Poxviruses
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Genus *Parapoxvirus*

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Abstract

Highly contagious pustular skin infections of sheep, goats and cattle that were unwittingly transmitted to humans from close contact with infected animals, have been the scourge of shepherds, herdsmen and dairy farmers for centuries. In more recent times we recognise that these proliferative pustular lesions are likely to be caused by a group of zoonotic viruses that are classified as parapoxviruses. In addition to infecting the above ungulates, parapoxviruses have more recently been isolated from seals, camels, red deer and reindeer and most have been shown to infect man. The parapoxviruses have one of the smallest genomes of the poxvirus family (140 kb) yet share over 70% of their genes with the most virulent members. Like other poxviruses, the central core of the genomes encode factors for virus transcription and replication, and structural proteins, whereas the terminal regions encode accessory factors that give the parapoxvirus group many of its unique features. Several genes of parapoxviruses are unique to this genus and encode factors that target inflammation, the innate immune responses and the development of acquired immunity. These factors include a homologue of mammalian interleukin (IL)-10, a chemokine binding protein and a granulocyte-macrophage colony stimulating factor /IL-2 binding protein. The ability of this group to reinfect their hosts, even though a cell-mediated memory response is induced during primary infection, may be related to their epitheliotropic niche and the immunomodulators they produce. In this highly localised environment, the secreted immunomodulators only interfere with the local immune response and thus do not compromise the host's immune system. The discovery of a vascular endothelial growth factor-like gene may explain the highly vascular nature of parapoxvirus lesions. There are many genes of parapoxviruses which do not encode polypeptides with significant matches with protein sequences in public databases, separating this genus from most other mammalian poxviruses. These genes appear to be involved in inhibiting apoptosis, manipulating cell cycle progression and degradation of cellular proteins that may be involved in the stress response, thus allowing the virus to subvert intracellular antiviral mechanisms and enhance the availability of cellular molecules required for replication. Parapoxviruses in common with *Molluscum contagiosum virus* lack a number of genes that are highly conserved in other poxviruses, including factors for nucleotide metabolism, serine protease inhibitors and kelch-like proteins. It is apparent that parapoxviruses have evolved a unique repertoire of genes that have allowed adaptation to the highly specialised environment of the epidermis.

Taxonomy

The classification of the parapoxviruses has evolved slowly and there has been much confusion over the nomenclature used [1]. Members of the genus of the *Chordopoxvirus* subfamily that we now call the parapoxviruses, although having long been recognised as causing poxvirus-like disease, were not classified as poxviruses until 1956 [2]. At this time the members were few and included pustular dermatitis of sheep and goats and milkers nodes. In 1964, three viruses we now recognise as parapoxviruses,were listed as Group II poxviruses in "Viruses of Vertebrates" by Andrews 1964 [3]. Here the diseases of orf (synonyms: contagious pustular dermatitis, contagious ecthyma of sheep, sore mouth, scabby mouth, contagious pustular stomatitis; infectious labial dermatitis), milkers nodes (synonyms: pseudocowpox, paravaccinia), and stomatitis papulosa (of cattle) were included. The viruses associated with these diseases had a similar morphology and the "ball of yarn" appearance was described. The classification of the above as a distinct group within the poxviruses was described by the International Committee on Nomenclature of Viruses in 1971 but in addition to bovine papular dermatitis virus, milkers nodes and orf, the group also included chamois contagious ecthyma virus [4]. In 1976, the International Committee on Taxonomy of Viruses (ICTV) introduced the scheme of genera within families of viruses and here the subgroup became the *Parapoxvirus* genus within the family *Poxviridae* [5]. The genus was described as viruses of ungulates that infect man and *Orf virus* (ORFV) was chosen as the type species. The ICTV classification of the parapoxvirus genus now includes ORFV (syn. contagious pustular dermatitis virus and contagious ecthyma virus), *Bovine papular stomatitis virus* (BPSV) [syn bovine papular (pustular) stomatitis virus], *Pseudocowpox virus* (PCPV) (syn milker's nodule virus, paravaccinia virus) and the more recent member, *Parapoxvirus of red deer in New Zealand* (PVNZ) [6]. Tentative species of the genus include seal parapoxvirus, parapoxvirus of camels (contagious ecthyma of camels or Ausdyk disease), and chamois contagious ecthyma virus. A poxvirus able to cause fatal disease in red squirrels was initially considered a possible parapoxvirus, largely on the basis of its virion morphology. More recent DNA sequence data indicate that this virus is not a parapoxvirus and it remains an unclassified poxvirus [7].

Features that have proven useful in classifying viruses as parapoxviruses include the distinctive virion morphology when viewed by electron microscopy, the high G+C content of the genome and host range. However, each of these features have now been associated with poxviruses that are not parapoxviruses and further analysis, particularly DNA sequence, will be required to support the inclusion of additional viruses in the genus.

History of parapoxviruses

The history of the parapoxviruses has been reviewed by Robinson and Lyttle [1] and is covered here only briefly. Contagious pustular dermatitis or ORFV was first described in 1787, although shepherds have long recognised scabby disease of sheep [8]. However, it was another 100 years before the contagious nature of ORFV was recognised [9] and the susceptibility of humans to infection was reported [10]. In 1923, it was reported that the disease could be transmitted between sheep, by an agent that was smaller than most bacteria and could be included in the group of "filterable" viruses [11]. Aynaud also demonstrated that ORFV could be distinguished from *Vaccinia virus* (VACV) in that it did not cross-protect and he described the natural disease, its histological appearance, the susceptibility of the virus to solvents and chemicals, the development and duration of immunity, the lack of protection afforded by passive transfer of serum and the development of a vaccine which was live unattenuated ORFV. Today the vaccine against ORFV is still live unattenuated ORFV but it is commonly propagated in cell culture.

Although the disease we now know as pseudocowpox was present in cattle for a long time prior to the 1930s, it could not be readily distinguished from cowpox. In a review of the early literature Bonnevie [12] recognised that some smallpox vaccinations called "vaccine rouge" produced forms of lesions different from that caused by true cowpox and that vaccination material from these lesions did not protect from cowpox. Such infections were called false cowpox, paravaccine, milkers warts and milkers nodules and did not protect against smallpox. Lipschutz referred to the disease in humans as paravaccinia and along with others could distinguish the agent of milkers nodules from that causing cowpox by the application of the Pauls test, which involved the inoculation of the infectious material onto the cornea of rabbits [13]. Unlike cowpox, milkers nodules did not produce a lesion. In 1963, the virus was isolated in cell cultures from teat lesions in cattle and from milkers nodules and the virions were shown by electron microscopy to resemble those of ORFV and BPSV.

The early literature describing the disease caused by BPSV was reviewed by Griesemer and Cole [14], while the more recent literature has been reviewed by Robinson and Lyttle [1]. The disease was first described in Belgium in 1884 and given the name "la stomatite papillaire ou papillomateuse" (papular or papillomatous stomatitis) [15]. The definitive characterisation of bovine papular stomatitis was performed by Plowright and Ferris [16] and Griesemer and Cole [17] who reported the isolation of the virus from cell culture and the reproduction of the disease in calves. The first report of transmission of bovine papular stomatitis to humans was that of Carson and Kerr [18].

Reports of parapoxvirus diseases in camels [19–24], seals [25–33], red deer [34, 35], and reindeer [36–38] have only appeared since the late 1960s;

however, these diseases have probably been in existence for as long as the parapoxviruses described above.

Epidemiology

ORFV, BPSV, and PCPV are ubiquitous in sheep-producing and cattle-producing countries worldwide [1, 39]. Reports of parapoxviruses of camels and seals have increased in recent years suggesting a wider distribution than previously thought. It is believed that the spread and maintenance of the infections in each species is related to the resistant nature of the virions in the environment and the short-lived immunity to reinfection [1].

ORFV primarily infects animals less than 1 year old, affecting lambs and kids shortly after birth and at 3–4 months [40, 41]. Adults may also be affected and outbreaks have been observed at all times of the year. The incidence in a flock may reach 90%, but mortality is usually low. Spread in a flock is rapid and occurs by contact with affected animals or shed scabs [41, 42]. Lambs may spread the virus to the udder and teats of the ewe while suckling [40]. The virus may survive in chronically infected animals [40, 43, 44].

Recently, the prevalence of parapoxvirus infections of Japanese serows (*Capricornis crispus*) and Japanese deer (*Cervus nippon centralis*) was reported [45]. The serological survey suggested that parapoxviruses of Japanese serows is widespread in Japan. Characterisation of the DNA of isolates circulating in Japanese serows suggests that they are likely to be ORFV [46].

The parapoxvirus disease of camels called Ausdk or camel contagious ecthyma was first described in the Soviet Union in 1972 [19]. The disease has since been recorded in Mongolia [20, 21], Somalia [47], Kenya [22] and Libya [23]. In the Turkans district of Kenya outbreaks were only detected in camel calves (*Camelus dromedaries*) [48]. Mortality among camel calves is one of the most serious problems faced by camel herdsmen. Evidence suggests that parapoxvirus of camels is ORFV. Cases of contagious ecthyma of sheep occurring in camels have been reported [24] and more recently serological analysis of infected camels in Libya suggest that these infections are also caused by ORFV [23].

Parapoxvirus of reindeer (*Rangifer tarandus tarandus*) has been reported in Finland and Norway. A severe outbreak occurred in Finland during the winter of 1992–93 when approximately 400 reindeer died and 2800 showed clinical signs of disease [36]. Sporadic outbreaks have been reported since [38]. More recently, parapoxvirus infections of reindeer have been reported in semi-domesticated reindeer in Norway [37]. It has been shown that the parapoxvirus infections in Norway were likely caused by ORFV [49]. Genomic comparisons of one standard ORFV strain NZ2 ($ORFV_{NZ2}$) and the reindeer isolates, employing restriction fragment length polymorphism, random amplified polymorphic DNA analysis and partial DNA sequencing

of specific genes demonstrated high similarity between the reindeer viruses and known ORFV strains. It has been suggested that the virus may have been transferred from sheep and goats to reindeer *via* people, equipment and common use of pastures and corrals. Analysis of the viruses recovered from reindeer in Finland suggest that one disease outbreak was caused by ORFV and another by PCPV [38].

PVNZ is clearly distinct from the other recognised species of parapoxviruses. Curiously, it has only ever been reported in New Zealand [34, 35] even though red deer in New Zealand are derived from animals introduced from Europe in the 19th century and the country has no indigenous ungulate species. These observations suggest that PVNZ is probably present in other countries. The recent determination of the genome sequence of deerpox virus confirmed that it is likely to represent a new genus, and that it is clearly not a parapoxvirus and is distinct from PVNZ [50].

It is now evident that infections of seal species and other pinnipeds by parapox-like viruses are widespread. The first report appeared in 1969 and described the infection of Californian sea lions (*Zaophus californianus*) [26]. Since this time infections attributed to parapoxviruses have been reported in South American sea lions (*Otaria bryonia*) [25], harbour seals (*Phoca vitulina*) in the German North sea [27, 28], Northern fur seals (*Callorhinus ursinus*) [29], grey seals (*Halichoerus grypus*) around the coast of Cornwall [30] and other parts of the world [31, 32] and the Weddell seal (*Leptonychotes weddellii*) in Queen Maud Land Antarctica [33]. Early reports were generally confined to observations of the histopathology of the lesions and electron microscopy of the virus particles. More recent reports have included *in situ* hybridisation with parapoxvirus-specific DNA probes and sequence analysis of PCR products. The emerging picture is that the viruses infecting pinnipeds are likely to form one or more new species within the *Parapoxvirus* genus, although the separation between pinnipeds and the currently recognized hosts for parapoxviruses (ungulates) introduces a note of caution.

Pathogenesis

In general the pathology of parapoxvirus infection of mammals is confined to the epithelium and oral mucosa. The virus usually infects through abrasions and breaks to the skin, and the clinical pathology observed at sites of infection is typically the formation of pustules and scabs [1, 17, 22, 26, 34, 39, 42]. There is little evidence that parapoxviruses can spread systemically [1].

Parapoxvirus lesions evolve through the stages of macule, papule, vesicle, pustule, scab and resolution. The infection begins as reddening and swelling around the sites of inoculation and small vesicles develop within 24 h. The lesions take on a pustular appearance as they develop. The pustular nature of the lesions is due to a large infiltration of polymorphonucleocytes. Adjacent lesions may coalesce as the disease progresses eventually forming

a scab. Underlying the scabby lesions, the dermis becomes oedematous and proliferative, which gives a granulomatous appearance to the lesion. The lifting and cracking of scabs can result in the discharge of blood. Usually the resolution of the lesions takes up to 4–6 weeks, but there have been cases of persistent infection of ORFV in East Friesian sheep in New Zealand in which large tumour-like growths have developed (unpublished observation) and a report of a severe long-lasting contagious ecthyma in a goat's kid that lasted for 6 months [51].

In ORFV-infected sheep and goats, the lesions most often form around the muzzle and buccal cavity [1, 52]. ORFV lesions are normally benign; however, more serious complications can arise with secondary infections by bacteria or fungi. ORFV infections often cause a debilitating disease in young lambs or kids affecting the animals ability to feed. The lesions of PCPV are normally found on the teats of cattle and spread to mouths of calves [1]. With red deer (*Cervus elaphus*) lesions usually occur around the muzzle and face and multifocal scabby lesions on the velvet of stags have been recorded [34]. Parapoxvirus infections of fawns can be more serious and in addition to encrustations around the face and mouth, lesions covering 60–90% of the body have been seen [34]. Studies in infected Finnish reindeer (*R. tarandus tarandus*) have noted erosions, papules, pustules and ulcers in the mouth [36, 38]. In harbour seals (*P. vitulina*) lesions of the skin and mucosa of the oral cavity have been reported [28] and in infections of grey seals (*H. grypus*) cutaneous pocks have progressed to involve extensive regions of the skin [53]. An elevated skin lesion of 3 cm in diameter has been observed in the Weddell seal consisting of partly fresh and partly necrotic tissue and proliferative papilloma-like structures [33].

The histopathological features of natural and experimental infections of ORFV [1, 54–56], PCPV [57] and BPSV [14, 17] have been described and many of the features are common to all three viruses. Parapoxvirus infections are markedly proliferative. The infected epidermis is characterised by vacuolation and swelling of keratinocytes in the stratum spinosum, reticular degeneration, marked epidermal proliferation, intra-epidermal microabscesses and accumulation of scale-crust. Intracytoplasmic eosinophilic inclusion bodies may be visible in ballooning keratinocytes 72 h after infection. Epidermal proliferation leads to markedly elongated rete pegs. Neutrophils migrate into areas of reticular degeneration and form microabscesses that subsequently rupture on the surface. A thick layer of scale crust is built up, composed of hyperkeratosis, proteinaceous fluid, degenerating neutrophils, cellular debris and bacteria. Dermal lesions include oedema, marked capillary dilation and infiltration of inflammatory cells. Papillomatous growths that consist of pseudoepitheliomatous hyperplasia and granuloma formation often develop in natural ORFV infections and may become extensive [40].

Many, perhaps all, parapoxviruses can infect humans. Infections of humans have been reported for ORFV, BPSV and PCPV [1], as well as unclassified parapoxviruses isolated from reindeer [36] and seals [32].

Human infection by PVNZ has yet to be reported. Milkers nodules and ORFV infections of humans have been noted for centuries. The virus often infects the hands of people coming in close contact with infected animals. ORFV lesions are relatively common in people working in the sheep industry; however, recently it has been reported that ORFV infections coincided with the Islamic practice "feast of sacrifice" in which sheep are manipulated for slaughter with bare hands [58]. Unlike the lesions of animals they remain localised as foci of infection. The progression of the disease caused by ORFV from infection to resolution has been divided into six stages [1, 59]. A maculopapular stage (days 1–7), which is characterised by vacuolisation of the cells of the upper epidermis; a target stage (days 7–14) macroscopically having a red centre surrounded by a white ring of maculopapular stage cells, which is further surrounded by a red halo of inflammation; an acute stage (days 14–21) where the epidermis has disappeared, and in some areas hair follicles are dilated and full of pycnotic cells; a regenerative stage (days 21–28) where the epithelium is regenerating; a papilloma stage (days 28–35) characterised by a raised epidermal lesion with finger-like projections of epidermis extending down into the dermis; and a regressive phase (after 35 days) in which the skin returns to its normal thickness and appearance, often without scarring. The appearance of milkers' nodules are similar, with lesions beginning as reddish purple, raised nodules turning bullous or pustular, and surrounded by a red halo of inflammation. The lesions resolve in 5–6 weeks [60]. Parapoxvirus infections reported in handlers of reindeer and musk-oxen in Norway are markedly granulomatous and, unlike orf and milkers nodules, may take many months to heal [61]. More serious complications of orf in humans are large highly vascularised tumour-like lesions of the skin. These tumour-like lesions have been noted in immunocompromised people [62, 63], but have also been seen in people with apparently normal immune systems. ORFV infections can cause complications such as erythema multiforme reactions and in these cases individuals present with rashes on the backs of hands, legs and ankles [64–67]. Cases of severe forms of erythema multiforma, known as Stevens-Johnson syndrome, have been reported and involve rashes on mucous membranes and skin [68]. In immune-impaired individuals severe progressive disease can develop and cases have been reported presenting with multiple lesions [69]. The apparently successful use of cidofovir to treat a giant non-resolving ORFV lesion in an immunocompromised patient has been reported [70]. Immunity against ORFV is short lived and both animals and humans are susceptible to reinfection.

Virion structure

The virions of parapoxviruses have a characteristic ovoid structure and this unique morphology has formed the basis for their inclusion as a separate

group in the poxvirus family [1]. Electron microscopy of ORFV reveals a virion with a long axis of approximately 260 nm and a short axis of 160 nm [71–75]. Negatively stained preparations of parapoxviruses appear in two forms. In the capsular form where the stain has penetrated the virion, a finely crenelate membrane appears to surround an inner amorphous core, whereas virions that are impervious to the stain reveal a regular array of tubule-like structures arranged in a criss-cross manner along the length of the particle [73, 76]. Where the virus has been propagated in cell culture, virions that appear in the medium are surrounded by a membranous structure 9–18 nm thick. It has been suggested that this membrane, by analogy to VACV, has been derived from the Golgi. The criss-cross pattern seen by electron microscopy is apparently due to superimposed images of the tubule-like structure as it winds its way in a spiral around the viral particle much like a ball of wool. More recently, the surface ultrastructure of ORFV has been described using ultra high resolution scanning electron microscopy where spirally arranged protrusions are visible on the surface of the virion [77].

Few studies have been performed to characterise the polypeptides that make up the virion particles of the parapoxviruses. Preliminary characterisation of ORFV virion polypeptides [78] showed that up to 35 polypeptides could be resolved by polyacrylamide gel electrophoresis. Analysis of ORFV virion polypeptides solubilized by treatment with NP-40 and 2-mercaptoethanol showed that 13 of 35 polypeptide bands distinguishable in whole virion preparations, were found in supernatant fractions after detergent treatment. There appeared to be considerable enrichment for a polypeptide of 38.5 kDa. There were varying degrees of enrichment for the other 12 polypeptides. The major band in virus preparations was 64.5 kDa and was thought to be a major core polypeptide. Others have detected about 30-40 structural proteins of ORFV [79–81]. Studies on PCPV have shown that up to 40 polypeptides can be resolved by SDS-PAGE [82].

Monoclonal antibodies raised against ORFV particles have helped to identify the proteins that make up the virion structure. The majority of these antibodies have reacted with proteins of 65, 39 or 22 kDa [83, 84]. The gene encoding the immunodominant 39-kDa protein is a homologue of the VACV gene H3L gene [83–85]. VAC H3L encodes an immunodominant virion membrane protein of 35 kDa [86] that is a member of the C-terminal anchor proteins [87] and has a role in virus maturation [88] and intracellular mature virus (IMV) adsorption to mammalian cells [89]. There is strong evidence that a polypeptide of about 40 kDa is the major component of the surface tubule [78–80, 82, 90].

DNA analysis of parapoxvirus genomes has revealed further homologues of VACV structural proteins. VACV has two infectious forms; IMV that are particles that have an outer membrane derived from the intermediate compartment, and extracellular enveloped virus (EEV), which are IMV particles that have an outer membrane derived from the trans-Golgi net-

work (reviewed in [91]). Viral encoded proteins that have been incorporated into these membranes of VACV have been identified. ORFV and BPSV have homologues of the IMV-associated proteins as well as homologues of several of the EEV-associated proteins (see below). The discovery of such homologues in conjunction with the morphology of the virus suggests that the structure and morphogenesis of the parapoxviruses and VACV may be similar.

Immune response to parapoxviruses

Most of our knowledge of immunity to parapoxviruses comes from studies with ORFV. The current ORFV vaccine is live unattenuated virus that elicits protective immunity for approximately 6–8 months [92]. In view of the deficiencies of the current vaccine, an in-depth understanding of the protective immune response against ORFV has been undertaken by several laboratories.

Although the current evidence strongly suggests that cell-mediated immunity is likely to play a major role in conferring protective immunity against ORFV infection, the role of antibody in protection is less clear. Understanding the mechanisms of protective immunity to ORFV is complicated by the fact that immunity is short lived, whether it is induced by natural or experimental infection. Although there is general agreement that ORFV is able to reinfect its host, albeit the lesions are smaller and resolve sooner, there has been much debate over the role of humoral immunity in preventing or reducing the severity of lesions during reinfection. There are a number of reports that suggest that antibody is not important in protection or recovery. Early studies reported by Aynaud in 1923 showed that serum from immune animals was not protective [11] and many years later it was shown that colostrum passed onto lambs from their immune dams did not confer protection [93–95]. Others have shown that there was no relationship between antibody titre and severity of lesions. In experiments carried out by McKeever et al. [80], it was observed that lambs that were seropositive were not protected from infection. Some investigators dispute the lack of importance of antibody. Lloyd [41] showed a strong correlation between IgG2 and the resolution of lesions subsequent to challenge, suggesting that a specific isotype was important in defence against ORFV infection. Lloyd suggested that the involvement of IgG2 in the immune response might explain observations by Buddle and Pulford [93] that colostral antibody failed to protect lambs from ORFV infection since IgG1 but not IgG2 is selectively transported in milk of ruminants.

Other approaches to examine the inflammatory and immune responses to ORFV infection have involved histology of infected tissue where the cell types that infiltrate into lesions have been analysed [55, 56, 96–99]. These studies showed that neutrophils accumulated in a biphasic manner with an

initial influx at 24 h, followed by a second phase at 4 days post-infection, which coincided with the appearance of viral antigen in the epidermis [55, 96]. An influx of basophils also coincided with the appearance of antigen. A dense mass of MHC class II^+ dendritic cells (DC) developed in the necrotising dermis adjacent to infected hair follicles and under infected degenerating epidermis [56]. The MHC class II^+ cells accumulating in the dermis were shown to be $CD1^-$ cells (acetylcholine esterase negative) that could be further subdivided based on Factor XIII expression [98]. These cells appear to form a barrier to invasion and may be involved in the immune response or wound repair [56, 98]. There was no evidence of epidermal Langerhans cell ($CD1^+$, acetylcholine esterase positive) involvement in the response [56]. In addition, different classes of T cells also accumulated including $CD4^+$, $CD8^+$ and $T19/WC^+$ cells [97]. Anderson et al. [99] showed that $CD4^+$ cells and DC accumulated to greater numbers than other cell types over the first 8 days. $CD4^+$ cells concentrated in the papillary dermis. $CD8^+$ cells were seen throughout the dermis and occasionally in the epidermis proximal to virus-infected epithelium. Studies of $CD8^+$ T cells in ORFV lesions have suggested that, although these cells are recruited to the site of virus infection, they become trapped underneath the ORFV lesion and are unable to gain access to virus-infected cells [100]. In spite of the presence of activated cytolytic $CD8^+$ T cells, the virus was able to replicate for several days. B cells were generally restricted to the reticular dermis underlying the virus-infected epithelium. $T19^+$ cells were distributed throughout the dermis and occasionally in the epidermis.

The dynamics of the local immune response to ORFV infection has been studied by examining the cells and soluble mediators in afferent and efferent lymph draining from the site of infection. These studies have involved cannulating the afferent and efferent lymph ducts of prefemoral or popliteal lymph nodes draining an infection site in the hind flank of sheep [92, 101–105]. Acquired immunity to pathogens that infect skin is initiated in the peripheral lymph nodes. Antigen is carried to lymph nodes by antigen-presenting cells (APC) *via* the afferent lymphatic ducts. Antibody and cytotoxic T cells produced by the lymph nodes leave *via* the efferent lymph ducts and migrate to the infected site. In addition lymphocytes migrating from blood to the site of infection, cycle through the lymph node by passive movement and become activated during this process. Studies in sheep have shown that the local immune response to ORFV in reinfected animals was a biphasic lymph cell response involving $CD4^+$ T cells, $CD8^+$ T cells, B cells and DC (reviewed in [92]). The studies showed that $CD4^+$ T cells were the most numerous lymphocyte subset in afferent lymph and peaked on days 4 and 12 post infection in reinfected sheep [104]. A similar pattern was also seen in the production of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-1 (IL-1), IL-8, IL-2 and interferon-γ (IFN-γ) in lymph cells cultured from afferent lymph collected at various times post infection. The study also showed that reinfected animals produce a strong memory

response when given inactivated ORFV, which is essentially a delayed-type hypersensitivity response as shown by Buddle and Pulford [93]. Cytokine analysis of ORFV-infected tissue has also shown that the immune response to ORFV is predominantly a Th1 response [99]. Cells expressing tumour necrosis factor-α (TNF-α) included epidermal cells, vascular endothelium and uncharacterised cells that had lymphocyte morphology. The numbers of these cells increased more rapidly in skin after reinfection. Cells expressing IFN-γ mRNA were only detected after reinfection and these cells had lymphocyte morphology.

The components of the immune response that are important in protection from ORFV have been studied by immunosuppressing animals with the drug cyclosporin-A prior to infection [106], and more recently by the depletion of specific lymphocyte subtypes [107]. Cyclosporin-A caused severe ORFV lesions to develop and was associated with the inhibition of IFN-γ and IL-2. The lymphocyte depletion studies revealed that $CD4^+$ T cells and antibody to a lesser extent are important for clearance of ORFV. The depletion of $CD4^+$ cells was associated with virus persistence and correlated with previous studies showing that $CD4^+$ T cells were the predominant T cells in skin and draining lymph node [108]. Although there was no correlation between $CD8^+$ T cell depletion and size and resolution of ORFV lesions in this study, the role of $CD8^+$ T cells cannot be excluded, since not all $CD8^+$ T cells were depleted. The study suggested that the function of $CD4^+$ T cells as helper cells for antibody could be important because of the correlation seen between antibody titres and lesion size.

The conclusion from these studies is that sheep produce a normal antiviral immune and inflammatory response to ORFV in spite of the fact that ORFV is able to repeatedly reinfect sheep and replicate over a short period of time. Furthermore, a memory response is indicated by a delayed-type hypersensitivity reaction to ORFV antigen in previously exposed animals. The discovery of immune modulators encoded by ORFV (see below) [109–114] may explain how ORFV is able to avoid, at least temporarily, the effects of host immunity. The accumulating evidence suggests that the immune evasion strategies of the parapoxviruses are similar across the genus ([115] and unpublished data).

Molecular biology

Genome

The first reports on the molecular analysis of parapoxviruses showed that the genome of BPSV comprised linear double-stranded (ds)DNA of approximately 135 kbp with cross-linked ends [116]. Also at this time the nucleotide composition of the DNAs of BPSV, ORFV and PCPV were determined and the genomes of these viruses were found to be unusually G+C rich compared

with other poxviruses, approximately 63% [117]. Restriction endonuclease cleavage analyses of their genomes showed marked variability, although DNA/DNA hybridisation revealed strong inter-species homology between regions within the central core of the genomes, suggesting that, although there were sequence differences, their genomes were genetically conserved. There was a lack of cross-hybridisation between the terminal fragments of the parapoxviral genomes, suggesting significant differences in these areas [118, 119]. The genomic studies generally supported the classification of the above as separate species of parapoxviruses, which hitherto had been based on host range and pathology.

Over the last two decades, most studies on the genetic structure of the parapoxviruses were carried out with ORFV [39, 52, 120–124]. Detailed restriction endonuclease cleavage maps were produced for 16 New Zealand isolates and the complete genome of ORFV strain NZ2 was cloned [125–127]. Sequencing of selected regions across the $ORFV_{NZ2}$ genome revealed homologues of VACV genes. These included a dUTPase [128], homologues of VACV H4L (RNA polymerase-associated protein RAP94), H5R (35-kDa virion envelope antigen) [123] H6R (topoisomerase) [129] and the 14K fusion protein [130]. The distribution of these genes suggested that ORFV and VACV were colinear [123]. Further "spot sequencing" over the entire genome identified further homologues of VACV and supported the notion that many genes in ORFV conserved the order, orientation and spacing of genes seen in VACV [124]. Sequencing of the terminal regions identified genes that did not have counterparts in VACV and were likely to be involved in pathogenesis and virulence. These included a vascular endothelial growth factor (VEGF) [112], a homologue of IL-10 [110], a chemokine binding protein (CBP) [131] and a GM-CSF/IL-2 inhibitory factor (GIF) [109] at the right end of the genome. Moreover, there were a number of open reading frames that did not show matches with protein sequences in public databases, some of which had ankyrin repeats [132] and some of which appeared to be homologues of VACV genes with no known function. In addition, early and late promoter sequences were identified by transcriptional mapping [123, 133–136]. The early transcriptional termination motif, TTTTTNT, discovered in VACV, is conserved in ORFV [133, 137]. Furthermore, it was shown with VACV recombinants in which multi-gene ORFV fragments were inserted, that early ORFV genes were faithfully transcribed, demonstrating the conservation of transcriptional regulation between ORFV and orthopoxviruses [134, 135]. In addition, the inverted terminal repeat (ITR) of the $ORFV_{NZ2}$ genome was described [112].

Only recently have the genomes of ORFV (three strains) and BPSV (strain BV-AR02) [115] been fully sequenced (see Tab. 1). The genome sequences have provided further insights into the unique characteristics of the parapoxviruses and have allowed comparisons to be made within the genus and with members of the *Chordopoxviridae* subfamily.

Table 1. Species within the genus parapoxvirus

Members of the genus	Host range	Virion morphology	Genome size	GenBank accession no.
Orf virus	Sheep, goats, Japanese serow, camels, humans	Ovoid 260–160 nm	138 kbp 64% G+C	OV-NZ2, DQ184476, OV-IA82, AY386263, OV-SA00, AY386264
Bovine papular stomatitis virus	Cattle, humans	Ovoid 260–160 nm	134 kbp	BV-AR02, AY386265
Pseudocowpox virus	Cattle, humans	Ovoid 260–160 nm		
Parapoxvirus of red deer in NZ	Red deer	Ovoid 260–160 nm		
Sealpox virus (tentative)	Seal sp, humans	Ovoid 260–160 nm		
Ausdyk virus (tentative)	Camels	Ovoid		
Parapoxvirus of reindeer (tentative)	Reindeer, humans	Ovoid		
Chamois contagious ecthyma virus (tentative)	Chamois	Ovoid		

Our analysis of the ORFV genome sequences predicts 132 genes in the 138-kbp genome [138], whereas BPSV lacks one ORFV gene of unknown function but has two additional ankyrin F-box genes, giving a total of 133 genes [115, 139]. Sequences of only single genes of PCPV and PVNZ have been published; however, comparisons of these sequences and of as-yet unpublished partial genome sequences from this laboratory have confirmed the identity of ORFV, BPSV, PCPV and PVNZ as separate species within the *Parapoxvirus* genus [28, 38, 140]. Phylogenetic analyses of these sequences have, surprisingly, indicated a closer relationship between PCPV and ORFV rather than between PCPV and the other bovine parapoxvirus, BPSV.

The central core of ORFV and BPSV genomes contains homologues of VACV genes involved in replication and transcription of the genome as well as genes encoding proteins associated with structure and morphogenesis, including homologues of proteins that are incorporated into the membrane of the IMV and EEV (Fig. 1). The central region of the ORFV and BPSV genomes lack two genes (VACV D9R, a putative nucleoside triphosphate pyrophosphohydrolase, and VACV F15R, unknown function) present in all other chordopoxviruses, indicating that the minimum essential chordopoxvirus genome is 88 genes [141, 142].

The terminal regions of the parapoxvirus genomes comprising approximately 20% of the genome show substantial variation from that seen in

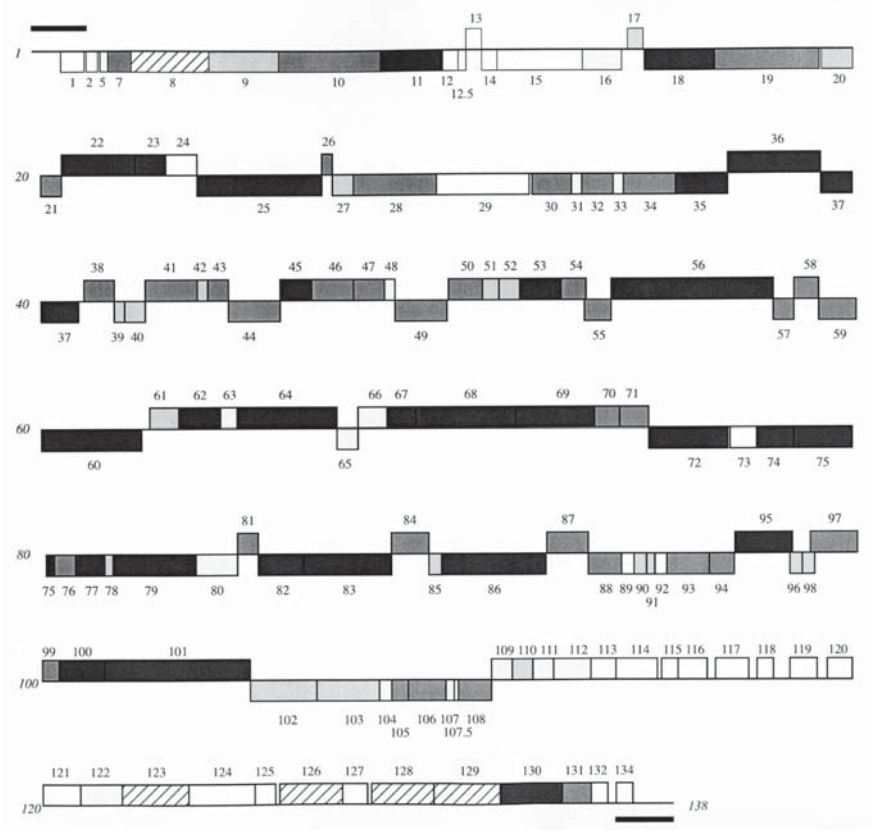

Figure 1. ORFV NZ2 genetic map. The assigned open reading frames of ORFV are shown as boxes on a line representing the genome. Boxes above the line represent open reading frames transcribed rightward and those below the line are transcribed leftward. Each line except the last corresponds to 20 kb as indicated by the numbers in italics at the left of each line. The boxes are shaded to indicate the approximate BLASTP bit score with the orthologous gene in VACV (Copenhagen). The four shades of grey from darkest to lightest correspond to bit scores of greater than 300, 100–300, 55–99 and 34–54, respectively. The speckled boxes represent scores of less than 34. White boxes are those ORFV genes for which no significant BLASTP match with a VACV protein was detected. White boxes with cross-hatching represent genes encoding ankyrin repeat proteins (see text). Figure from [138].

other poxviruses (Fig. 1). For example the ORFV genome includes 31 genes that lack clear matches in VACV, and 17 that have no significant homology with genes from all other poxvirus genera. Many of these are located in a 25-kbp region at the right terminus and are likely to encode factors that are involved in pathogenesis and virulence. Sequence analysis of approximately 25 kbp of the PCPV and PVNZ genomes has revealed the presence of this extended genus-specific region in these two other species (unpublished).

Comparisons of predicted protein sequences of the three fully sequenced ORFV isolates revealed a degree of inter-isolate sequence variation that is uncommon among chordopoxviruses. For instance, amongst the 20 most variant genes, the average predicted amino acid sequence identity was only 80% [138]. Most of these genes are not seen in other poxviruses and have unknown functions. However, two of the most variant proteins are orthologs of envelope glycoproteins present in all mammalian poxviruses (VACV A33R and A34R). A33R has been shown to be a target of neutralizing antibody, leading to the suggestion that inter-isolate variation might represent "escape mutants" and be linked to the ability of ORFV to reinfect previously infected animals [138]. It has also been proposed that some of the inter-isolate variation seen in these ORFV proteins might be associated with host-specific requirements for infection of different species, such as sheep and goat [115]. A homologue of the transcription factor VLTF-4 that shows little variability in capripoxviruses, *Sheeppox virus* and *Goatpox virus* is highly variable in ORFV and BPSV, suggesting that it may play a role in host range [115]. Both ORFV and BPSV encode proteins that are apparent homologues of orthopoxvirus proteins involved in the formation of A-type inclusions.

Parapoxviruses, in common with *Molluscum contagiosum virus* (MOCV), lack some genes that are highly conserved in other chordopoxviruses and that are likely involved in nucleotide metabolism, including ribonucleotide reductase, thymidine kinase, guanylate kinase, thymidylate kinase and a putative ribonucleotide reductase cofactor. Parapoxviruses also lack a Ser/Thr protein kinase and the serine protease inhibitor and the kelch-like gene families found in all other chordopoxviruses except MOCV [115]. These genes are known to affect host responses including inflammation, apoptosis, complement activation and coagulation and are associated with virulence. The absence of these genes may, at least in the case of parapoxviruses, be compensated for by an alternative set of genus-specific genes involved in host manipulation. Phylogenetic analysis suggests that parapoxviruses and MOCV, although clearly divergent from one another, share a separation from the other genera of mammalian poxviruses.

The full genomes of three ORFV isolates and one BPSV isolate have been shown to share an average nucleotide composition of 64% G+C. However, points of marked deviation from this average were observed in near-terminal regions in such a distinctive and uniform pattern as to form a signature [138]. In contrast, representatives of other poxvirus genera, including the high G+C of MOCV, display more uniform G+C contents across their genomes. In some cases the parapoxviral regions of uniform deviation in G+C content coincide with regions that show significant inter-isolate sequence variation. These results suggest a selective pressure to maintain unusually low regions of G+C content in specific regions of the parapoxvirus genome, independent of coding potential.

The ORFV genome is subject to rearrangement of terminal sequences when the virus is passaged in cell culture [143]. Similar rearrangements have

been reported in other poxviruses [144–147]. Such terminal rearrangements have occurred in the highly passaged and attenuated strain of $ORFV_{D1701}$, that has given rise to a considerably enlarged ITR compared with low passage field isolates of ORFV [39, 148]. This has resulted in the duplication of some genes and the loss of others. We have observed spontaneous terminal transposition-deletion variants of $ORFV_{NZ2}$ that emerged during serial passage in bovine testis cells. Characterisation of one of the variants revealed that 6.6 kb of DNA at the left end of the genome had been replaced by 19.3 kb from the right end. The transposition resulted in the deletion at the left end of 3.3 kb encoding three genes and the terminal sequence of a fourth [143].

Virulence genes

IL-10

The discovery of an IL-10-like gene in a poxvirus was first reported in ORFV [110]. Since then, IL-10-like genes have been found in other parapoxviruses including BPSV [115] and PVNZ (unpublished) and yatapoxviruses (*Yaba-like disease virus* (YLDV)) [149] and capripoxviruses (*Lumpy skin disease virus*, *Goatpox virus* and *Sheeppox virus*) [150]. Mammalian IL-10 is a multifunctional cytokine that has suppressive effects on inflammation, anti-viral responses and T helper type 1 (Th1) effector function [151]. The inhibition of a Th1 response occurs indirectly through antigen-presenting macrophages and DC. In addition, IL-10 has co-stimulatory functions and is a costimulator of T lymphocytes associated with Th2 responses, mast cells and B cells.

$ORFV_{NZ2}$-IL-10 is 186 amino acid peptide with a molecular mass of 21.7 kDa and BPSV-IL-10 has 185 amino acids making the parapoxvirus IL-10s slightly larger than their mammalian counterparts. The gene is expressed early and is flanked by typical poxvirus early transcriptional sequences [110]. The homologies of the predicted polypeptide sequences of $ORFV_{NZ2}$-IL-10 with IL-10s of mammals and herpesviruses are: ovine 80%, bovine 75%, human 67% mouse 64%, Epstein-Barr virus (EBV) 63% and equine herpesvirus 2 67%. The homology of BPSV with bovine IL-10 is 75%. It is clear from sequence alignments of both ORFV-IL-10 and BPSV-IL-10, that they most closely resemble their natural host. The amino acid sequence identity of the parapoxvirus IL-10 with mammalian IL-10 is highest over the C-terminal two thirds of the polypeptide, although the relatedness at the nucleotide level is less apparent and reflects differences in codon usage and the high G+C content of the parapoxvirus genomes in general. Curiously, the N-terminal region of the parapoxviral IL-10 contains little similarity to mammalian or herpesvirus IL-10s [110].

The close similarity between the IL-10s of herpesviruses and their hosts suggested there were selective pressures for the viral IL-10 to resemble

their eukaryotic counterparts, which is also apparent with the parapoxviruses. However, the discovery of IL-10-like genes in members of two other poxvirus genera (*Yatapoxvirus* and *Capripoxvirus*) and in the herpesvirus, cytomegalovirus (CMV) [152, 153], which are only approximately 20% identical to mammalian IL-10, would seem to refute this notion, although few studies have been performed on the functional characterisation of these IL-10 variants. It is possible that some of the viral IL-10s may have evolved from captured host gene(s) other than IL-10 but which are structurally similar to mammalian IL-10. It has recently been shown that YLDV-IL-10 is functionally more similar to IL-24 than IL-10 [154].

The functional characterisation of parapoxvirus IL-10 has thus far been carried out with ORFV-IL-10. These studies have revealed that it appears to have all the activities of mammalian IL-10. Using murine, ovine and human cells ORFV-IL-10 has been shown to inhibit the production of TNF-α and IL-8 by LPS-activated macrophages and PMA/calcium ionophore-activated keratinocytes, and IFN-γ and GM-CSF from Con A-activated peripheral blood lymphocytes [122, 155]. In addition, ORFV-IL-10 costimulates mast cells and thymocyte proliferation [110, 122, 155]. It is active on APC and has been shown to inhibit maturation and antigen presentation of murine bone marrow-derived DC [156] and human blood-derived monocyte DC (Chan, Baird, Mercer, Fleming, unpublished). These activities suggest that the parapoxviral IL-10 will have a role in suppressing inflammation and the development of the innate responses and acquired immunity.

Studies on viral IL-10-like molecules have provided insights into structure - function aspects of cellular IL-10. The active form of mammalian IL-10 is a homodimer. The three-dimensional structure of human IL-10 [157–159] and its interaction with its receptor at the molecular level has been determined [160]. It has been shown that a total of 27 amino acids of IL-10 contact the binding interface of the IL-10 receptor. These amino acids are located within both the near N-terminal and the C-terminal regions of IL-10. Examination of ORFV-IL-10 reveals that it conserves 11 of the 16 N-terminal amino acids of human IL-10 that make contact with the receptor and 10 of the 11 C-terminal residues. Although ORFV-IL-10 and EBV-IL-10 resemble their host counterparts only ORFV-IL-10 has all the functional activities of mammalian IL-10, whereas EBV-IL-10 has only a subset of activities, having evolved a more immunosuppressive form [161–164]. It was originally thought that EBV IL-10 lacked immunostimulatory activities because of differences at the N terminus, which had been suggested to contain the immunostimulatory domain; however, studies on ORFV-IL-10 showed that it was also different within this region, although it costimulated thymocytes and mast cells [122, 155]. The concept that IL-10 has multiple domains is no longer accepted and it is believed that essentially two regions bind to the receptor, one region at the near N terminus and the other at the C terminus [165]. It is intriguing that ORFV has not evolved a more immunosuppressive form of this virokine, like EBV IL-10. This may suggest that

mutations of the ORFV-IL-10 to a potentially more immunosuppressive form may compromise the coexistence of host and virus.

Chemokine binding protein

In common with orthopoxviruses and leporipoxviruses, the parapoxviruses encode a CBP [111, 115, 131, 166, 167]. Chemokines are a large family of molecules that recruit and activate immune cells at sites of inflammation and infection [168, 169]. Thus far, a CBP has been found in ORFV, BPSV, PVNZ (unpublished). The most well characterised of these is the CBP of $ORFV_{NZ2}$, which is a protein of 286 amino acids with a predicted molecular mass of 31.2 kDa [111]. $ORFV_{NZ2}$-CBP shows surprisingly low similarity to CBPs of orthopoxviruses or leporipoxviruses with only approximately 16% amino acid identity. The parapoxvirus CBPs are more similar to ORFV-GIF at the polypeptide level than to other poxvirus proteins (approximately 20% identical at the amino acid level). This suggests that the *Parapoxvirus*, *Orthopoxvirus* and *Leporipoxvirus* genera share a common ancestral gene that became modified during the course of evolution to create different binding specificities [111]. The orthopoxvirus and leporipoxvirus CBPs share a high level of identity and many of the highly conserved regions of sequence within these CBPs are not apparent in the parapoxvirus CBPs. In addition, only six of the eight cysteine residues present in other poxvirus CBPs are found in parapoxvirus CBPs. The viral CBPs, including those found in the herpesvirus group, bear no sequence or structural homology to any known G-protein coupled receptors or mammalian proteins.

The ORFV-CBP has been shown to have a unique binding profile amongst the poxvirus CBPs with the most significant difference being that in addition to binding a number of CC inflammatory chemokines, it also binds the C chemokine lymphotactin that the other poxviruses do not bind [111]. To date, it has been shown that the ORFV-CBP binds the CC chemokines eotaxin, MCP-3, MCP-1, MIP-1b and MIP-1a and I309 and lymphotactin with high affinity. In common with the other poxvirus CBPs, ORFV-CBP does not bind the homeostatic chemokines MDC or TARC.

The binding site of ORFV-CBP to human MCP-1 has been examined at the molecular level using single amino acid mutants of MCP-1. The studies revealed that ORFV-CBP binds to residues that are critical for the interaction of MCP-1 with CCR2b (MCP-1 receptor) and demonstrates that ORFV-CBP occludes the receptor binding site of the chemokine in a similar manner to other poxvirus CBP [111]. The findings suggest that viral CBPs are likely to act as competitive inhibitors *in vivo*. In addition, it has been shown that ORFV-CBP inhibits chemokine-induced signalling in a dose-dependent manner in a calcium flux assay [111].

The binding activities of ORFV-CBP suggest that it blocks the recruitment of monocytes, macrophages, DC, natural killer (NK) cells and T cells

to sites of infection. ORFV-CBP does not bind the homeostatic chemokines such as monocyte-derived or thymus- and activation-regulated chemokines, suggesting the importance of inhibiting the inflammatory CC-chemokines rather than the homeostatic CC-chemokines. In addition, lymphotactin has been implicated in the chemotaxis of T cells, neutrophils and B cells that express the lymphotactin receptor XCR1 [111]. Furthermore, the binding spectrum of ORFV-CBP suggests that in addition to inhibiting chemokine-induced chemotaxis, it has evolved to target Th1 antiviral responses. MIP-1α, MIP-1β, RANTES and lymphotactin can function in concert with IFN-γ as Th1 cytokines that can coactivate macrophages and promote NK cells and $CD8^+$ T cells in driving Th1 responses [111].

GM-CSF/IL-2 inhibitory factor

The GM-CSF/IL-2 inhibitory factor (GIF) discovered in ORFV has not been reported in any virus other than the parapoxviruses [109, 170]. GIF was originally identified as an activity produced from primary ovine skin keratinocytes infected with ORFV. Although GM-CSF was up-regulated at the transcriptional level, the secreted protein could not be detected in ORFV-infected cell culture supernatants, whereas the inflammatory cytokines IL-1β and TNF-α were detected. GIF is highly conserved within ORFV strains. ORFV strains orf 11 and MRI scab are predicted to encode proteins with 98% identity to $ORFV_{NZ2}$ GIF. Homologues of GIF have more recently been identified in BPSV [115] and PVNZ (unpublished). GIF has sequence similarities with poxvirus CBPs (see above), suggesting extensive divergence from a poxvirus ancestral gene. GIF has 32% amino acid similarity with the VACV A41L protein but that protein does not bind GM-CSF, IL-2 or a range of chemokines and, while it appears to be involved in reducing the migration of inflammatory cells, its function has not been fully defined [171].

GIF exists in solution as either a dimer or a tetramer and both forms are functionally active. It has been shown to bind ovine GM-CSF and ovine IL-2 with high affinity with Kd of 369 pM and 1.04 nM, respectively; however, it does not bind the human equivalents of these molecules, highlighting the adaptation of ORFV to its ovine host [109]. In biological assays, GIF has been shown to inhibit the haemopoietic activity of GM-CSF in a soft-agar bone marrow cell colony assay and ovine IL-2 in a T cell proliferation assay [109]. Furthermore, the activity has been detected in cannulated afferent lymph from ORFV-infected sheep, in which the highest GIF activity detected corresponded to the time of maximum growth of the virus [109]. Sequence comparisons of IL-2 and GM-CSF have not revealed a potential binding domain shared by these cytokines. The only common feature is that they are members of the short-chain, four-α-helical bundle family of cytokines that also includes IL-4; however, GIF does not bind IL-4 [109].

Vascular endothelial growth factor

A gene encoding a polypeptide with homology to mammalian VEGF has been identified in ORFV, [112, 172–174], BPSV [115], PCPV [175], and PVNZ (unpublished data). The viral VEGF is thought to explain the extensive proliferation of vascular endothelial cells, dilation of blood vessels and dermal swelling seen in parapoxvirus lesions. Indeed, reports from as early as 1890 use terms such as "readily bleed" in describing ORFV lesions [9]. VEGF-like factors are not encoded by any other poxviruses and the only other occurrence of a potential viral VEGF is in two closely related iridoviruses of fish [176, 177].

Members of the mammalian VEGF family are major regulators of the formation of new blood vessels during embryogenesis and angiogenesis. The family currently comprises VEGF-A, placental growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D [178]. These factors mediate endothelial cell proliferation, vascular permeability, angiogenesis and lymphangiogenesis *via* the tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk1), and VEGFR-3 (Flt-4) [179]. In general VEGFR-1 plays a role in haematopoietic cell differentiation and migration, VEGFR-2 is involved in vascular endothelial cell mitogenesis, and VEGFR-3 is involved in the regulation of lymphangiogenesis.

The viral VEGFs bind and induce autophosphorylation of VEGFR-2 but do not bind VEGFR-3 and show little recognition of VEGFR-1 [172–174]. This receptor binding spectrum differs from that of all mammalian VEGF family members and the viral VEGFs have been classified as a new subgroup of the family, called VEGF-E [172]. The ORFV and PCPV VEGF have been shown to share a disulphide-linked homodimeric structure with mammalian VEGF, to be mitogenic for endothelial cells and to induce vascular permeability.

Variants of VEGF-E were revealed during the genetic analysis of different strains of ORFV. $ORFV_{NZ2}$ encodes a polypeptide of 14.7 kDa, whereas the $ORFV_{NZ7}$ encodes a polypeptide of 16 kDa. Both forms of VEGF-E show low amino acid sequence identity to mammalian VEGF with $ORFV_{NZ2}$ VEGF-E showing 35% and ORFVNZ7 25% identity to human VEGF-A. Intriguingly, these two viral VEGFs are only 41% identical to each other. The VEGF of PCPV shows 27% amino acid identity to human VEGF-A and 41% and 61% amino acid identity to VEGF encoded by ORFV strains NZ2 and NZ7, respectively [175]. Similar levels of sequence relatedness are observed for the VEGF of BPSV [115] and PVNZ (unpublished).

The sequence disparity of the NZ2 and NZ7 VEGFs has been examined further by sequence analysis of 21 ORFV isolates [180]. It was found that most carried the NZ2-like version but their amino acid sequences varied by up to 31%. Despite the sequence variations, structural predictions for the viral VEGFs were similar to the structure determined for VEGF-A. In addition, the viral VEGFs are all equally active mitogens, stimulating prolifera-

tion of human endothelial cells *in vitro* and dermal vascularisation of sheep *in vivo* with potencies equivalent to VEGF-A [181]. It has been suggested that the extensive sequence divergence seen in at least the ORFV VEGF may have been generated primarily by selection against VEGFR-1 binding and its associated recruitment and activation of cells involved in antiviral responses [181].

A recombinant ORFV, in which the VEGF gene was deleted, has been used to assess the contribution of this gene to the vascular responses in infected sheep. The striking proliferation of blood vessels within the dermis underlying the site of infection was absent in sheep infected with the VEGF deletion mutant; however, viral replication in the early stages was not impaired but appeared reduced later in infection [182]. Epidermal hyperplasia is a feature of the response to ORFV infection and this feature was also reduced in infections with the VEGF deletion mutant. The epidermal and vascular responses seen in ORFV lesions are reminiscent of a sustained wound healing response and extravagantly proliferative ORFV lesions have been reported in immunocompromised individuals. Expression of a viral VEGF might assist in maintaining a regenerative response and thereby support extended viral growth. Parapoxviruses do not encode the epidermal growth factor seen in several other poxviruses and which has been associated with localised cellular proliferation.

Another possible role for the viral VEGF relates to the extensive scab formation seen in ORFV lesions. Scab shed from ORFV lesions contains substantial amounts of infectious virus and the scab provides protection from environmental inactivation. In this way the virus remains available to infect naïve animals as much as a year after being shed. The viral VEGF is able to induce vascular permeability and would seem to contribute to scab formation since lesions induced by VEGF-deleted ORFV have essentially no scab [182].

IFN-resistance gene

ORFV is resistant to type 1 and type 2 IFN. A homologue of the VACV IFN resistance factor E3L has been described for ORFV [113, 114] and homologues have since been discovered in BPSV [115]. The E3L gene product inhibits IFN-mediated down-regulation of protein synthesis by binding dsRNA thus preventing the activation of the dsRNA-dependent IFN inducible protein kinase (PKR) [183]. During the antiviral response, PKR phosphorylates itself and the translation initiation factor eIF2-2, thus blocking protein translation and viral replication. The ORFV E3L homologue (ORFV 020) is 31% identical and 57% similar to VACV E3L at the protein level and is expressed early [113]. A predicted dsRNA binding motif is present in ORFV 020 and it has been shown to bind specifically to dsRNA and to competitively inhibit the activation (phosphorylation) of the

ovine dsRNA-dependent PKR gene. In addition, cell lysates from ORFV-infected cells diminished PKR phosphorylation, which was also observed in the presence of cytosine arabinoside, indicating that the inhibitory activity is encoded by an early gene [114]. Further, transient expression of ORFV 020 protected Semliki forest virus from the inhibitory effects of IFN-α.

The predicted protein sequence of the BPSV E3L homologue (BPSV-020) is 53% identical to the ORFV protein and it includes a predicted dsRNA binding motif. Both BPSV and ORFV 020 proteins have evidence of a N-terminal Z-DNA binding domain that, in the case of VACV E3L, has been linked to pathogenicity in mouse infection models [184].

Anti-apoptosis

Recently the VACV IFN resistance factor, the E3L protein, has been shown to have anti-apoptotic properties and it has been suggested that these functions are linked to its N-terminal Z-DNA binding domain acting as a transcriptional transactivator of a range of cellular genes [185]. Inspection of the ORFV and BPSV genomes reveals that the VACV E3L homologues are the only parapoxvirus proteins with clear links to the inhibitors of apoptosis identified in other poxviruses. Despite this, investigations in this laboratory have revealed that ORFV is a potent inhibitor of apoptosis and we have identified a mitochondrial-targeted ORFV protein that blocks UV-induced apoptosis and which shows some similarities to Bcl-2 family members (unpublished). Related proteins are present in each of BPSV, PCPV and PVNZ.

Ankyrin repeat, F-box-like proteins

In common with all other chordopoxviruses except MOCV, parapoxviruses encode several proteins carrying the ankyrin repeat motif. This motif is named after the cytoskeleton protein, ankyrin, which contains 24 copies of the motif. The motif is recognised as a mediator of protein-protein interactions. ORFV encodes five such proteins and BPSV seven. Each of the ORFV genes can be paired with a corresponding gene in BPSV, but the direct relationships between the parapoxvirus proteins and other chordopoxvirus ankyrin repeat genes are less clear. In fact, any one of the parapoxvirus ankyrin repeat proteins shows more similarity to all of the other parapoxvirus ankyrin repeat proteins than to any of the ankyrin repeat proteins in other poxviruses. However, all five ORFV and seven BPSV ankyrin repeat proteins carry the C-terminal F-box-like domain present in most poxviral ankyrin repeat proteins and this has led to the suggestion that these proteins may function within the ubiquitin–proteasome system by acting as recognition subunits of cellular ubiquitin ligase complexes [139]. The

proteins targeted by the viral ankyrin-F-box proteins might be involved in modulating cellular stress responses or cell cycle regulation [186, 187].

Parapoxviruses and immune evasion

It has become apparent over the last decade that parapoxviruses, like other poxviruses, encode an arsenal of weapons that allows this group to temporarily suppress the host's defences and thus create a window of opportunity in which to replicate. The immunomodulators that have been discovered thus far suggest that this group has the capability to target inflammatory processes, the innate responses such as apoptosis, NK cell activity and antiviral effects of IFN, and the development of adaptive immunity. Although these targets are common to almost all of the poxviruses, the virulence factors encoded by the parapoxviruses in many cases are unique.

As described above, parapoxviruses replicate exclusively within the epidermis and in the case of ORFV within keratinocytes. The skin is the largest organ of the body and has evolved a highly specialised defence system to respond rapidly to invading organisms. Keratinocytes are the principle immune cell within the epidermis and act as proinflammatory signal transducers responding to non-specific stimuli by secreting inflammatory cytokines, chemotactic factors and adhesion molecules into the extracellular fluid of the epidermal compartment [188]. In the initial phase of non-specific cutaneous inflammation, keratinocytes release IL-1β and TNF-α. IL-1β and TNF-α activate dermal vascular endothelium, which up-regulates the expression of adhesion molecules involved in the recruitment of leukocytes to the endothelium. In conjunction with chemokines, these cytokines direct the migration of leukocytes from the circulatory system into the epidermis. TNF-α is down-regulated in activated keratinocytes by cellular IL-10 [189], suggesting that the production of proinflammatory cytokines produced by these cells may be the main targets of viral IL-10 during the early stages of cutaneous inflammation. There is no evidence that parapoxviruses produce receptor-like homologues of IL-1β or TNF-α to sequester these cytokines. Furthermore, there is no evidence at this time to suggest that parapoxviruses produce factors other than IL-10 that disrupt the induction of the proinflammatory signalling cascade in virus infected cells. Poxviruses in general have developed multiple strategies to minimize the deleterious effects of proinflammatory cytokines but few encode a viral IL-10. It is likely that parapoxvirus factors will be discovered in the future that specifically target pathways that lead to the induction of proinflammatory cytokines in virus-infected cells. In addition, the production of proinflammatory cytokines secreted by immigrating macrophages and $CD8^+$ cells are likely to be blocked by viral IL-10.

Apoptosis can be induced by a variety of extracellular inducers including TNF, FAS, IFN, NK cells and cytotoxic T lymphocytes (CTL), as well as

agents such as UV light, serum growth factor deprivation and hypoxia and within the cell by macromolecular synthesis such as viral dsRNA. Thus far the parapoxviruses have only been shown to encode one factor that directly inhibits apoptosis and evidence suggests that it acts *via* the mitochondria by inhibiting the release of cytochrome c (unpublished). There is no evidence that parapoxviruses produce factors that bind caspases and factors that disrupt the cellular death effector domains of the TNF or FAS receptor mechanisms common to other poxviruses [190–193]. Furthermore, serpins have not been found, such as CrmA that protects cells from perforin-dependent apoptosis induced by CTL and NK cells [194, 195].

The activities of poxvirus anti-apoptotic proteins are closely interrelated with strategies that target intracellular elements in the IFN response pathway, including the PKR and 2',5' oligoadenylate synthetase [196, 197]. Both of these enzymes are activated by dsRNA. In addition, dsRNA is known to initiate cascades that inhibit protein synthesis and induce apoptosis by activating caspase-8 [198]. As described above, it has been established that ORFV produces a homologue of VAC E3L that binds dsRNA and blocks PKR activation. It has not been established, however, that it blocks other elements in the IFN response pathway that have been reported for VAC E3L, such as inhibiting the induction of IFN-α/β [199], reducing adenosine deaminase editing activity and mediating virus host range [200].

The binding activities of ORFV-CBP (inflammatory CC chemokines and lymphotactin) [111] suggest that parapoxviruses have the capability to establish a blockade to prevent the recruitment of inflammatory cells to the site of infection, in particular monocytes, NK cells, T cells and DC. In addition, the ability of ORFV-CBP to bind lymphotactin suggests that lymphocytes, B cells, DC and NK cells are of particular significance in the immune response to parapoxviruses. Although lymphotactin is also a chemotactic factor for neutrophils, the heavy infiltration of polymorphs into ORFV lesions suggests that these cells are being recruited to the site of infection predominantly by the CXC chemokines to which CBP does not bind. It has recently been reported that $CD8^+$ cytotoxic T cell infiltration into tumours is enhanced by transgene expression of lymphotactin by $CD8^+$ T cells [201]. In light of this observation, it is interesting to note that activated $CD8^+$ cells appear to become trapped under ORFV lesions [100], suggesting that the specificity of CBP for lymphotactin may provide an explanation for this observation.

The discovery of a secreted ORFV GM-CSF/IL-2 binding protein signals the importance of these cytokines in the immune response to parapoxviruses. GM-CSF is produced by a variety of cell types including T cells and keratinocytes [109] and stimulates the recruitment and/or activation of neutrophils, monocytes and eosinophils in tissues [202]. In addition, GM-CSF is involved in the maturation of DC. IL-2 stimulates T cell and NK cell activation and proliferation. It also stimulates the proliferation of activated B cells and may play a role in the survival of cytotoxic T cells at the site of the

infection. It is tempting to speculate that $CD8^+$ cytotoxic T cells are a major target of ORFV immunomodulators and that their proliferation, migration, activation and survival are affected by these factors. In addition, GIF could also be interfering with the maturation of DC as discussed below.

IFN-γ is associated with Th1 anti-viral immune responses and all poxviruses have evolved mechanisms to limit its actions [196]. IFN-γ is produced by $CD4^+$, $CD8^+$ and NK cells and has various effects on cells. It stimulates the production of IgG2a synthesis in B cells, inhibits Th2 cell growth, activates MHC class I and class II in macrophages and activates NK cells. Most poxviruses sequester extracellular IFN-γ by producing soluble IFN-γ receptor-like proteins [197]. There is no evidence that parapoxviruses encode such factors. However, parapoxviruses have the potential to suppress the production of IFN-γ since this cytokine is inhibited in NK cells, $CD4^+$ Th1 cells and $CD8^+$ cells by IL-10. In addition to their role in inflammation, TNF-α and IFN-γ are involved in the antiviral innate responses and specific early immune responses. IFN-γ acts synergistically to enhance antiviral cytotoxic activity of TNF-α and the anti-viral activities of IFN-α and IFN-β.

A further point of intervention by parapoxviruses could involve APC, which could be of particular significance during reinfection and persistent infections. It is apparent that the host produces a memory response to ORFV [93, 104], inferring that ORFV has the capability to replicate, albeit temporarily, in the immune host. The question is how ORFV is able to subvert the reactivation of memory T cells during reinfection. The evidence suggests that dermal DC or blood-derived DC are involved in initiating the immune response to ORFV, since Langerhans cells do not appear to play a role [56]. In the normal course of events DC are recruited to the site of infection, capture antigen, mature and migrate to the lymph node in response to constitutive chemokines where they present antigen to naïve T cells or memory T cells. APC other than DC may be involved during reinfection. Parapoxviruses have the potential to disrupt this process at multiple points. It is possible that the viral secreted immunomodulators GIF, IL-10, CBP, and VEGF work in concert to inhibit this process. DC have been observed to accumulate at the site of infection in ORFV-infected animals [56] and it has been observed that there is a reduction in DC trafficking from the epidermis to the lymph node at a point when ORFV replication is maximal, suggesting intervention by viral immunomodulators [92]. Although GIF and viral IL-10 have the potential to disrupt antigen presentation and activation of $CD4^+$ and $CD8^+$ T cells in the lymph node, we have no evidence that the viral cytokines are carried into the lymph node by passive movement. If this is the case, viral IL-10 could potentially block the development of the acquired immune response and may have effects on the development of immune memory, such as the induction of tolerogenic T cells. The IL-2 binding properties of GIF could block clonal expansion of $CD8^+$ cells and the sequestering of lymphotactin could all impact on the development of a Th1 response [131]. It has also been reported that ORFV induces apoptosis

in APC *via* the CD95 pathway in a mouse model [203] and CMV-IL-10 has been shown to increase apoptosis associated with DC maturation [204]. This may be a further mechanism that the parapoxviruses exploit to reduce or delay the development of the acquired immune response. It is also possible that parapoxviral IL-10 could skew the immune response towards a Th2 response during the early phase of infection, as proposed for CMV *via* viral IL-10 [204] and that may also involve the induction of immunological tolerance. Such a response might explain persistent parapoxvirus infections.

In conclusion, the accumulated evidence suggests that the parapoxviruses have evolved mechanisms to temporarily delay viral clearance by a Th1 immune response. It is possible that this strategy is only successful where virus replication is localised and restricted to specific tissues, such as the skin epithelium. The short range effects of the secreted viral immunomodulators in this instance are unlikely to compromise the general integrity of the host's immune system such as might occur with a more generalised infection.

Parapoxviruses in immunotherapy and recombinant vaccines

Although ORFV encodes potential immune-evasion factors, the attenuated strain D1701 possesses a variety of immunostimulatory properties [205–209]. Moreover, the massive accumulation of DC that occurs around the lesion in the natural host [98] is thought to indicate some chemoattractive activities. These properties lead to the development of the so-called 'paraimmunity inducer' licensed as immunomodulator Baypamun® [203, 205, 206]. Recent studies with inactivated $ORFV_{D1701}$ have shown that it induces a complex autoregulatory cytokine response that involves the up-regulation of IL-12, IL-18, IFN-γ and other Th1 cytokines and their subsequent down-regulation that is accompanied by the induction of IL-4 [210]. The powerful immune enhancing effect of D1701 may have application in immunotherapy. ORFV has been shown to mediate anti-viral activity and is effective in mice infected with herpes simplex virus type 1, in a guinea pig model of recurrent genital herpes disease and in a transgenic mouse model of human HBV replication, without any signs of inflammation or other side effects [210].

The potential and development of parapoxviruses as recombinant vaccines in permissive and non-permissive hosts has been described [1, 211–215]. The use of parapoxviruses in permissive hosts may have advantages over other recombinant viral vaccines since parapoxviruses only cause localised skin lesions that resolve within weeks and they do not cause systemic infection [1]. In addition, ORFV represents a promising candidate as a novel vaccine vector due to its immunomodulating properties even in non-permissive hosts [211]. Recombinant parapoxvirus vectors have been shown to induce protective immunity against the lethal alphaherpes virus of swine and pseudorabies virus in a non-permissive mouse model [215]. $ORFV_{D1701}$

recombinants have been produced that express the glycoproteins gC and gD of pseudorabies virus. The study demonstrated the potential of the parapoxvirus recombinant vector vaccines to efficiently prime both protective humoral and cell-mediated immune mechanisms in a non-permissive host species for the virus. In a further study, intramuscular injection of recombinant $ORFV_{D1701}$ expressing the nucleoprotein p40 of Borna disease virus was shown to protect rats against Borna disease virus infection of the brain [211]. The results of investigations thus far suggest that recombinant parapoxviruses have exciting potential as new vaccine vector.

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