproliferative responses to mitogens (Fig. 6). This is in marked contrast to the inhibition of mitogen-stimulated proliferation observed in the three pigs inoculated with L60 by the o.n. route. The latter animals died from acute ASF between days 6 to 9 p.i. and their PBMC taken on day 4 p.i. exhibited a profound reduction in lymphoproliferative response to ConA and PHA (data not shown). In fact, the levels of proliferation observed in response to both concentrations of ConA and PHA in the animals exposed to NHV and L60 were significantly ($P < 0.05$) above the response levels in the two controls. Responses of control and inoculated pigs to PWM, on the other hand, were not so clearly differentiated ($P > 0.05$) (Fig. 6). Proliferative responses of PBMC from four animals taken at 0, 2 and 4 days post-i.m. inoculation with L60 were normal, relative to responses obtained with two control, uninfected pigs (data not shown).

Both control and NHV infected pigs developed similar levels of NK cell activity after inoculation with L60 (data not shown).

Discussion

This work reveals a positive association between the stimulation of NK cell activity and the development of protective immunity to pathogenic ASFV upon inoculation of pigs with a naturally occurring, non-haemadsorbing, non-fatal ASFV/NH/P68 isolate (NHV). Such non-haemadsorbing viruses display different levels of virulence (Thomson et al., 1979; Pan & Hess, 1984) and provide useful models for the study of protective immune mechanisms. The presence of haemadsorption alone, however, is probably not directly related to virus virulence as deletion of the two genes mediating haemadsorption, CD2 homologue (Rodriguez et al., 1993; Ruiz-Gonzalvo et al., 1996) and ORF EP153R (Galindo et al., 2000), did not alter the characteristics of the virus other than loss of haemadsorption (Borca et al., 1998; Galindo et al., 2000).

Inoculation (i.m. or o.n.) of pigs with NHV established two clinical groups: pigs developing chronic type ASF lesions and pigs remaining asymptomatic. Those developing lesions showed viraemia and fever in a late phase of infection (after day 14 p.i.), NK activity levels similar to control animals, high levels of anti-ASFV specific antibodies and increasing concentrations of total IgG1, IgG2, IgM and IgA. Pigs remaining asymptomatic after infection, on the other hand, were neither viraemic nor febrile after day 14 p.i., had normal plasma immunoglobulin concentrations throughout the duration of the experiments, relatively low levels of anti-virus antibody titres, and markedly high levels of NK activity. The appearance of anti-ASFV antibodies and specific sensitized lymphocytes in the circulation was first detected from days 7 to 18 p.i., in agreement with previous observations (reviewed by Wardley et al., 1987).

The correlation of high levels of NK cell activity with the development of resistance is particularly interesting as it implies a functional role for this lymphocyte subset in protection. Thus, there was a clearly enhanced level of NK at day 7 p.i. in those animals remaining healthy after inoculation with NHV and developing resistance to the L60 strain of ASFV. In some of these pigs high levels of NK cell activity were observed throughout the duration of the experiments. In striking contrast, the animals that developed chronic lesions of ASF showed NK levels similar to or only slightly above the control animals. In contrast, a moderately virulent ASFV apparently depresses NK activity in pigs (Norley & Wardley, 1983), and in vitro the NK activity of porcine mononuclear cells was inhibited by both a low and a highly virulent ASFV (Mendonza et al., 1991). Although the phenotype of porcine NK cells has been described (Saalmuller et al., 1994; Yang & Parkhouse, 1996), there is little information on their role against viral diseases of pigs. Indeed, the foregoing and
potential role of NK cells in the activation of immune responses in both mouse and man (review by Biron et al., 1999) provide an urgent argument for further work on these cells as possible modulators of porcine immune responses against ASFV, particularly as IFN-α and IFN-γ are known to inhibit ASFV replication in a synergistic manner in vitro (Esparza et al., 1988; Paez et al., 1990). The other obvious arm of cellular immunity, the cytotoxic T cell, is also a candidate for future investigation in this experimental model, in which the generation of CD8+ T cells able to lyse ASFV-infected macrophages has already been demonstrated (Martins et al., 1993).

In negative correlation with the NK activity results, hypergammaglobulinemia was only found in animals developing ASF chronic type lesions, and not in animals remaining asymptomatic and developing resistance after virus inoculation. Thus, Ig concentrations in inoculated animals remaining asymptomatic showed identical profiles to control animals, independent of the route of inoculation used. In contrast, the development of chronic ASF type lesions was associated with a significant increase in IgG levels (data not shown) in agreement with the across-the-board increased levels of IgG1, IgG2, IgM and IgA in the same animals. Consistent with the increased total Ig levels, anti-ASFV specific antibody titres observed in pigs developing chronic infection were much higher than those of asymptomatic animals. Although the IgG2/IgG1 ratio largely remained constant throughout the experiment in all the animal groups (controls, chronically infected and asymptomatic), this ratio was considerably higher at the beginning of the experiment in the group of animals that developed lesions, perhaps suggesting a predisposition for the establishment of chronic ASF in animals with high IgG2/IgG1 ratios. Further work is required to confirm this possible correlation, but this work does not provide positive evidence for a role for antibodies in protective immunity to ASFV.

The demonstration of hypergammaglobulinemia in pigs developing chronic ASF confirms earlier work (Pan et al., 1970; Pan, 1987) and may be related to the systemic immune activation and associated increase in macrophages, B- and CD8+ T-cells that occurs in pigs with chronic persistent infection (Ramiro-Ibañez et al., 1997). Other authors have demonstrated that infection with ASFV isolates of different virulence may result in over-stimulation of B-lymphocytes in vitro and in vivo (Wardley, 1982; Takamatsu et al., 1999). Finally, a specific immunosuppression due to overstimulation of CD4+ and CD8+ T-cells, and mediated by IL-4 and IL-10, has been described in mice inoculated with ASFV protein p36 (Arala-Chaves et al., 1988; Ribeiro et al., 1991; Vilanova et al., 1999).

A variety of results has been obtained when mitogen responses of lymphocytes from pigs infected with ASFV have been investigated. In contrast to some previous work (Sanchez-Vizcaíno et al., 1981; Childerstone et al., 1998), but in agreement with other studies (Wardley & Wilkinson 1980; Knudsen & Genovesi, 1987; Scholl et al., 1989) there was no depression of lymphoproliferative responses to virus antigens or mitogens (ConA, PHA and PWM) in any of the animals infected with NHV. It is possible that this variation is due to differences in the systems investigated, e.g. PBMC versus spleen, strain of virus. Furthermore, the mitogen responses of PBMC were not depressed after a subsequent inoculation with the highly virulent L60 isolate, although, PBMC from pigs infected with only the virulent L60 isolate had reduced lymphoproliferative responses. An inhibitory effect of ASFV on mitogenic responses of normal lymphocytes in vitro has also been noted (Wardley, 1982; Gonzalez et al., 1990), and should be distinguished from the effect of ASFV infection upon mitogenic responses of lymphocytes subsequently taken from the infected animals. Relevant to this work, Borca et al. (1998) similarly demonstrated normal mitogen responses in PBMC from pigs infected with a virulent ASFV isolate with the CD2 homologue deleted.

Finally, the question of protective serological immunity to ASFV remains controversial. These data, with a negative correlation between antibodies and pathology, on the one hand, versus the positive correlation between increased NK cell activity and the development of protective immunity in the absence of pathological symptoms, on the other, support the notion that immunity to ASFV depends, at least in part, on cellular mechanisms, in particular NK cells. Thus, pigs inoculated with the NHV isolate developed significantly high levels of NK cell activity in the absence of clinical symptoms, and survived subsequent infection with the fatal highly virulent L60. Further studies with this infection model, particularly future work on NK cells and cytotoxic T cells, may provide new insights on mechanisms of protective immunity to ASFV.

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