The pathogenesis of African swine fever in the resistant bushpig

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Bushpigs and warthogs are natural reservoir hosts of African swine fever virus (ASFV) in the wild, showing no clinical signs of disease when infected with the same highly virulent isolates of ASFV that induce rapid, haemorrhagic death in domestic pigs. In contrast to domestic pigs, infection of bushpigs with Malawi isolate results in low levels of virus replication and lymphocyte apoptosis within the spleen, and a relatively low spread of virus to other lymphoid tissues. However, at 10 days post-infection, a high degree of apoptosis was seen in B lymphocytes of the B cell follicles in bushpig lymph nodes. Virus infected cells were present amongst the apoptotic B cells of these follicles, suggesting that indirect factors released from ASFV infected macrophages signal surrounding lymphocytes to enter apoptosis. The susceptibility/resistance of domestic pigs/bushpigs to ASFV may serve as a unique veterinary model for the recently emerging haemorrhagic disease of man.

African swine fever virus (ASFV) is a non-pathogenic DNA virus of warthogs and bushpigs which persists in Africa via a natural cycle of transmission between the warthog (*Phacochoerus aethiopicus*), bushpig (*Potamchoerus porcus*) and the soft tick (*Ornithodorus moubata*). Warthogs are born free from infection and may be infected early in life following the bite of an infected tick. Virus replicates in the warthog and produces a low degree of viraemia for a few weeks, which is sufficient to infect a proportion of ticks that feed on the viraemic young warthogs (Thomson, 1985). Domestic pigs in Africa acquire infection from wildlife reservoirs of the virus primarily by the bite of an infected tick (Plowright, 1977). Also, under experimental conditions, domestic pigs have been infected by

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eating warthog tissue or whole ticks containing virus (Thomson *et al.*, 1980). The susceptibility of bushpigs to ASFV was first demonstrated by Montgomery (1921). Since then, however, the one further study on bushpigs was limited to the isolation of ASFV from several animals (DeTray, 1963). Bushpigs are thought to be natural reservoir hosts of ASFV. They show no clinical signs of disease when infected with virulent and haemorrhagic isolates of ASFV that kill domestic pigs within 5–7 days of infection. The aim of this study was to investigate the role of the bushpig as a reservoir host of ASFV and to compare the pathogenesis of ASF in bushpigs and domestic pigs. Specifically, the levels of ASFV replication in tissues of bushpigs and pigs were assessed by virus titrations; the pathology, cell tropism and organ distribution of ASFV were investigated by immunocytochemistry, and compared to similar studies in the domestic pig which are reported in the accompanying paper (Oura *et al.*, 1998*a*). Such comparisons of disease immunopathology may lead to an understanding of why bushpigs survive infections with ASFV whereas domestic pigs die, and provide important clues for the design of future control strategies, such as a vaccine.

All bushpig infection studies were carried out on an island on Lake Kariba, Northern Zimbabwe, with two separate groups of bushpigs. The bushpigs were captured from an ASFV-free area in Zimbabwe. Of the first group of four bushpigs, around 6 months old, three were infected intramuscularly with 10^4 HAD$_{50}$ (50% haemadsorbing units per g tissue or per ml blood) Malawi isolate (Haresnape *et al.*, 1984) of ASFV and sacrificed 5, 10 and 15 days post-infection (p.i.), and the fourth was an uninfected control. The second group of two bushpigs, also 6 months old, was infected intramuscularly with 10^4 HAD$_{50}$ Malawi isolate of ASFV and sacrificed at 5 and 10 days p.i. Blood, liver, spleen, kidney, gastro-hepatic lymph node, mesenteric lymph node, parotid lymph node, submandibular lymph node, thymus, lung and tonsil were removed and rapidly frozen (liquid N$_2$) in OCT compound (Tissue Tec) or fixed in 4% paraformaldehyde in PBS for 18 h, then paraffin embedded. Sections (5 μm) were placed onto Superfrost Plus slides (BDH). As a direct comparison, Large White × Landrace pigs weighing 30 kg were inoculated intramuscularly with the highly virulent, 100% fatal and haemadsorbent Malawi isolate of ASFV (10^4 HAD$_{50}$ per pig). One
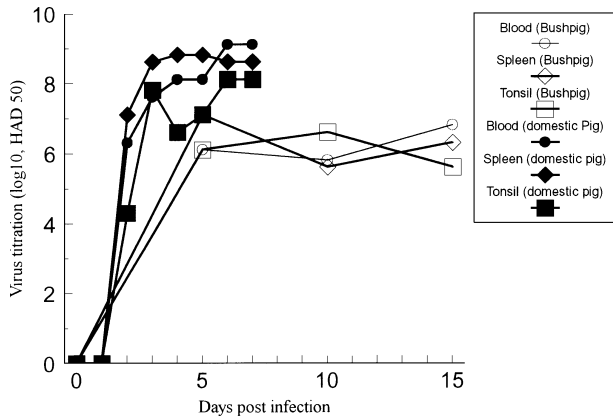
Fig. 1. Comparison of virus titrations in bushpigs and domestic pigs infected with the Malawi isolate of ASFV. Virus titrations ($\log_{10}$ 50% haemadsorbing units per g tissue or per ml blood, HAD_{50}) in blood, spleen and tonsil removed from bushpigs and domestic pigs infected with Malawi isolate of ASFV.

pig was used as the uninfected control, one pig was sacrificed at 2 days p.i., and pigs were sacrificed in pairs at 3, 4, 5, 6 and 7 days p.i.
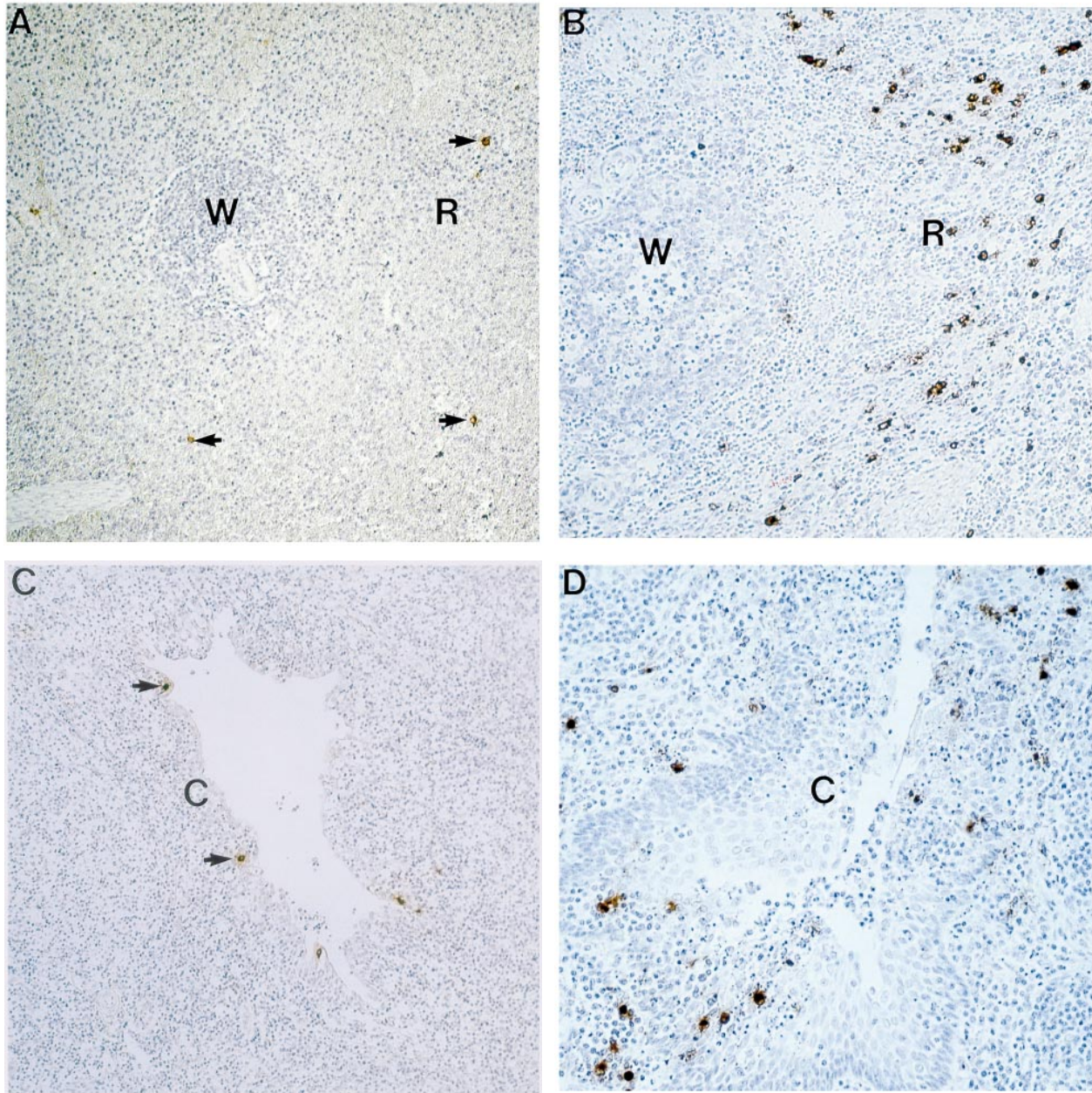
Virus levels in bushpig and domestic pig tissues and blood were titrated by the haemadsorption assay described previously by Wilkinson *et al.* (1977). Titres of between 5 and 7 $\log_{10} HAD_{50}$ were measured in bushpig tissues (blood, spleen, liver, tonsil, lymph nodes, kidney and lung) at 5, 10 and 15 days p.i. When compared with tissue virus titres in Malawi infected domestic pig tissues, the bushpig titres were around 2 $\log_{10} HAD_{50}$ lower (Fig. 1). These results indicate that considerably less virus replication occurred in ASFV infected bushpig tissues compared to domestic pig tissues. Similar levels of blood and tissue virus titrations ($\pm 1 \log_{10} HAD_{50}$) were measured in the two separate groups of infected bushpigs.

Cell tropism and organ distribution of ASFV Malawi isolate were determined by immunocytochemistry on bushpig tissues taken from two bushpigs at 5 and 10 days p.i. and one bushpig at 15 days p.i. Standard immunohistochemical techniques were carried out as described previously (Oura *et al.*, 1998*b*). The levels of virus replication in tissues stained with anti-vp73 MAb 4H3 (Cobbold *et al.*, 1996) were quantified by counting the number of infected cells per mm². The mean number of infected cells and standard error from 30 fields were calculated. As might have been predicted from the virus titrations, results reveal that there was considerably less virus replication in bushpig tissues compared to domestic pig tissues. There was an initial low level of virus replication of ($3\cdot5 \pm 0\cdot5$ infected cells/mm²) at 5 days p.i. in cells in the red pulp of the spleen (Fig. 2 A). At this time-point there was no detectable replication in the lymph nodes; however, in the tonsil there was virus replication ($3\cdot2 \pm 0\cdot5$ infected cells/mm²) in cells located within the epithelium of tonsillar crypts (Fig. 2 C). This was in marked contrast to the domestic pig in which, at 5 days p.i., there was extensive replication of ASFV (Malawi isolate) in all lymphoid

tissue. A high degree of virus replication (87 ± 5 infected cells/mm²) was seen in the red pulp of the spleen (Fig. 2 B) and in cells located both outside and within the epithelium of crypts in the tonsil ($13 \pm 1\cdot1$ infected cells/mm²) (Fig. 2 D). Interestingly, in the bushpig ASFV replication was seen in cells of the tonsillar crypt at 5, 10 and 15 days p.i. These infected cells are shed into the pharynx and, if they are coughed up, may be a mode of transmission of the virus. Indeed, direct transmission of ASFV (Malawi isolate) from bushpig to domestic pig has been achieved under experimental conditions (Anderson *et al.*, 1998).

The organ distribution of the virus was significantly different in bushpigs compared to domestic pigs. In bushpig lymphoid tissues, virus replication was seen in the spleen but there was very limited escape of the virus to other lymphoid organs. This was in marked contrast to the domestic pig, where initial high levels of virus replication in the spleen were rapidly followed by escape of the virus and high levels of virus replication in other lymphoid organs (Oura *et al.*, 1998*a*). The higher degree of infection in the lymphoid tissue of domestic pigs was associated with severe irreversible pathological damage and apoptosis of lymphocytes which were not seen in the bushpig lymphoid tissues. The number of ASFV infected cells correlates with the degree of lymphocyte apoptosis, indicating that ASFV infected macrophages, possibly through the release of cytokines or other apoptotic mediators, may be responsible for the extensive lymphocyte apoptosis. Therefore, the reason why bushpigs survive infection with virulent isolates of ASFV may be because the virus is not so extensively disseminated throughout the mononuclear phagocytic system as clearly occurs in the domestic pig. With fewer infected macrophages in the peripheral lymphoid tissue there would be less release of cytokines or vasoactive substances such as reactive oxygen intermediates or nitric oxide, and thus less destructive pathology and lymphocyte apoptosis.

There was a striking difference between bushpig lymph nodes at 5 and 10 days p.i. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP end labelling technique (TUNEL) was used to label DNA strand breaks in apoptotic cells in tissue sections (Oura *et al.*, 1998*a*). At 10 days p.i. many B cells in many of the follicles had pyknotic nuclei and were apoptotic (Fig. 3 A, B). In some B cell follicles 70–80% of B lymphocytes were apoptotic. Also, interestingly, virus replication was detected in cells in the B cell follicles (Fig. 3 C). The most likely phenotypes of infected cells in the follicles are tingible body macrophages or follicular dendritic cells. In order to identify the phenotype of the apoptotic cells in the follicles, lymph node cryosections from bushpigs, 10 days p.i. with Malawi, were immunostained with anti-CD21 B cell specific MAb C35 (Saalmuller, 1996). The vast majority of the cells in the follicles were B cells (Fig. 3 D). This demonstrated that the apoptotic cells in the follicles were B lymphocytes. The degree of apoptosis of B lymphocytes in B cell follicles of bushpig lymph nodes, at 10 days p.i., was considerably higher than the

Fig. 2. Immunocytochemical localization of vp73 protein in bushpig and domestic pig tissues infected with ASFV (Malawi isolate). Paraffin embedded sections, stained with anti-vp73 MAb 4H3, visualized with DAB (brown, arrow) and counterstained with haematoxylin. (A) Bushpig spleen, 5 days p.i. with Malawi ($\times 80$); (B) domestic pig spleen, 5 days p.i. with Malawi ($\times 80$); (C) bushpig tonsil, 5 days p.i. with Malawi ($\times 80$); (D) domestic pig tonsil, 5 days p.i. with Malawi ($\times 160$). W, White pulp; R, red pulp; C, crypt.

degree of apoptosis seen in follicles of lymph nodes from domestic pigs, 10 days p.i. with the moderately virulent Malta isolate of ASFV (Oura *et al.*, 1998*a*). In order to stimulate an exuberant immune response in lymph node germinal centres, a pig was vaccinated twice with foot-and-mouth disease virus vaccine (O1 serotype, Bayer) 3 weeks apart. Seven days after the second vaccine the pig was sacrificed and the draining lymph node was removed, paraffin embedded and stained with

TUNEL. There was considerably less apoptosis observed in germinal centres of the vaccinated pig compared to the bushpig at 10 days p.i. (data not shown). While not conclusive, this indicates that the observed exuberant apoptosis is more likely to result from a pathological component than a normal immune activation.

Apoptosis of lymphocytes and ASFV infection of cells in bushpig lymph nodes were mostly confined to the B cell
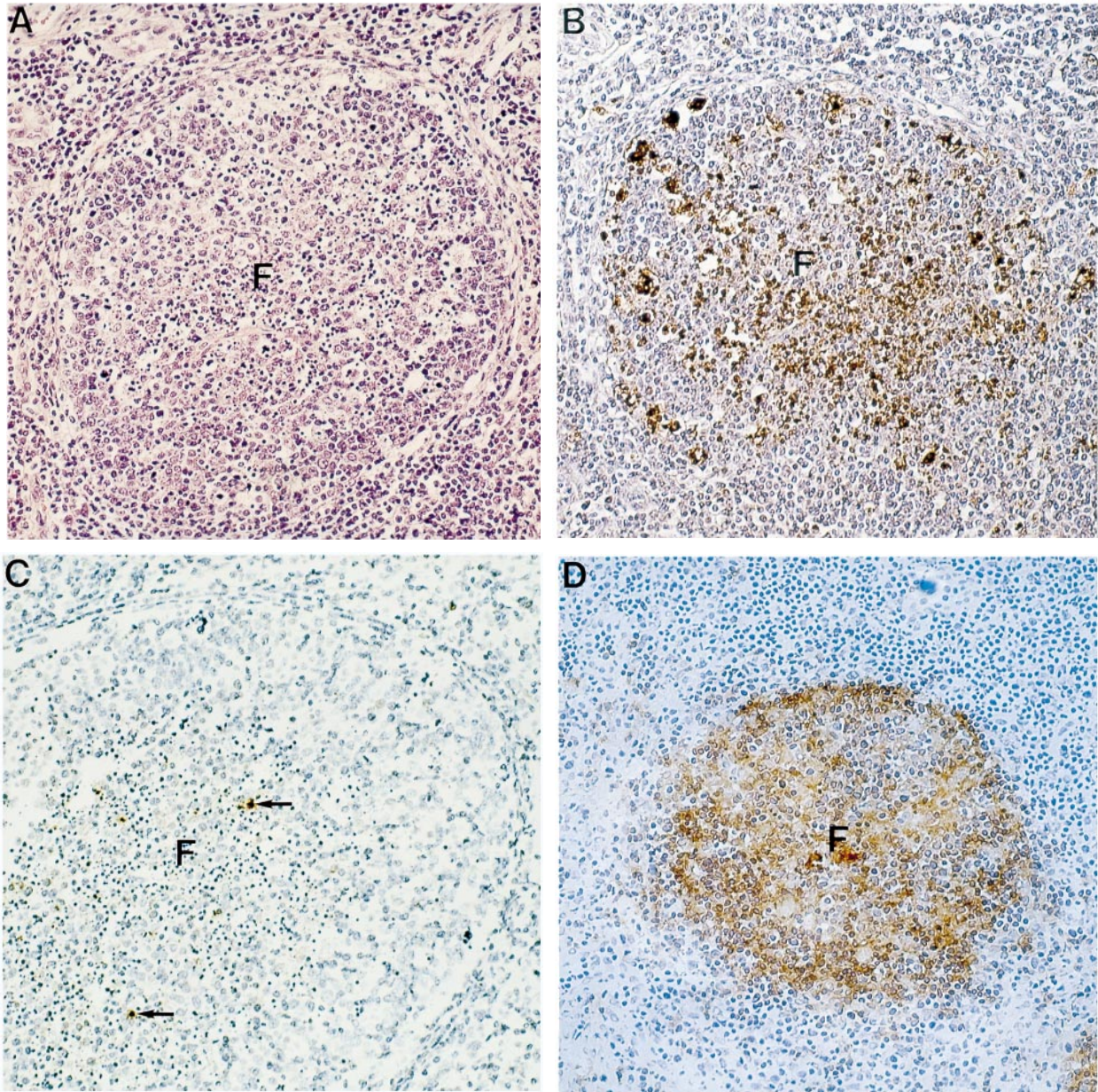
Fig. 3. Apoptosis in bushpig lymph node infected with ASFV (Malawi isolate). Paraffin embedded lymph node sections from a bushpig, 10 days p.i. with Malawi isolate ($\times 160$). (A) Stained with haematoxylin and eosin; (B) stained with TUNEL; (C) immunostained with anti-vp73 MAb 4H3; (D) lymph node cryosection from a bushpig, 10 days p.i. with Malawi isolate, immunostained with anti-CD21 MAb C35. In immunohistochemical and TUNEL staining, signal was visualized with DAB substrate (brown) and sections were counterstained with haematoxylin. F, B cell follicle.

follicles, with low levels of apoptosis and ASFV infection occurring in T cell zones of the cortex and paracortex. This observation is in marked contrast to the situation in domestic pigs infected with Malawi isolate where the majority of ASFV infected cells and apoptosis of lymphocytes were located in the T cell areas (Oura *et al.*, 1998 *a*; Carrasco *et al.*, 1996). There are many possible causes of this B lymphocyte apoptosis which include: (1) ASFV infected tingible body macrophages are indirectly, perhaps through the release of cytokines or other apoptotic mediators, inducing surrounding B cells to enter apoptosis; (2) the lymphocyte selection process is impaired through damage to follicular dendritic cells so most of the B cells in the germinal centres are selected for programmed cell death, with few lymphocytes being positively selected for continued survival; or (3) the process of affinity maturation is compromised so that only low affinity antibody-producing B lymphocytes are produced. Because only high affinity antibody-producing lymphocytes are selected for survival in

the germinal centres, the majority of B lymphocytes enter apoptosis.

ASFV has evolved in a natural cycle between the reservoir hosts: the bushpig, the warthog and the soft tick. Sufficiently high viraemias occur in the bushpig to allow infection of feeding ticks, but ASFV replication in lymphoid tissues is kept in check, and pathological damage and apoptosis in lymphoid tissue are kept to a minimum so that the hosts survive infection with no clinical signs of disease. Also, a controlled level of apoptosis in uninfected lymphocytes may result in a diminished immune response enabling ASFV infected cells to survive the immune system for sufficient time to produce a productive infection. In addition, bushpigs may also control the apoptosis of B lymphocytes at the time of selection of high affinity antibody producing B lymphocytes (10 days p.i.). This would halt the production of high affinity anti-ASFV antibody and facilitate long term survival of extracellular virus adsorbed to the surface of red blood cells, enabling its transmission to feeding ticks. This may also have important implications for the persistence of the ASFV in the bushpig and suggests that antibody mediated immunity may not be critically important for bushpig survival. Characterization of bushpig and domestic pig antibody responses are being carried out.

In contrast, the domestic pig is not a natural host of ASFV. The factors that keep both the virus and apoptosis in check in bushpigs are 'out of control' in domestic pigs. High levels of virus replication in lymphoid tissue result in uncontrolled apoptosis of lymphocytes, a severely impaired immune response, and perhaps aberrant levels of cytokines contributing to the haemorrhagic pathology. Therefore domestic pigs die within 5–7 days of infection.

ASF with its differential pathology, haemorrhagic in the susceptible domestic pig versus non-pathogenic in the resistant bushpig, may be considered as a veterinary model for the recently emerging haemorrhagic diseases of man.

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