INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book. These are also available as one exposure on a standard 35mm slide or as a 17" x 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9000710

Molecular and biological characterization of deletion mutants of vaccinia virus

Lai, Alexander Chi-Keung, Ph.D.

City University of New York, 1989

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Molecular and biological characterization of deletion mutants of vaccinia virus

by

Alexander Chi-Keung Lai

A dissertation presented to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1989

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in the satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/24/89.
Date

Dr. Beatriz G.-T. Pogo
(Chairperson of Examining Committee)

4/25/89
Date

Dr. Terry Krulwich
(Executive Officer)

Dr. Constantin C. Bona

Dr. Jerome L. Schulman

Dr. Peter Palese

Dr. Enzo Paoletti

(Supervisory Committee)

The City University of New York

Abstract

Molecular and biological characterization

of deletion mutants of vaccinia virus

by

Alexander Chi-Keung Lai

Advisor: Dr. Beatriz G.-T. Pogo

Although smallpox has been eradicated, studies with vaccinia virus continue, due to the recent advance in vaccinia recombinants. However, before these vaccinia-based vectors are used as human vaccines, a better understanding of: a) the molecular basis of virulence; and b) the immune response during vaccinia virus infection is required.

Several deletion mutants of vaccinia virus were generated from a persistently infected Friend erythroleukemia cell line. Repeated plaque-purification resulted in the isolation of two stable deletion mutants, designated Z4 and Z19. The deletion was mapped to the left terminal fragments HindIII C and N, and calculated to be 22 kb (or 12% of the genome) for Z19. The deletions extended into the tandem repeats (TR) of the inverted terminal repeats (ITR), and the difference in the size of deletions bewteen Z19 and Z4 is probably due to further deletion of sets of TR in Z19. Also, rearrangements in the right terminal fragment were found.

Phenotypic characterization of the deletion mutants revealed that they were similar to the wild-type (IHD-W strain) when compared in vitro, but different when compared in vivo. The deletion mutants were highly attenuated. Results from experiments of inoculation by different routes indicated that the LD_{50} of the mutants were at least 1000-fold higher than

the wild-type. Titration of tissue infectivity for mice inoculated with the wild-type or with the mutants viruses revealed that the mutants were unable to replicate in vivo. Southern blot hybridization of viral DNA with labeled cloned vaccinia growth factor (VGF) gene demonstrated that this gene is located in the unique region of HindIII C fragment of the wild-type, and hence the deletion mutants lack this gene due to the deletion.

The lack of ability to replicate in vivo may also be the basis for the less efficient induction of protective immunity by the mutants after 3 days or 4 weeks of primary immunization. However, after 7 and 10 days of immunization, 10^2 pfu of Z19 elicited protective immunity. Specific cytotoxic T cells (T-CTL) were induced by the wild-type after 7 days of immunization, but not by Z19 at both low (10^3 pfu) or high (10^6 pfu) inocula although protective immunity was elicited. Induction of delayed-type hypersensitivity (DTH) was readily demonstrated with both the wild-type and the mutant, suggesting that T-DTH may be associated with protection. These results also suggested that T-CTL plays a minor, if any, role in protection.

Another orthopoxvirus, Indiana virus, was found to have two copies of the VGF gene, one at each of the terminal fragments and to be more virulent than the wild-type IHD-W. Rescue of the deletion mutant Z19 using IHD-W wild-type HindIII C fragment resulted in isolation of recombinant viruses with the VGF gene. The partial recovery of virulence of these recombinants, and the results obtained with Indiana, strongly indicated that the expression of the VGF gene is correlated with virulence.

The significance of this study is that it provides information for the design of a safe vaccinia-based recombinant (more attenuated), and of a better protocol for immunization.

Aknowledgements

This dissertation could not have been completed without the constant encouragement and support of many people. This is especially true for me since I am a foreign student, my family and most of my friends are far away.

I am especially indebted to Dr. K-P Wong, who encouraged me to stay in this country and to pursue graduate training.

I would like to thank Dr. Peter Palese, who gave me the opportunity to enroll in his department, even though I was one year late, and as an examining member for this dissertation.

I would also like to thank Dr. Jerome Schulman and Dr. Constantin Bona, who not only taught me immunology, but also served in my advisory committee.

I am grateful to Dr. Enzo Paoletti, not only served in my examining committee, but also gave me the cloned fragments and made this dissertation research possible.

Of course, I owe so much to Dr. Beatriz Pogo, who not only gave me the opportunity to work in her laboratory, but also taught me what science is about.

I would also thank Dr. Jim Cox and Mrs. Betty Cox for their 'mid-western hospitality' and many thoughtful discussions; and to Dr. Bert Johansson, for giving me a guiding hand all along.

Last, but not the least, I am indebted to my parents whose constant support and understanding (that I have to defer some of the family commitments) made this dissertation possible.

Format of thesis

This thesis is written in accordance with the new guidelines of the City University of New York which permitted direct incorporation of published research articles as chapters. This thesis has a general introduction, followed by three chapters, two of which are published papers and one is a manuscript in preparation. Chapter 5 includes results not yet published in any of the previous chapters. Each chapter has its own introduction, results, discussion, materials and method, and references. A general discussion is provided in chapter 6.

Table of Contents

List of tables

List of figures

Chapter 1

Introduction

I. Introduction

Vaccinia virus, the best studied member of the poxviruses, is unique in human history, since it had been used as a safe and effective vaccine to eradicate smallpox. Ancient Chinese used scab material from smallpox patients and inoculated naive recipients to induce protection. Although this practice of variolation resulted in 1/10 case fatality rate of the naturally acquired disease, it caused about 1% mortality in the recipients. Also, the recipients were capable of spreading the virus and could start an epidemic in a previously uninfected community. It was Jenner who first demonstrated that inoculation of material from persons infected with cowpox, which caused a mild disease, resulted in protection against smallpox (Jenner 1798). In fact, it was Pasteur (1881) who used the term 'vaccination' to denote preventive inoculation as a way to induce a resistant state to other pathogens.

Smallpox has now been eradicated (W.H.O. 1980), and therefore, vaccinia is the first agent to date to have been used to eradicate a human disease.

The demonstration of genetic recombination in poxviruses by Fenner and Comben (1958, Fenner 1959) and later by Sam and Dumbell (1981) and Nakano et al (1982) paved the way for the construction of vaccinia recombinants in which foreign DNAs are inserted into vaccinia genome. These recombinants have been used as eukaryotic expression vectors and possibly as candidates for human vaccines. Research in vaccinia virus has been since then re-invigorated, and in Fenner's word: 'resurrected' (Fenner 1985).

II. Classification

The poxviruses, of which vaccinia is a prototype virus, is a large group of viruses that infects both the vertebrates and invertebrates.

Earlier classification schemes were based on symptomatology. Molluscum contagiosum and myxoma viruses were grouped into a different genus from that of other poxviruses by Holmes (1948) in the sixth edition of Bergey's Manual of Determinative Bacteriology. However, histopathology of poxvirus-infected tissues demonstrated distinctive inclusion bodies, which led Goodpasture (1933) to propose the grouping of vaccinia-variola, fowlpox, horsepox, sheep-pox, goat-pox, swine-pox and molluscum contagiosum viruses into the same genus. It was Fenner and Burnet's short description of poxvirus at the Sixth International Congress for Microbiology that provided the basis of subsequent classification with respect to the criteria used and subdivision adopted (Fenner and Burnet 1957).

The current classification is based on the proposal by the International Committee on Taxonomy of Viruses (Matthews 1982). Poxvirudae are divided into two families: the Chordopoxviruses (poxviruses of vertebrate) and Entomopoxviruses (insect poxviruses). The criteria for classification of the poxvirudae include cytoplasmic site of replication, genome of large double-stranded DNA and large oval-shaped virions. The vertebrate poxviruses all share a group-specific nucleoprotein (NP) antigen (Takahashi et al 1959; Woodroofe and Fenner 1962) and are able to rescue other heat-inactivated member by non-genetic recombination (to be described later) (Fenner and Woodroofe 1960; Hanafusa et al 1959). The members of the same genus usually are more related antigenically, have similar morphology and host range, and as added recently, have similar restriction patterns (Esposito et al 1978; Mackett and Archard 1979; Schnitzlain et al 1988) and DNA homology (Black et al 1986).

III. General characteristics

A. Morphology

The poxviruses are large brick-shaped particles 200 to 400 nm long with axial ratios of 1.2 to 1.7, and are surrounded by a lipid membrane. They are the largest of animal viruses. Although they could be seen by light microscopy, detailed morphology was not well characterized until the introduction of electron microscopy (EM). Green et al (1942) observed a dense internal structure of the unstained elementary bodies (EB). Peters employed metal- shadowing and mild enzymatic treatments which allowed observation for the first time many of the subviral components (Peters 1956). Negative staining revealed that the virions are covered by tubular structures (Gold and Dales 1968). Thin sections show that the virion consists of a biconcave core which contains the viral genome, two lateral bodies and an outer membrane (Pogo and Dales 1969). Virions released spontaneosly from cells also contain an outer envelope which is of host cell origin, however, it also contains viral polypeptides (Payne and Norrby 1976).

B. Virion composition

The principle components of vaccinia virus virions are protein, lipid and DNA comprising 90%, 5% and 3.2% of the dry weight, repectively (Zwartouw 1964). Traces of RNA may be detected (Joklik 1962). Because of the cytoplasmic site of replication (Dales and Pogo 1981), vaccinia virus carry enzymes to initiate its replication. There are more than 100 structural polypeptides (Essani and Dales 1979) constituting the virions, a number of which are identified as virion associated enzymes (Moss 1986). In fact, a DNA-dependent RNA polymerase was the first enzyme of this kind identified in viral cores by Kates and McAuslan (1967). These enzymes can complement

other members of the group and reactivate co-infected heat-inactivated poxvirus (Fenner and Woodroofe 1960). Since no DNA recombination takes place (Joklik et al 1960), this phenomenon was termed non-genetic reactivation.

C. DNA genome

i. Structure

The genome of vaccinia is linear double-stranded DNA of about 187 kb. The terminii contain unpaired hairpin loops of 104 nucleotides (Baroudy et al 1982), thus the two strands of the DNA are covalently linked (Geshelin and Berns 1974). Sequence data from Baroudy et al (1982) indicated that the loops exist in two forms, and probably flip-flopped during DNA replication. There are two inverted terminal repeats (ITR) of variable size (Garon et al 1978, Wittek et al 1978, Mackett and Archard 1979). Within the ITR, at the terminal 3 kb non-transcribed region, there are sets of tandem repeats of 70 and 125 base-pairs (bp) which are interrupted by spacers (Wittek and Moss 1980). The tandem repeats allowed unequal cross-over (Baroudy and Moss 1982) and may be the basis of the instability and heterogeneity of the vaccinia genome (Moss et al 1981a). Similar DNA structures have recently been found in raccoonpox virus (Parsons and Pickup 1987) and in fowlpox virus (Campbell et al 1989).

Restriction analysis and DNA homology studies (Mackett and Archard 1979; Black et al,1986; Muller et al 1977) indicated that the central part of the poxvirus genomes is highly conserved, suggesting that this region is critical for functions that are essential for viral replication. Physical mapping of temperature-sensitive mutants further support this view (Thompson and Condit 1986, Drillien and Spehner 1983). By contrast, the terminal portions of the genome are more variable (Mackett and Archard 1979). These

regions contain genes coding for non-essential functions, host range and pathogenicity.

ii. Genetic map

A restriction map of the WR strain was constructed by DeFilippes (1982). The use of a variety of mutants, either in temperature-sensitivity, drug resistance, host range or deletions have helped to mapped many loci. A detailed genetic map of vaccinia has been provided recently by Earl and Moss (1987). Cloning of the various fragments (Niles et al 1986, Rosel et al 1986), hybrid arrest (Venkatesan et al 1982) and sequencing resulted in accumulation of DNA sequence data. This approach also lead to the discovery of a vaccinia growth factor (VGF) gene which has a domain with 35% aminoacid identity to human epidermal growth factor (EGF) (Reisner 1985, Brown et al 1985, Twardzik et al 1985, Stroobant et al 1985). Similar genes were also found in Shope fibroma virus (Chang et al 1987), in myxoma virus (Upton et al 1987) and in Molluscum contagiosum virus (Porter and Archard 1987). More recently, sequences homologous to the platelet-derived growth factor (PDGF) gene has been identified in fowlpoxvirus (Campbell et al 1989). Coding sequences similar to other proteins such as a complement C4b binding protein (Kotwal and Moss 1988a), and two plasma serine protease inhibitors (Kotwal and Moss 1989) were also identified by comparing DNA sequences with data banks.

IV. Replication

The replication of poxviruses takes place exclusively within the host cytoplasm (Dales and Pogo 1981). Involvement of the host nucleus is controversial, as recently reviewed by Moyer (1987). Prescott et al (1971)

demonstrated the occurrence of vaccinia DNA replication in enucleated mouse L cells, however, Penington and Follett (1974) showed that no infectious progeny resulted from enucleated BSC-40 cells.

Hruby et al (1979) observed that although early functions of vaccinia virus were not affected by ultra-violet (uv) irradiation and alpha-amanitin treatment of BSC-40 cells, assembly of progeny virions was suppressed. The authors suggested that there was active involvement of host transcription apparatus for viral replication. Experiments performed by Silver et al (1979), however, indicated that only host nuclear DNA-dependent RNA polymerase (Pol II) was required. This interpretation has recently been further supported by the observation that viral replication depended on the relative availability of Pol II in different types of cells (Wilton and Dales 1989).

Recently, Moyer's group demonstrated that during vaccinia replication, a large subunit of cellular Pol II (Morrison and Moyer 1986) as well as a lamin-like protein (Bloom et al 1989) were recruited from the host cell nucleus to the virosomes where replication of the virus occurred. The significance of these findings is not clear at the moment, since other cell components can be found in purified viruses (Franke and Hruby 1987, Obom et al 1988). However, current evidence indicates that some host functions are required for viral replication during late stages.

Vaccinia can infect a large range of cells. The isolation of host range mutants (Drillien et al 1981) and the use of specific fragments in marker rescue experiments helped to identify DNA sequences required for the virus to infect human cells (Gillard et al 1985, Gillard et al 1986). Infection of vaccinia in macrophages had been shown to be abortive, although certain viral polypeptides were found present on the host membrane (Natuk and Holowczak

1985). In our laboratory, it was found that the abortive infection of hemopoietic cell lines such as HL60 can be reversed by differentiation (Pogo et al 1988), implying that factors present in differentiated cells are required for replication. Although there was evidence of early viral transcription in untreated infected cells, no viral polypeptides were detected (Pogo et al 1988).

A. Adsorption and penetration

It was generally acccepted that vaccinia virus enters host cell by 'viropexis' or phagocytosis (Dales 1965). However, recent studies suggested that fusion events might also be responsible for viral penetration. The rapid dispersion of viral proteins into host cell membrane soon after infection suggested a fusion event (Chang and Metz 1976). Indeed, a 14 kDa virion polypeptide has been identified as a fusion protein (Rodriguez et al 1987), although monoclonal antibodies (Mab) to this protein did not neutralize virus infectivity. Mab to epidermal growth factor (EGF) receptors (Marsh and Eppstein 1987) or presence of free EGF (Eppstein et al 1985) decreased the yield of progeny virus suggesting that EGF receptors may be a portal of entry for the virus. Therefore, viral entry could be via several mechanisms.

B. Uncoating

The uncoating of poxviruses has been studied by Dales (1963) and Joklik (1964a, 1964b). Following entry of the virion into host cytoplasm, the lipid membrane is released leaving the nake core. The first stage of uncoating involves the rapid hydrolysis of some virion proteins and viral phopholipids. The input genome is still DNase resistant. Early viral transcription occurs and an 'uncoating protein' is made. This uncoating protein can mediate non-genetic reactivation (Pedley and Cooper 1987). Whether input viral DNA is

released from the core remains uncertain. The DNase sensitivity of input DNA (Joklik 1962, 1964a) suggested that the viral DNA dissociated from the core early in infection. However, Dahl and Kates (1970a, 1970b) showed that viral DNA could be protected from DNase, and suggested that the parental DNA associated with cellular components. More recently, Zaslavsky (1985) demonstrated that the core polypeptides were associated with input DNA in the cytoplasmic structures of infected cells, and that both transcription and DNA replication took place in these structures, suggesting that viral DNA was not released from the virion during uncoating.

C. Transcription and translation

The transcription of vaccinia DNA is temporally regulated. That is, it can be divided into immediate early, early, delayed early, and after DNA replication, late transcription. About half of the genome is transcribed before DNA replication and classified as early genes. They are distributed throughout the genome (Belle et al 1981). Transcription and translation mapping of the HindIII D fragment indicated that both early and late genes are present and are closely packed. Potential interference of expression of these closely spaced genes is minimized by intercalation of the early and late genes, and by the difference in direction of transcription (Lee-Chen and Niles 1988).

The transcripts are capped (Venkatesan and Moss 1981), polyadenylated (Nevins and Joklik 1975) and there is no evidence for splicing (Wittek and Moss 1982). Early genes have AT rich regions upstream of the coding sequences, and using a transient functional assay, Yuen and Moss (1987) found a TTTTT(N)T terminator motif downstream. Sequence analysis of late genes indicated a conserved TAAATG sequence at the transcription and translation initiation site (Rosel et al 1986). Therefore, it appears that the temporal

regulation of vaccinia transcription utilize different regulatory sequences.

Late transcripts are synthesized discontinously (Bertholet et al 1987) and contain a 5'poly(A) leader sequence which is not encoded in the genome (Schwer et al 1987, Patel and Pickup 1987). The poly(A) head perhaps enhances stability of these late messages. Interestingly, in vitro transcription of late genes also contained the 5'poly(A) head (Wright and Moss 1987, Schwer and Stunnenberg 1988), suggesting a 'slippage' model of the RNA polymerase.

Although vaccinia virus uses the same translation apparatus as the host, host protein synthesis is shutoff early in infection. The selective inhibition of host protein synthesis has been shown to be by the virus-core-synthesized poly (riboadenylic acids) (Bablanian et al 1987).

D. DNA replication

The replication of viral DNA occurs between 2 and 5 h after infection and is completed before mature progeny virus is formed. Based on EM observations of ds DNA loops at one end, Esteban et al (1977) proposed a model of replication in which DNA replication initiated at one terminus by RNA primers which were probably synthesized by a viral DNA-dependent RNA polymerase. DNA synthesis proceeded until both parental strands were replicated and the terminal cross-links formed after completion of DNA replication.

Pogo et al (1981, 1984) had shown that initiation and termination sites were located at both ends, these results were compatible with the model proposed by Moyer and Graves (1981) in which the terminal hairpin loops were utilized as self primers for DNA replication. This model also can explain flip-floping of the hairpin sequence (Baroudy et al 1982). Detection of

concatameric terminal fragments and resolution into mature viral terminii have recently been shown (Merchlinsky and Moss 1986, 1989). The presence of head to tail concatamers suggested that recombinational events ocurred during DNA replication (Merchlinsky and Moss 1989).

E. Assembly and release

The assembly process followed by EM indicated that the lipid membrane was first assembled, then DNA and late proteins were inserted to form the core and lateral bodies. The process appears to be dependent on the rigidity of the membrane, which in turn was provided by spicules (Nagayama et al 1970). The spicules were later replaced by surface tubular elements (STE) (Stern and Dales 1974, 1976).

Assembly of viral structural proteins depends on late gene sequences, as demonstrated recently by Huang et al (1988) who showed that fusion of 11 kDa late gene 5' to E. coli LacZ gene resulted in incorporation of the fusion protein into virions. Beta-galactosidase activity was easily assayed with purified virions. Removal of the vaccinia sequence resulted in non-incorporation. However, other cellular components may be packaged non-specifically (Franke and Hruby 1987, Obom et al 1988).

EM work also revealed that release of mature virions take place by a mechanism of budding (Tsutsui 1983). Only 1 to 10% of the progeny virus is released (Appleyad et al 1971), acquiring an extra envelope from host cell. The extracellular virus had been shown to penetrate and disseminated better into new host cells (Payne and Norrby 1978, Payne 1980).

F. Inhibitors of replication

The replication cycle of vaccinia is complex, therefore, there are a few inhibitors with activity at different stage of the replication cycle.

Hydroxyurea (HU) inhibits DNA replication, leading to the accumulation of early transcripts. Overproduction of viral encoded ribonucleotide reductase conferred HU resistance (Slabaugh and Mathews 1986). Rifampicin is a specific inhibitor for viral assembly (Moss et al 1969) and the gene for rifampicin-resistance has been mapped to the HindIII D fragment (Tartaglia and Paoletti 1985, Baldick and Moss 1987) which encodes a late gene product of 63 kDa (Tartaglia et al 1986). Detection of a phosphonoacetate-resistant enzyme provided evidence for a viral encoded DNA polymerase (Moss and Cooper 1982) and the locus had been mapped to the HindIII E fragment (Jones and Moss 1984, Traktman et al 1984).

Replication of vaccinia virus in L cells is resistant to interferon (IFN), however, the treatment resulted in defective progeny viruses which have decreased phosphorylation of the core polypeptides (Esteban 1984). Although high level of 2'-5' linked adenylate oligomers (2-5 A) was detected in IFN treated infected cells in the absence of other inhibitors, viral replication was not affected (Rice et al 1984). Specific kinase inhibitory factors had been shown to be induced by vaccinia virus which specifically interferes with the IFN-induced ds RNA-dependent protein kinase (Whitaker-Dowling and Youngner 1983).

V. Pathogenicity and Immunogenicity

A. Cytolytic poxviruses

The most virulent of poxviruses is of course the smallpox virus. An excellent description of the clinical course of the disease was recently published by Fenner et al (1988). Other poxvirus infections such as monkeypox (Ladnyi et al 1972) and parapoxvirus such as orf virus (Robinson and Peterson

1983) have occasionally been reported to infect humans.

Although vaccinia is considered non-pathogenic for humans, the high complication rate reported in immunocompromised vaccinees (Fulginiti et al 1968) and the recent case of generalized vaccinia in a patient with the human immunuodificiency disease virus (HIV) (Redfield et al 1987), have changed this perception.

B. Tumorigenic poxviruses

i. in vivo

All poxviruses (including vaccinia) induce an initial hyperplasia response in early infection in vivo, which has been shown to be due to the expression of the VGF gene (Buller et al 1988b). Molluscum contagiosum causes benign tumors in human and has not been cultured in vitro. Yaba monkey tumor poxvirus and Shope fibroma virus induce benign tumors in their respective hosts. The weak tumorigenic nature of Shope fibroma virus was illustrated by the fact that SFV produces malignant disease in neonates or in adult rabbits treated with immunosuppressive drugs (Smith et al 1973, Allison and Friedman 1966), whereas only benign, transient tumors are induced in immunocompetent adult rabbits (Shope 1932).

ii. in vitro

The transformation of mouse embryo cells by vaccinia virus has been reported (Koziorowska et al 1971). Yaba monkey virus (Rouhandeh and Vafai 1982) and Molluscum contagiosum (Barbanti-Brodano et al 1974) can transform cells in vitro. Obom and Pogo (1988) recently showed that infection with uv-inactivated Shope fibroma virus in vitro resulted in a transient transformation, as the malignant phenotype of the transformed cell was lost after serial passages in vitro. Some of the observations described above can

be explained by the expression of the viral growth factor.

C. Mechanism of pathogenicity

Most of the studies on poxvirus pathogenicity were performed with ectromelia (mousepox)(Fenner 1948). The attenuated phenotype of vaccinia recombinants (Buller et al 1985) and the recent demonstration of correlation between mutation of the VGF gene and attenuation (Buller et al 1988a) suggested that an understanding of the molecular basis of virulence is possible.

D. Adaptation of poxvirus

The introduction of myxoma virus, a highly virulent poxvirus in rabbits, into the Australian bush to eliminate wild rabbits illustrated the rapid evolution of both the virus and the host (Mykytowycz 1953, Marshall and Fenner 1958, 1960, Fenner and woodroofe 1965). The appearance of myxoma viruses less virulent in laboratory rabbits, and the emergence of rabbits resistant to the virus made this kind of biological control less desirable.

Earlier workers used to passage vaccinia virus in different tissues or cell cultures to select for more 'mild' strains to be used as vaccine. A much attenuated variant, LC16m8, was selected by Hashizume et al (1985) from the Lister (Elstree) strain. No severe complications were observed in a field trial of more than 100,000 children in Japan, as oppose to its parental Lister strain [approximately 10-20 post-vaccinal encephalitis per million (Arita and Fenner, 1985)]. However, the eradication of smallpox and the cessation of vaccination halted further studies of this vaccine strain.

E. Immunogenicity

All members of the orthopox genus share a common nucleoprotein (NP) antigen (Takahashi et al 1959; Woodroofe and Fenner 1962), and that was the

basis of cross protection by vaccinia against variola. However, not all strains of vaccinia induced the same degree of immunity. CV-1, developed by River and Ward (1933) was less virulent, however, it was less potent (McIntosh 1985), as compared to the Lister or Copenhagen strains which were incidently more virulent. Since uv-inactivated viruses had been shown to be less effective to elicit protective immunity (Boulter et al 1971), therefore, live viruses have to be used to induce protective immunity.

An immunodominant antigen Ag35 identified by Gordon et al (1988) has a domain highly homologous to the G glycoprotein of respiratory syncytial virus (RSV). The significance of this finding has not been delineated. Maa et al (1987) have identified another structural 39 kDa immunodominant protein, however, Mab to it did not neutralize virus infectivity.

VI. Recombination

A. Homologous recombinations

Experiments by Ball (1987) indicated that poxviruses have high frequency of homologous recombination. Evans et al (1988) showed that in poxvirus infected cells, high frequency of recombination could be induced to heterologous DNA in a trans-acting manner, suggesting a putative recombinase with trans-activity. The cytoplasmic mode of replication and the easiness by which heterologous gene(s) can be inserted by homologous recombination (Panicali and Paoletti 1982), made vaccinia virus a useful vector.

B. Recombinants

i. Construction

Most of the vaccinia recombinants have been constructed utilizing the thymidine kinase (TK) gene as an insertion site. A general scheme

(Mackett et al 1982) is described below. Foreign gene(s) with complete open reading frame (ORF) are first ligated downstream of a vaccinia promoter. The construct is then inserted into a cloned HIndIII J fragment which contains the TK gene. The plasmid is then transfected into cell culture infected with wild-type vaccinia. When homologous recombination occurs, the TK gene of the wild-type is interrupted and isolation of the recombinants is carried out by selection of TK- virus. Chakrabarti et al (1985) and Panicali et al (1986) incorporated beta-galatosidase gene in the TK vector, so that the recombinants were easily isolated by the blue color of the plaques.

Other loci, such as the HindIII F fragment (Panicali and Paoletti 1982), the hemagglutinin in HindIII A fragment (Siomi et al 1988), and the locus determining plaque size (Rodriguez and Esteban 1989) had been used. Recently, fowlpox recombinants had been constructed and used as poultry vaccines (Boyle and Coupar 1988).

ii. Expression vectors

Most recombinants were constructed to be used as expression vectors, that is, to elicit immune response to the recombinant expressing the heterologous antigen. Excellent reviews had been published by Mackett and Smith (1986), Piccini et al (1987), Moss and Flexner (1987). It is worth mentioning the susscessful vaccination against rabies by oral administration (by bate) with vectors expressing the rabies glycoprotein (Tolson et al 1988, Blancou et al 1986) in foxes, and in raccoons (Rupprecht 1986). More exciting, a vector expressing an oncogene has been shown to elicit protective immunity against the tumor (Bernards et al 1987).

Not all recombinants are immunogenic. A recombinant expressing feline leukemia virus env gene (gp70) was found to be non-immunogenic (Gilbert et

al 1987), the lack of anti-gp70 antbodies was specific, since neutralizing antibodies to vaccinia virus was detected in the inoculated mice. Similarly, a vaccinia recombinant expressing dengue fever viral polypeptide was found to be weakly immunogenic (Zhao et al 1987).

Vaccinia recombinants were also used as a tool to study other biological functions. For example, Thomas et al (1986) and Edwards and Rutter (1988) used a vector to study the processing of a neuropeptide; Coupar et al (1986) studied cytotoxic T-cell reponse to influenza virus and Smith et al (1987) studied the synthesis and cellular location of influenza viral polypeptides in cells infected with recombinants virus; Rodriguez et al (1988) used a vector expressing luciferase to follow the dissemination of vaccinia virus in the host during an infection; Gowda et al (1989) and Flexner et al (1988) studied the processing of gag and pol genes of the human immunodificiency virus type 1 (HIV-1); Falkner et al (1988) studied the synthesis, intracellular location and activities of the trans-activation protein of HIV expressed in vaccinia recombinants.

In general, the heterologous protein expressed in these recombinants behaved as the native protein. This approach is particular useful when the protein being studied is rare, or the protein is from a hazardous pathogen such as HIV or dengue fever virus.

VII. **Deletion mutants**

Several deletion mutants of vaccinia have been previously described. A host range mutant was isolated by Drillien et al (1981) after nitrous acid mutagenesis of the parental Copenhagen strain. This mutant had lost its ability to replicate in human cell lines. By using marker rescue technique, the

same authors subsequently located a short region in the HindIII M and N fragments which is responsible for replication in human cells (Gillard et al 1986).

Panicali et al (1981) noticed that in their serially propagated wild-type stock of the WR strain of vaccinia virus, submolar quantities of certain restriction fragments were detected. On repeated plaque-purification, they isolated two 'variants', a large DNA variant (L) and a small DNA variant (S). Restriction analysis revealed that the S-variant had a deletion of 6.3 MDa. The deletion was mapped to a site 6.8 MDa from the left terminus, just beyond the ITR. The additional DNA in the L-variant was found to be unique viral sequences which were transcribed in vitro and in vivo. In spite of the large difference in their genome, there were no major difference in the phenotype of the variants. A similar heterogeneity of the left terminal fragment was observed by Moss et al (1981a) in their wild-type stock of WR strain. However, these variants with the shortened terminal fragments were unstable since about 20% of them reverted back to the longer and stable form just after one round of plaque-purification. Even after repeated plaque-purification, these variants still gave an array of terminal fragments with differences in length of 1.65 kb increments, probably due to variations in the number of copies of a reiterated sequence. Restriction analysis of the stable variants showed that there was also a deletion of 9000 bp in these variants. The deletion was also mapped just beyond the terminal repetition in the left terminus (Moss et al 1981b). In fact, the deletion might be similar to, if not exactly, to the variant of Panicali et al (1981).

Kotwal and Moss (1988b) have recently shown that the deletion of the short variant designated 6/2 was actually more than 9 kb, because part of the

right terminal fragment was translocated to the left. Since the mutant has a ITR of 12 kb, a model in which the remaining of the HindIII C fragment in the mutant was from the HindIII B fragment (10 kb of the ITR plus 2 kb unique sequence) was proposed by the authors.

In a Friend erythroleukemia cell line persistently infected with vaccinia virus (Pogo and Friend 1982), we have reported the isolation of deletion mutants of vaccinia, of the IHD-W strain (Lai and Pogo 1989). Using a similar cell system but a different strain of vaccinia (WR), Paez et al (1985) previously reported the isolation of vaccinia mutants with a 8 MDa deletion at the left terminus. The generation of these deletion mutants seemed to be regulated by interferon, although the precise mechanism remains to be determined (Paez and Esteban 1985).

The mechanism by which deletion mutants of vaccinia were generated is unknown. However, they could be useful for genetic mapping, such as the host range mutant of Drillien et al (1981), and for the construction of expression vectors (Panicali and Paoletti 1982, Rodriguez et al 1989) In fact, double deletions have been constructed from the S-variant by Perkus et al (1986) in which the DNA was manipulated in vitro first, then by in vivo recombination. Thus a larger deletion of 'non-essential' regions was created. This facilitate insertion of more foreign genes in the construction of expression vectors.

VIII. **Goal of research**

The molecular basis of virulence of poxvirus infection is poorly understood. Since the deletion mutants isolated in our laboratory are highly attenuated, they provide a good system to study the molecular basis of

virulence of vaccinia virus.

Since more than 21 kb of DNA of the mutants had been deleted, a number of genes must have been affected. A comparison of the deletion mutants published so far (Fig. 1, chapter 6) suggested that gene(s) residing in the region where the deletion occured is(are) important for virulence.

The goal of this dissertation research was first to identify which gene(s) is/are missing in these mutants, and to investigate what effects the lack of these genes have on the mutants. And finally, to test which gene play an important role in virulence by insertion of the test gene into the mutants by marker rescue technique or by site specific homologous recombination.

These deletion mutants are also less immunogenic than the wild-type, thus providing a system to study the immune response during poxvirus infection. Utilizing the mutants as a tool, the immune response could be dissected to assess their relative importance in protection. Understanding of these responses will facilitate the formulation of strategies for immunization with vaccinia-based recombinants.

It is expected that the results from this study will provide significant information for the construction of a more attenuated vaccinia vector (thus a safe vaccine) and a better protocol for immunization.

Reference

Allison AC, Friedman RM. (1966) Effect of immunosuppression on Shope rabbit fibroma. J Nat Cancer Inst 36: 859-868.

Appleyard G, Hapel A, Boulter EA. (1971) An antigenic difference between intracellular and extracellular rabbitpox virus. J Gen Virol 13:9-17.

Arita I, Fenner F. (1985) Complications of smallpox vaccination. In: Vaccinia viruses as vectors for vaccine antigen. Ed. Quinnan, Jr. GV. Elsevier, New York. p. 49-60.

Bablanian R, Goswami SK, Esteban M, Banerjee AK. (1987) Selective inhibition of protein synthesis by synthetic and vaccinia virus-core synthesized poly(riboadenylic acids). Virology. 61:366-73.

Baldick CJ Jr, Moss B.(1987) Resistance of vaccinia virus to rifampicin conferred by a single nucleotide substitution near the predicted NH2 terminus of a gene encoding an Mr 62,000 polypeptide. Virology.156:138-45.

Ball LA.(1987) High-Frequency homologous recombination in vaccinia virus DNA. J Virol 61:1788-95.

Barbanti-Brodano G, Mannini-Palenzona A, Vardi O, Portolani M, LaPlaca M. (1974) Abortive infection and transformation of human embryo fibroblasts by Molluscum contagiosum virus. J Gen Virol 24:237-246.

Baroudy BM, Moss B.(1982) Sequence homologies of diverse length tandem repetitions near ends of vaccinia virus genome suggest unequal crossing over. Nucleic Acids Res 10:5673-5679.

Baroudy BM, Venkatesan S, Moss B.(1982) Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell 28:315-324.

Belle IH, Venkatesan S, Moss B. (1981) Cell-free translation of early and late mRNAs selected by hybridization to cloned DNA fragments derived from the left 14 million to 72 million daltons of the vaccinia virus genome. Virology 112:306-317.

Bernards R, Destree A, McKenzie S, Gordon E, Weinberg RA, Panicali D.(1987) Effective tumor immunotherapy directed against an oncogene-encoded product using a vaccinia virus vector. Proc Natl Acad Sci USA 84:6854-6858.

Bertholet C, Van Meir E, ten Heggeler Bordier B, Wittek R. (1987) Vaccinia virus produces late mRNAs by discontinuous synthesis. Cell.50:153-62.

Black DN, Hammond JM, Kitching RP (1986) Genomic relationship between capripoxviruses. Virus Res. 5:277-292.

Blancou J, Kieny MP, Lathe R, Lecocq JP, Pastoret PP, Soulebot JP, Desmettre P.(1986) Oral vaccination of the fox against rabies using a live

recombinant vaccinia virus. Nature. 322:373-5.

Bloom DC, Massung R, Savage L, Morrison DK, Moyer RW.(1989) Recruitment to the cytoplasm of a cellular lamin-like protein from the nucleus during a poxvirus infection. Virology 169:115-126.

Boulter EA, Zwartouw HT, Titmuss DHJ, Maber HB.(1971) The nature of the immune state produced by inactivated vaccinia virus in rabbits. American J Epidemiology 94:612-20.

Boyle DB, Coupar BE.(1988) Construction of recombinant fowlpox viruses as vectors for poultry vaccines. Virus Res. 10:343-56.

Brown JP, Twardzik DR, Marquardt H, Todaro GJ (1985). Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming growth factor. Nature 313:491-492.

Buller RML, Smith GL, Cremer K, Notkins AL, Moss B.(1985) Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinas-negative phenotype. Nature 317:813-5.

Buller RML, Chakrabarti S, Cooper JA, Twardzik DR.,Moss B.(1988a) Deletion of the vaccinia virus growth factor gene reduces virus virulence. J Virol 62:866-874.

Buller RML, Chakrabarti S, Moss B, Fredrickson T.(1988b) Cell proliferative response to vaccinia virus is mediated by VGF. Virology 164:182-92.

Campbell JIA, Binns MM, Tomley FM. Boursnell MEG (1989) Tandem repeat sequences within the terminal region of the fowlpox virus genome. J Gen Virol 70:145-154.

Chakrabarti S, Brechling K, Moss B.(1985) Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. Mol Cell Biol 5:3403-3409.

Chang A, Metz DH.(1976) Further investigation on the mode of entry of vaccinia virus into cells. J Gen Virol 32:275-82.

Chang W, Upton C, Hu SL, Purchio AF, McFadden G.(1987). The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and transforming growth factor alpha. Mol Cell Biol 7:535-540.

Coupar BE, Andrew ME, Both GW, Boyle DB.(1986) Temporal regulation of influenza hemagglutinin expression in vaccinia virus recombinants and effects on the immune response.
Eur J Immunol 16:1479-1487.

Dahl R, Kates JR. (1970a) Intracellular structures containing vaccinia DNA: isolation and characterization. Virology 42:453-462.

Dahl R, Kates JR. (1970b) Synthesis of vaccinia virus 'early' and 'late' messenger RNA in vitro with nucleoprotein structures isolated from infected cells. Virology 42:463-472.

Dales S. (1963) Uptake and development of vaccinia virus in strain L cells followed with labeled viral DNA. J Cell Biol 18:51-72.

Dales S. (1965) Penetration of animal viruses into cells. Progress in Medical Virology 7:1-43.

Dales, S. and Pogo, B.G.T. (1981). In: Biology of Poxviruses. Virology Monographs 18. Springer-Verlag, New York.

DeFilippes F.M. (1982) Restriction enzyme mapping of vaccinia virus DNA. J Virol 43:136-149.

Drillien R, Koehren F, Kirn A.(1981) Host range deletion mutant of vaccinia virus defective in human cells. Virology 111:488-499.

Drillien R, Spehner D.(1983) Physical mapping of vaccinia virus temperature-sensitive mutations. Virology 131:385-93.

Earl PL, Moss B.(1987) Vaccinia virus. In:Genetic maps 1987. A compilation of linkage and restriction maps of genetically studied organisms. Volume 4. Ed. O'Brien SJ. Cold Spring Harbor Press, Cold Spring Harbor. p.116-123.

Edwards RH, Rutter WJ. (1988) Use of vaccinia virus vectors to study protein processing in human disease : Normal nerve growth factor processing and secretion in cultured fibroblasts from patients with familial dysautonomia. J Clin Invest 82:44-47.

Eppstein DA, Marsh YV, Schreiber AB, Newman SR, Todaro GJ, Nestor JJ Jr. (1985) Epidermal growth factor receptor occupancy inhibits vaccinia virus infection. Nature 318:663-5.

Esposito JJ, Obijeski JF, Nakano JH. (1978) Orthopoxvirus DNA: Strain differentiation by electrophoresis of restriction endonuclease fragmented virion DNA. Virology 89:53-66.

Essani K, Dales S.(1979) Biogenesis of vaccinia virus:Evidence for more than 100 polypeptides. Virology 95:385-94.

Esteban M, Flores L, Holowczak JA. (1977) Model for vaccinia DNA replication. Virology 83:467-473.

Esteban M. (1984) Defective vaccinia virus particles in interferon-treated infected cells. Virology 133:220-227.

Evans DH, Stuart D, McFadden G.(1988) High levels of genetic recombination among cotransfected plasmid DNAs in poxvirus-infected mammalian cells. J Virol.62:367-75.

Falkner FG, Fuerst TR, Moss B. (1988) Use of vaccinia vectors to study the synthesis, intracellular localization, and action of the human immunodificiency virus trans-activator protein. Virology 164:450-457.

Fenner F. (1948) The pathogenesis of the acute exanthems; an interpretation based on experimental investigation with mousepox (infectious ectromelia of mice). Lancet ii:9915-920.

Fenner F, Burnet FM. (1957) A short description of the poxvirus group (vaccinia and related viruses). Virology 4:305-314.

Fenner F, Comben BM.(1958) Genetic studies with mammalian poxvirus. I. Demonstration of recombination between two strains of vaccinia virus. Virology 5:530-548.

Fenner F. (1959) Genetic studies with mammalian poxviruses. II. recombination between two strains of vaccinia virus in single HeLa cells. Virology 8:499-507.

Fenner F, Woodroofe GM. (1960) The reactivation of poxviruses. II. The range of reactivating viruses. Virology 11:185-201.

Fenner F, Woodroofe GM (1965) Changes in virulence and antigenic structure of strains of myxoma virus recovered from Australian wild rabbits between 1950 and 1964. Aust. J Exp Med Sci 43:359-370.

Fenner F. (1985) Vaccination: Its birth, death and ressurection. Eight Burnet Lecture of the Australian Academy of Science,1985. Aust. J Exp Med Sci 63:607-22.

Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. (1988) Smallpox and its eradication. World Health Organization,Geneva. Chapter 1,p 1-68.

Flexner C, Broyles SS, Earl P, Chakrabarti S, Moss B. (1988) Characterization of human immunodeficiency virus gag/pol gene products expressed by recombinant vaccinia viruses. Virology 166:339-49.

Franke CA, Hruby DE.(1987) Association of non-viral proteins with recombinant vaccinia virus virions. Arch Virol 94:347-351.

Fulginiti VA, Kempe CH, Hathaway WE, et al.(1968) Progressive vaccinia in immunological dificient individuals. In: Immunological dificient diseases in man. Ed. Bergsma D, Good RA. New York, National Foundation, 1968.

Garon CF, Barbosa E, Moss B. (1978) Visualization of an inverted terminal repetition in vaccinia virus DNA. Proc. Natl. Acad. Sci. USA 75:4863-4867.

Geshelin P, Berns KI. (1974) Characterization and localization of naturally occurring crosslinks in vaccinia virus DNA. J Mol Biol 88:785-796.

Gilbert JH, Pedersen NC, Nunberg JH (1987) Feline leukemia virus envelope protein expression encoded by a recombinant vaccinia virus: apparent lack

of immunogenicity in vaccinated animals. Virus Res 7:49-67.

Gillard S, Spehner D, Drillien R. (1985) Mapping of a vaccinia host range sequence by insertion into the viral thymidine kinase gene. J Virol. 53:316-8.

Gillard S, Spehner D, Drillien R, Kirn A. (1986) Localization and seuence of a vaccinia virus gene required for multiplication in human cells. Pro. Natl. Acad. Sci. USA 83:5573-5577.

Gold P, Dales S. (1968) Localization of nucleotide phosphohydrolase activity within vaccinia. Proc. Natl. Acad. Sci. USA 60:845-852.

Goodpasture EW.(1933) Borreliotoses: fowlpox, molluscum contagiosum, variola-vaccinia. Science 77:119-121.

Gordon J, Kovala T, Dales S. (1988) Molecular characterization of a prominant antigen of the vaccinia virus envelope. Virology. 167:361-9.

Gowda SD, Stein BS, Steimer KS, Engleman EG. (1989) Expression and processing of human immunodificiency virus type 1 gag and pol genes by cells infected with a recombinant vaccinia virus. J Virol. 63:1451-1454.

Green RH, Anderson TF, Smadel JE. (1942) Morphological structure of the virus of vaccinia. J Exp Med 75:651-655.

Hanufusa H, Hanafusa T, Kamahora J. (1959) Transforming phenomena in the pox group of viruses. II. Transformation between several members of group. Biken J:85-91.

Hashizume S, Yoshizawa H, Morita M, Suzuki K. (1985) Properties of attenuated mutant of vaccinia virus, LC16m8, derived from Lister strain. In: Vaccinia viruses as expression vectors for vaccine antigens. Ed. Quinan GV, Elsevier, New York, p.87-99.

Holmes FO. (1948) Filterable viruses; in Breed, Murray and Hitchens, Bergey's Manual of determinative bacteriolgy;6th ed. Williams and Wilkins, Baltimore.p.1127-1186.

Hruby D, Lynn D, Kates J. (1979) Vaccinia virus replication requires active participation of the host cell transcriptional apparatus. Proc Natl Acad Sci USA 76:1887-1890.

Huang C, Samsonoff WA, Grzelecki A. (1988) Vaccinia virus recombinants expressing an 11-Kilodalton beta-galactosidase fusion protein incorporate active beta-galactosidase in virus particles. J Virol 62:3855-3861.

Jenner E.(1798) An inquiry into the causes and effects of the variolae vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire and known by the name of the cow pox. London [Reprinted in Camac CNB ed. Classics of Medicine and Surgery. New York, Dover, 1959.]

Joklik WK, Abel P, Holmes IH. (1960) Reactivation of poxviruses by a non-genetic mechanism. Nature 186:992-993.

Joklik WK.(1962) Preparation and characterization of highly purified radioactively labeled poxvirus. Biochem. Biophysics Acta 61:290-301.

Joklik WK (1964a) The intracellular uncoating of poxvirus DNA. I. The fate of radioactively labeled rabbitpox virus. J Mol Biol 8:262-276.

Joklik WK.(1964b) The intracellular uncoating of poxvirus DNA. II. the molecular basis of the uncoating process. J Mol Biol 8:277-288.

Jones EV, Moss B.(1984) Mapping of vaccinia virus DNA polymerase gene by marker rescue and cell-free translation of selected RNA. J Virol 53:312-315.

Kates JR, McAuslan BR (1967) Poxvirus DNA-dependent RNA polymerase. Proc. Natl. Acad. sci. USA 58:134-141.

Kotwal GJ, Moss B. (1988a) Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 335:176-8.

Kotwal GJ, Moss B. (1988b) Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. Virology 167:524-537.

Kotwal GJ, Moss B. (1989) Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitors superfamily. J Virol 63:600-6.

Koziorowska J, Wlodarski K, Mazurowa N (1971) transformation of mouse embryo cells by vaccinia virus. J Natl Cancer Inst. 46:225-241.

Ladnyi ID, Ziegler P, Kima E. (1972) A human infection caused by monkeypoxvirus in Basanusu Territory, Democratic Republic of the Congo. Bull. W.H.O. 46:593-597.

Lai ACK, Pogo BGT. (1989) Characterization of vaccinia virus deletion mutants isolated from persistently infected Friend erythroleukemia cells. Virus Res 12:239-50.

Lee-Chen GJ, Niles EG. (1988) Transcription and translation of the 13 genes in the vaccinia virus HindIII D fragment. Virology 163:52-63.

Maa J-S, Esteban M. (1987) Structural and functional studies of a 39,000-Mr immunodominant protein of vaccinia virus. J Virol 61:3910-9.

Mackett M, Archard LC. (1979) Conservation and variation in orthopoxvirus genome structure, J Gen Virol 45:683-701.

Mackett M, Smith GL, Moss B.(1982) Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci USA 79:7415-7419.

Mackett M, Smith GL.(1986) Vaccinia virus expression vectors. J Gen Virol 67:2067-82.

Marsh YV, Eppstein DA. (1987) Vaccinia virus and the EGF receptor:A portal for infectivity. J Cell Biochem 34:239-45.

Marshall ID, Fenner F. (1958) Studies in the epidemiology of infectious myxomatosis of rabbits. V. Changes in the innate resistance of Australian wild rabbits exposed to myxomatosis. J Hyg, Camb 56:288-302.

Marshall ID, Fenner F. (1960) Studies in the epidemiology of infectious myxomatosis of rabbits. VII. The virulence of strains of myxoma virus recovered from Australian wild rabbits between 1951 and 1959. J Hyg, Camb 58:485-488.

Matthews REF.(1982) Classificatin and Nomenclature of viruses. Intervirology 17:42-46.

McIntosh K.(1985) A comparative study of four smallpox vaccines in children. In: Vaccinia virus as vectors for vaccine antigen. Ed. Quinnan Jr, GV. Elsevier, New York. p.77-84.

Merchlinsky M, Moss B.(1986) Resolution of linear minichromosomes with hairpin ends from circular plasmids containing vaccinia virus concatemer junctions. Cell. 45:879-84.

Merchlinsky M, Moss B.(1989) Resolution of vaccinia virus DNA concatamer junctions requires late-gene expression. J Virol 63:1593-1603.

Morrison DK, Moyer RW.(1986) Detection of a subunit of cellular Pol II within highly purified preparations of RNA polymerase isolated from rabbit poxvirus virions.Cell 44:587-96.

Moss B, Rosenblum EN, Katz E. (1969) Rifampicin: a specific inhibitor of vaccinia virus assembly. Nature 224:1280-1284.

Moss B, Winters E, Cooper N.(1981a) Instability and reiteration of DNA sequences within the vaccinia virus genome. Proc Natl Acad Sci USA 78:1614-1618.

Moss B, Winters E, Cooper JA.(1981b) Deletion of a 9000-Base-Pair segment of vaccinia virus genome that encodes nonessential polypeptides. J Virol 40:387-395.

Moss B, Cooper N. (1982) Genetic evidence for vaccinia virus-encoded DNA polymerase: isolation of phosphonoacetate-resistant enzyme from the cytoplasm of cells infected with the mutant virus. J Virol 43:673-678.

Moss B. (1986) Replication of poxviruses. In: Virology (Fields BN. ed),Raven Press, New York.p.685-703.

Moss B, Flexner C.(1987) Vaccinia virus expression vectors Annu Rev Immunol 5:305-324.

Moyer RW, Graves RL. (1981) The mechanism of cytoplasmic orthopoxvirus DNA replication. Cell 27:391-401.

Moyer RW.(1987) The role of the host cell nucleus in vaccinia virus morphogenesis. Virus Res 8:173-191.

Muller HK, Wittek R, Schaffner W, Schumperli D, Menna A, Wyler R (1977) Comparison of five poxvirus genomes by analysis with endonucleases HindIII, BamHI and EcoRI. J Gen Virol 38:135-147.

Mykytowycz R (1953) An attenuated strain of myxomatosis virus recovered from the field. Nature 172:448-449.

Nakano E, Panicali D, Paoletti E. (1982) Molecular genetics of vaccinia virus: demonstration of marker rescue. Proc. Natl. Acad. Sci. USA 79:1593-6.

Nakayama A, Pogo BGT, Dales S.(1970) Biogenesis of vaccinia: Separation of early stages from maturation by means of rifampicin. Virology 40:1039-1051.

Natuk RJ, Holowczak JA. (1985) Vaccinia virus proteins on the plasma membrane of infected cells.III. Infection of peritoneal macrophages. Virology 147:354-372.

Nevin JR, Joklik WK. (1975) Poly(A) sequences of vaccinia virus messenger RNA: nature mode of addition and formation during translation in vitro and in vivo. Virology 63:1-14.

Niles EG, Condit RC, Caro P, Davidson K, Matusick L, Seto J. (1986) Nucleotide sequence and genetic map of the 16-kb vaccinia virus HindIII D fragment. Virology 153:96-112.

Obom KM, Hoyos B, Pogo BGT.(1988) Association of mitochondria DNA with viral DNA in purified preparation of poxviruses. Virus Res 11:165-174.

Obom KM, Pogo BGT. (1988) Characterization of the transformation properties of Shope fibroma virus. Virus Res 9:33-48.

Paez E, Dallo S, Esteban M. (1985) Generation of a dominant 8-MDa deletion at the left terminus of vaccinia virus DNA. Pro. Natl. Acad. Sci. USA 82:3365-3369.

Paez E, Esteban M.(1985) Interferon prevents the generation of spontaneous deletions at the left terminus of vaccinia virus DNA. J Virol 56:75-84.

Panicali D, Davis S W, Mercer SR, Paoletti E (1981) Two major DNA variants present in serially propagated stocks of the WR strain of vaccinia virus. J Virol 37:1000-1010.

Panicali D, Paoletti E.(1982) Construction of poxviruses as cloning vectors:

insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci USA 79:4927-4931.

Panicali D, Grzelecki A, Huang C.(1986) Vaccinia virus vectors utilizing the beta-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression. Gene. 47:193-9.

Parsons BL, Pickup DJ.(1987) Tandemly repeated sequences are present at the ends of the DNA of raccoonpox virus. Virology 161:45-53.

Pasteur L.(1881) Vaccination in relation to chicken-cholera and splenic fever. Transactions of the International Medical Congress, Seven Session, London, August 2-9,1881:85-90.[Reproduced in vallery-Radot P ed. Oeuvres de Pasteur. Paris, Masson, 6:370-378,1933.]

Patel DD, Pickup DJ. (1987) Messenger RNAs of a strongly expressed late gene of cowpox virus contain 5'-terminal poly(A) sequences. The EMBO J 6:3787-3794.

Payne LG, Norrby E. (1976) Presence of haemagglutinin in the envelope of extracellular vaccinia virus particles. J Gen Virol. 32:63-72.

Payne LG, Norrby E.(1978) Adsorption and penetration of enveloped and naked vaccinia virus particles. J Virol 27:19-27.

Payne LG.(1980) Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol 50:89-100.

Pedley CB, Cooper RJ. (1987) The assay, purification and properties of vaccinia virus-induced uncoating protein. J Gen Virol 68:1021-1028.

Pennington TH, Follett EAC. (1974) Vaccinia virus replication in enucleated BSC-40 cells: particle production and synthesis of viral DNA and proteins. J Virol 13:488-493.

Perkus ME, Panicali D, Mercer S, Paoletti E. (1986) Insertion and deletion mutants of vaccinia virus. Virology 152:285-297.

Peters D. (1956) Morphology of resting vaccinia virus. Nature 178:1453-1455.

Piccini A, Perkus ME, Paoletti E. (1987) Vaccinia virus as an expression vector. Methods Enzymol 153: 545-563.

Pogo BGT. Dales S. (1969) Two deoxyribonuclease activities within purified vaccinia virus. Proc. Natl. Acad. Sci. USA 63:820-827.

Pogo BGT, O'Shea, Freimuth P. (1981) Initiation and termination of vaccinia virus DNA replication. Virology 108:241-248.

Pogo BGT, Friend C. (1982) Persistent infection of Friend erythroleukemia cells with vaccinia virus. Pro. Natl. Acad. Sci. USA 79:4805-4809.

Pogo BGT, Berkowitz EM, Dales S. (1984) Investigation of vaccinia virus DNA replication employing a conditional lethal mutant defective in DNA. Virology 132:436-444.

Pogo BGT, Lai ACK, Holland JG, Friend C. (1988) Differences in the susceptibility of hematopoietic human cell lines to vaccinia virus. Intervirology 29:11-20.

Porter CD, Archard LC.(1987) Characterization and physical mapping of Molluscum contagiosum virus DNA and location of a sequence capable of encoding a conserved domain of epidermal growth factor. J Gen Virol 68:673-682.

Prescott DM, Kates J, Kirpatrick (1971) Replication of vaccinia virus DNA in enucleated L cells. J Mol Biol 59:505-508.

Redfield RR, Wright DC, James WD, Jones TS, Brown C, Burke DS.(1987) Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N Eng J Med 316:673-6.

Reisner AH (1985) Similarity between the vaccinia virus 19K early protein and epidermal growth factor. Nature 313:801-803.

Rice AP, Roberts WK, Kerr IM. (1984) 2-5 A accumulates to high levels in interferon-treated, vaccinia virus-infected cells in the absence of any inhibition of virus replication. J Virol 50:220-228.

Rivers TM, Ward SM.(1933) Further observation on the cultivation of vaccinia virus for Jennerian prophylaxis in man. J Exp Med 58:635-648.

Robinson AJ, Peterson GV (1983) Orf virus infection of workers in the meat industry. NZ Med J 96:81-85.

Rodriguez JF, Paez E, Esteban M. (1987) A 14,000-Mr envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. J Virology 61:359-404.

Rodriguez JF, Rodriguez D, Rodriguez JR, McGowan EB, Esteban M. (1988). Expression of the firefly luciferase gene in vaccinia virus: a highly sensitive gene marker to follow virus dissemination in tissues of infected animals. Proc Natl Acad Sci USA 85:1667-1671.

Rodriguez D, Rodriguez J-R, Rodriguez JF, Trauber D, Esteban M. (1989) High attenuated vaccinia virus mutants for the generation of safe recombinant viruses. Proc. Natl. Acad. Sci. USA 86:1287-1291.

Rodriguez JF, Esteban M. (1989) Plaque size phenotype as a selectable marker to generate vaccinia virus recombinants. J Virol. 63:997-1001.

Rosel JL, Earl PL, Weir JP, Moss B.(1986) Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII H genome

fragment. J Virol. 60:436-49.

Rouhandeh H, Vafai A. (1982) A novel cell transformation with DNA-containing cytoplasmic Yaba tumor poxvirus. Virology 120:77-92.

Rupprecht CE, Wiktor TJ, Johnston DH, Hamir AN, Dietzschold B, Wunner WH, Glickman LT, Koprowski H. (1986) Oral immunization and protection of raccoons (Procyon lotor) with a vaccinia-rabies glycoprotein recombinant virus vaccine. Proc Natl Acad Sci USA.83:7947-50.

Sam CK, Dumbell KR.(1981) Expression of poxvirus DNA in coinfected cells and marker rescue of thermosensitive mutants by subgenomic fragments of DNA. Ann. Virology, Paris 132E:490-5.

Schnitzlain WM, Ghildyal N, Tripathy DN. (1988) Genomic and antigenic characterization of avipoxviruses. Virus Res 10:65-76.

Schwer B, Visca P, Vos JC, Stunnenberg HG.(1987) Discontinuous transcription or RNA processing of vaccinia virus late messengers results in a 5' poly(A) leader. Cell.50:163-9.

Schwer B, Stunnenberg HG. (1988) Vaccinia virus late transcripts generated in vitro have a poly(A) head. The EMBO J 7:1183-1190.

Shope RE (1932) A transmissible tumor-like condition in rabbit. J Exp. Med. 56:793-802.

Silver M, McFadden G, Wilton S, Dales S. (1979) Biogenesis of poxviruses: Role for the DNA-dependent RNA polymerase of the host during expression of late functions. Proc Natl Acad Sci USA 76:4122-4125.

Siomi H, Shida H, Nam SH, Nosaka T, Maki M, Hatanaka M. (1988) Sequence requirements for nuclear localization of human T cell leukemia virus type 1 pX protein which regulates viral RNA processing. Cell 55:197-209.

Slabaugh MB, Mathews CK.(1986) Hydroxyurea-resistant vaccinia virus: overproduction of ribonucleotide reductase. J Virol 60:506-14.

Smith JW, Tevethia SS, Levy BM, Rawls WE.(1973) Comparative studies on host response to Shope fibroma virus in adult and newborn rabbits. J Natl Cancer Inst 50:1529-1539.

Smith GL,Levin JZ,Palese P, Moss B (1987) Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. Virology 160:336-345.

Stern W, Dales S. (1974) Biogenesis of vaccinia: Concerning the origin of the enveloped phospholipids. Virology 62:293-306.

Stern W, Dales S. (1976). Biogenesis of vaccinia: Relationship of the envelope to the virus assembly. Virology 75:242-255.

Stroobant P, Rice AP, Gullick WJ, Cheng DJ, Kerr IM, Waterfield MD (1985) Purification and characterization of vaccinia virus growth factor. Cell 42:383-393.

Takahashi M, Kameyama S, Kato S, Kamahora J. (1959) The immunological relationship of the poxvirus group. Biken J:27-29.

Tartaglia J, Paoletti E. (1985) Physical mapping and DNA sequence analysis of the rifampicin resistance locus in vaccinia virus. Virology 147:394-404.

Tartaglia J, Piccini A, Paoletti E. (1986) Vacccinia virus rifampicin-resistance locus specifies a late 63,000 Da gene product. Virology 150:45-54.

Thomas G, Herbert E, Hruby DE. (1986) Expression and cell type--specific processing of human preproenkephalin with a vaccinia recombinant. Science 232:1641-3.

Thompson CL, Condit RC.(1986) Marker rescue mapping of vaccinia virus temperature-sensitive mutants using overlapping cosmid clones representing the entire virus genome. Virology 150:10-20.

Tolson ND, Charlton KM, Casey GA, Knowles MK, Rupprecht CE, Lawson KF, Campbell JB (1988) Immunization of foxes against rabies with a vaccinia recombinant virus expressing the rabies glycoprotein. Arch Virol. 102:297-301.

Traktman P, Sridhar P, Condit RC, Roberts BE. (1984) Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J Virol 49:125-131.

Tsutsui K.(1983) Release of vaccinia virus from FL cells infected with the IHD-W strain. J Electron Microsc (Tokyo).32:125-40.

Twardzik DR, Brown JP, Ranchalis JE, Todaro GJ, Moss B.(1985) Vaccinia virus-infected cells release a novel polypeptide functionally related to transforming and epidermal growth factors. Proc Natl Acad Sci USA 82:5300-5304.

Upton C, Macen JL, McFadden G.(1987) Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. J Virol 61:1271-1275.

Venkatesan S, Moss B.(1981) In vitro transcription of the inverted terminal repetition of the vaccinia virus genome: correspondence of initiation and cap sites. J Virol 37:738-747.

Venkatesan S, Gershowitz A, Moss B. (1982) Complete nucleotide sequences of two adjacent early vaccinia virus genes located within the inverted terminal repetition. J Virol 44:637-646.

Whitaker-Dowling P, Youngner JS. (1983) Vaccinia rescue of VSV from interferon-induced resistance: Reversal of translation block and inhibition of protein kinase activity. Virology 131:128-136.

Wilton S, Dales S. (1989) Relationship between RNA polymerase II and effeciency of vaccinia virus replication. J Virol 63:1540-1548.

Wittek R, Menna A Muller K Schumperli D Bosley PG Wyler R.(1978) Inverted terminal repeats in rabbits poxvirus and vaccinia DNA. J Virol. 28:171-181.

Wittek R, Moss B. (1980) Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21:277-284.

Wittek R, Moss B.(1982) Colinearity of RNAs with the vaccinia virus genome: anomalies with two complementary early and late RNAs result from a small deletion or rearrangement within the inverted terminal repetition. J Virol.42:447-55.

Woodroofe GM, Fenner F.(1962) Serological relationships within the poxvirus group: An antigen common to all members of the group. Virology 16:334-341.

World Health Organization.(1980) The global eradication of smallpox. Final report of the global commission for the certification of smallpox eradication. In History of International Public Health. No. 4, WHO, Geneva.

Wright CF, Moss B. (1987) In vitro synthesis of vaccinia virus late mRNA containing a 5'poly(A) leader sequence. Proc Natl Acad Sci USA 84:8883-8887.

Yuen L, Moss B.(1987) Oligonucleotide sequence signalling transcription termination of vaccinia virus early genes. Proc Natl Acad Sci USA 84:6417-6421.

Zaslavsky V.(1985) Uncoating of vaccinia virus. J Virol 55:352-356.

Zhao BT, Prince G, Horswood R, Eckels K, Summers P, Chanock R, Lai CJ.(-1987) Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus.J Virol 61:4019-4022.

Zwartouw HT. (1964) The chemical composition of vaccinia virus. J Gen Microbial 34:115-123.

Chapter 2

Characterization of vaccinia virus deletion mutants isolated from persistently infected Friend erythroleukemia cells

***Alexander C.-K.Lai and **Beatriz G.-T. Pogo**

**Center for Experimental Cell Biology and *Department of Microbiology, Mount Sinai School of Medicine, City University of New York, New York, U.S.A.

Virus Research 1989;12:239-250

Address for correspondence: Beatriz G.-T. Pogo, Center for Experimental Cell Biology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New

Summary

Persistent viral infections in vitro are useful to study the evolution of virus populations in the absence of immunological pressure. Several deletion mutants have been isolated in this laboratory from Friend erythroleukemia cells persistently infected with vaccinia virus strain IHD-W, designated SQA_{vac}. Two of the mutants, which remains stable after serial passage in L cells, have been characterized. The deletion which range between 20 to 22 kb, has been localized at the left terminus comprising HindIII C and N. In addition, HindIII B fragment lost the sequences that hybridize to pAG5, a plasmid containing the 3.5 kb terminal sequences and acquired different restriction sites. Phenotypic characterization of these mutants revealed that they were not replication defective since they grew in all cell lines tested and produces plaques of the same size as the wild-type. However, they were less effective in suppressing host protein synthesis. The LD_{50} for the mutants titered in NIH-Swiss female mice was greater than 10^9 PFU as compared to 10^6 PFU for the wild-type, indicating reduced virulence in vivo. These mutants, which display different properties to previuosly described mutants with deletions at the left terminus, provide another valuable system to study the molecular basis of virulence of vaccinia.

Keywords: Vaccinia virus, deletion mutants

Introduction

Persistent viral infections are of extreme medical importance because of their implications in chronic and recurrent disease(s). Persistent viral infections in vitro provide a unique system to study the evolution of the virus population in the absence of immunological pressure as well as the modifications suffered by the cells which are chronically infected

We have previously reported that vaccinia virus, a highly cytolytic poxvirus, can establish persistent infections in Friend erythroleukemia (FEL) cells which are transformed by a retrovirus (Pogo and Friend, 1982) and in a human hematopoietic cell line (Pogo et al., 1988). During the course of the persistent infection, genetic changes in the vaccinia virus population resulting in the generation of mutants were observed (Paez et al., 1985, Dallo and Esteban, 1987). Furthermore, changes in the properties of the host cells such as high level of spontaneous differentiation, decreased tumorigenicitiy and resistance to superinfection were recorded (Pogo and Friend, 1982, Obom et al., 1986).

In addition to a previously described mutant with a deletion of 8 MDa at the left terminus (Paez et al., 1985), we have observed that mutants with larger size deletions are generated in a different FEL cell line designated SQA (Friend et al., 1984) when it is persistently infected with vaccinia virus. In this communication we report the isolation and characterization of these mutants.

Material and Methods

Cells and Virus

The establishment and maintenance of SQA cells were already described

(Friend et al., 1984). L cells, BSC-1, SIRC, and HeLa cells from the ATCC collection were propagated as monolayers in minimum essential medium (MEM) from Flow Laboratories, supplemented with 5% fetal bovine serum (FBS). Second passage chicken embryo fibroblast, a generous gift from Dr. Peter Palese, were propagated in F10 medium (Gibco Laboratories) and 10% FBS.

The IHD-W strain of vaccinia virus was used to initiate the persistent viral infections. Virus infectivity was monitored in L cells (NCTC clone 929) according to published procedures (Dales, 1963). Virus purification was performed following the method described by Stern and Dales (1974).

Analysis of viral DNA by restriction enzymes, Southern blotting and hybridization

DNA was extracted from purified virus and digested with the restriction enzymes following the conditions recommended by the manufacturer (Boehringer Mannheim Biochemicals). In general 1 ug of viral DNA was digested with 5 units of enzyme in a 20 ul reaction mixture for 4 h at 37 C. Restriction fragments were separated by electrophoresis in 0.5% or 0.2% agarose gels for 18 h at 50 V in a buffer containing 90 mM Tris-HCl pH 8.2, 90mM boric acid and 2.5 mM EDTA. After denaturation, the DNA fragments were transferred to nitrocellulose paper by the Southern procedure (Southern, 1975). Prehybridization and hybridization reactions were carried out according to the conditions described by Maniatis et al. (1982). Vaccinia virus, pAG5 and lambda DNA was labeled with [thio-^{35}S]dATP by nick-translation reaction (Rigby et al., 1977). Restriction fragments of vaccinia DNA cloned in pBR322 and pAG5, which contains the terminal 3.5 kb of the molecule, were kindly provided by Drs. E. Paoletti and B. Moss, respectively.

Preparation of labeled virus

^{32}P-labeled vaccinia virus was prepared by growing the virus in phosphate-free Eagle's medium supplemented with 2% FBS and 5 uCi per ml of [^{32}P] phosphate. Virus was purified, DNA extracted, digested with restriction enzymes and electrophoreses in agarose gels as described above. The gels were dried and exposed to Kodak-X-Omat-R film at -70°.

Virus labeled with [^{35}S]methionine was prepared by growing the virus in cells cultured in methionine-less medium supplemented with 3% FBS and 5 uCi/ml of [^{35}S]methionine (Stern and Dales, 1976). The virus was purified and aliquots were resuspended in Tris-HCl buffer pH 7.6 with 1% SDS and 1% ß-mercaptomethanol boiled for 5 minutes at 100° before loading onto 12% polyacrylamide slab gels containing SDS (Laemmli, 1970). Electrophoresis was carried out for 3 h at 100 V or for 8 h at 50 V. Gels were dried and exposed to films as described above. ^{14}C-labeled proteins of known molecular weight were used as standards to calculate the M_r of the bands.

Results

Analysis of vaccinia virus DNA by restriction enzymes

SQA cells (Friend et al., 1984) were persistently infected with vaccinia virus and designated SQA_{vac}. Once the persistent infection was well established, the cells were subcloned into two populations designated SQA_{vac} Z and SQA_{vac} Y and passaged independently (Obom et al., 1986). The viruses produced by these two cell lines were purified and their DNA digested with restriction enzymes. Viral DNA from the virus grown in SQA_{vac} Y cells showed a typical deletion of the left terminus, resulting in the absence of the

HindIII C fragment and the appearance of an extra fragment, F'. The size of this deletions, around 9 kb, is similar to those previously described by Panicali et al., (1981), Moss et al., (1981) and Paez et al., (1985) in the WR strain.

The virus isolated from the Z line was grown in L cells in the presence of [^{32}P]phosphate and the labeled viral DNA was compared with the labeled DNA of the wild-type by digestion with five restriction enzymes followed by electrophoresis and radioautography. Differences in the restriction pattern between the wild-type and the mutant DNA were observed in all five digests as shown in Fig. 1. The absence of some of the restriction fragments (indicated by open triangles) and the presence of several extra fragments (closed triangles) in the same digest suggest that within the Z virus there was a mixed population of virions probably with different size deletions.

After several rounds of plaque purification, several clones of vaccinia mutants were isolated. One of them, designated Z116-4 (Z-4), was used to map the deletion. As shown in Fig.2, comparison of HindIII (A, lanes 1 and 2) and BglI (A,lanes 3 and 4) digest of wild-type (lanes 1 and 3) and Z-4 (lanes 2 and 4) DNAs revealed that the mutant lacks HindIII C and BglI C fragments (23.5 kb) but it has an extra HindIII fragment close to K, designated K' (4.4 kb) and a larger BglI D fragment (12.5 kb). To map the deletion this gel was transferred to nitrocellulose paper and hybridized to pAG5, which comprise the 3.5 kb terminal the molecule. As shown in Fig. 2B, the pobe hybridized to both HindIII terminal fragments B and C of the wild-type as expected, but only to fragment HindIII K' and BglI D of the mutant. When cloned HindIII N fragment was used as a probe, it hybridized to fragments HindIII N and BglI D of the wild-type and to fragments HindIII K' and BglI D of the mutant.

These results indicated that the deletion of Z-4 mutant involves fragments HindIII C and N, or BgII C. The position of these fragments is shown in the map represented in Fig.2. From the size of the missing fragments and the resulting fragment K' this deletion was calculated to be 21 kb.

The DNA of another viral isolate designated Z-116-1-19 (Z-19) was compared to Z-4 DNA as shown in Fig.3. The size of the resulting HindIII K' fragment was smaller in Z-19, indicating a larger deletion of about 22 kb, or 12% of the genome. Hybridization with pAG5 and HindIII N gave similar results to those illustrated in Fig.2 (data not shown). Since both Z-4 and Z-19 deletion extended into the tandem repeats, it is highly probably that the difference between them reside in the number of tandem repeats present at the left boundary, as shown schematically in Fig.3. The lack of hybridization of the B fragment with pAG5 probe in the DNA of the mutant as seen in Fig.2 was an unexpected finding. To investigate the possibility of a deletion at the other terminus, the digests were electrophoresed in 0.2% agarose gel that allow a better separation of the large MW fragments. As shown in Fig.4A, there are no apparent difference in the migration of the fragments B between wild-type and the Z-19 mutant. However, when fragments B were electroeluted from the gels and subjected to digestion with EcoRI, it was found that the mutant DNA contained different restriction sites (Fig.4B, lane 2). The EcoRI fragments from the wild-type HindIII B (Fig. 4B, lane 1) are indicated in the figure according to the size reported by McFadden and Dales (1979) from the IHD-W strain. Extra bands are the result of unavoidable contamination with fragment C. Similar findings were observed with Z-4. The restriction pattern of both mutants have remained unchanged after serial passage in L cells.

Phenotypic characterization of the deletion mutant

The isolation of virus population with large deletions prompted us to investigate the phenotypic properties of these variants. The multiplication rate of the deletion mutant Z19 in L cells was compared to the wild-type. Both viruses reached maximum titers within 24 to 48 h after infection (Fig.5A). Other properties such as the growth of the mutant in other cell types and temperature sensitivity were also investigated. To find out whether there was a limitation in host range, the multiplication of the wild-type and Z19 were tested and found to be identical in human and chicken cells (Fig.5B and C). In addition, rabbit and monkey cells were also found to support the growth of the mutant. Similar results were obtained with the Z-4 mutant.

The temperature sensitivity of the Z-19 and Z-4 mutants were also explored by comparing the growth of the viruses at 39° C and at 37° C. The ratio of the multiplicity obtained at 39° C and 37° C was close to 1 for both, mutants and wild-type, indicating that no sensitivity to temperature was involved (data not shown). The rate of viral DNA synthesis was also by measuring [^{3}H]thymidine incorporation in the cytoplasmic fraction of infected cells. The kinetics of incorporation were similar for both mutants and wild-type, although the amount of thymidine incorporated was approximately 10% less in cells infected with either Z-4 or Z-19 mutants. This decreased uptake may be due to the fact that the genome of the mutants is 12% smaller than the wild-type (data not shown).

Analysis of virion protein by gel electrophoresis

A comparision of the virion proteins from the wild-type, Z-4 and Z-19

was carried out by polyacrylamide gel electrophoresis using [^{35}S]methionine-labeled virus preparations. Both stained gels and radioautographs revealed no major differences between the structural proteins of the wild-type and the two mutants (Fig. 6).

Pathogenicity of the deletion mutant

Serial passage of viruses may resulted in variants with decreased pathogenicity. although the Z mutants replicated in L cells at the same rate than the wild-type and produced plaques of similar size, they did not alter the growth of SQA_{vac} cells. It is therefore of importance to determine how host cell functions, mainly protein synthesis, was affected by the infection of these mutants. Pulse labeling experiments with [^{35}S]methionine were carried out in wild-type, Z-4 and Z-19 infected L cells. Infection with wild-type virus decreased [^{35}S]methionine incorporation by 50% between 3 to 4 hours after infection and by 90% at 8 h. Inhibition with both mutants was only recorded late in infection and to a lesser extent (30% at 8 h after infection). These results suggest that both mutants are less virulent than the wild-type in vitro.

To find out whether the lack of cytopathogenicity is also seen in vivo, Swiss and DBA/2J mice were inoculated i.p. with different multiplicities of the wild-type , Z-4 or Z-19. The results of three separate experiments indicated that the LD_{50} of the wild-type was 10^6 PFU, whereas the LD_{50} of the mutants was higher than 10^9 PFU, implying that both mutants expressed decreased virulence in vivo.

Discussion

SQA_{vac} cells generated mutants with different size deletions at the left terminus. One of the mutants (Y) is similar in size to that previously described by Panicali et al. (1981) and Moss et al.(1981) which appears spontaneously in stocks of the WR strain and similar to the mutant isolated by Paez et al. (1985) from other lines of FEL persistently infected cells. Deletion and rearangements near the ends of the genome have also been found in mutants of vaccinia virus (McFadden and Dales, 1979), rabbitpox (Moyer et al., 1980), cowpox (Archard and Mackett, 1979) and monkeypox (Esposito et al., 1981) and are believed to lead to alterations in the phenotype for the host range, cytopathology and virulence.

It is expected that under the dynamic conditions provided by a persistent infection, the virus population can be subjected to more profound changes than in an acute infection. Thus, SQA_{vac} line Z described here has generated more than one size deletion from around one kb to a few hundred bases pairs, suggesting that sets of tandem repeats may be involved. Extensive plaque purification lead to isolation of two mutants Z-4 and Z-19, with deletions, calculated to be 21 kb and 22 kb, respectively.

The deletion comprises most of the HindIII C fragment, which in the IHD-W strain is 23.5 kb and the HindIII N fragment (1.5 kb). These deletions are the largest described for spontaneously occurring mutants of vaccinia strain IHD-W. A mutant of the WR strain with a similar size deletion has been described by Perkus et al. (1986) although its phenotype has not been characterized. A host range mutant of the Copenhagen strain of vaccinia was shown to have a deletion of 18 kb at the left end of the genome involving HindIII fragments C, N and M (Gillard et al., 1986).

The presence of mutations with different size deletions in the same cell

line can be explained by the loss and accumulation of tandem repeats at the terminal ends, as have been recently demonstrated by Paez and Esteban (1988) in deletion mutants isolated from a different FEL cell line. However, in the Z mutants, we have failed to see accumulation of repeats on the right side terminus after serial passages. On the contrary, this end of the molecule seems to have loss the sequences that hybridized to pAG5 and to have acquired different restriction sites. The mechanism by which these changes have occurred is not clear at the moment.

Studies on the phenotype of Z-19 and Z-4 revealed that they can produce plaques and replicates in L cells to the same multiplicity to that of the wild-type. However, they inhibit host cell protein synthesis only at later times and to a lesser extent and they are thus less virulent in vitro. In contrast to the changes reported by Dallo et al. (1987) in the virion proteins of their mutants which are relevent for cytopathology, the Z mutants did not show major structural protein changes. This could be attributed by the difference in virus strains and cell lines used.

The significance of the findings reported here laid in the potential of these mutants for the studies on the identification of genes involved in the virulence of vaccinia virus in vivo and in vitro. The participation of the growth factor gene, which is present at both terminal fragments of the genome in vaccinia virus, in virulence has been recently demonstrated (Buller et al.,1988). Experiments are now in progress to established if this gene is involved in the determination of these mutants phenotype.

Acknowledgemants

Supported by Grant CA29262 and CA10000 from the National Institute of

Health and the Chemotherapy Foundation.

We are indebted to Dr. Kristine Obom for help and advice, to Mr. J. Gilbert Holland and Ms. Der Yi Cheng for efficient technical assistance and to Ms. Dolores Klaft for excellent typework.

References

Achard, L.C. and Mackett, M. (1979). Restriction endonuclease analysis of red cowpox virus and its white pock variant. J. Gen. Virol. 45, 51-63

Buller, R.M.L., Chakrabarti, S., Cooper, J.A., Twardzik, D.r., and Moss, B. (1988). Deletion of the vaccinia virus growth factor gene reduces virus virulence.J. Virol. 62, 866-874.

Dales, S. (1963). The uptake and development of vaccinia virus in strain L cells followed with labeled viral deoxyribonucleic acid. J. Cell Biol. 18, 51-72.

Dales, S. and Pogo, B.G.T. (1981). In: Biology of Poxviruses. Virology Monographs 18. Springer-Verlag, New York.

Dallo, S. and Esteban, M. (1987). Isolation and characterization of attenuated mutants of vaccinia virus. Virology 159, 408-422.

Dallo, S., Rodriguez, J.F. and Esteban, M. (1987). A 14K envelope protein of vaccinia virus with an important role in virus-host cell interactions is altered during virus persistence and determines the plaque size phenotype. Virology 159, 423-432.

DeFilippes F.M. (1982) Restriction enzyme mapping of vaccinia virus DNA. J. Virol. 43, 136-149.

Esposito, J.J., Cabradillo, C. D., Nakano, J.H. and Obijeski, J.F. (1981). Intragenic sequence transposition in monkeypox virus. Virology, 109, 231-243.

Friend, C., Pogo, B.G.T. and Holland, J.G. (1984). Characterization of leukemogenic virus produced by a new line of Friend erythroleukemia virus-transformed cells. Proc. Natl. Acad. Sci. U.S.A. 81, 1831-1834.

Gillard, S., Spehner, D., Drillien, R. and Kirn, A. (1986). Localization and sequence of a vaccinia virus gene required for multiplication in human cells. Proc. Natl. Acad. Sci. USA 83, 5573-5577.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) 227, 680-684.

Maniatis, T., Fritsch, E.F. and Sambrook, J.(1982). In: Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

McFadden, G. and Dales, S. (1979). Biogenesis of Poxviruses: Mirror image deletions in vaccinia virus DNA. Cell 18,101-108.

Moss, B., Winters, E. and Cooper, J.A. (1981). Deletion of a 9000-Base-Pair segment of vaccinia virus genome that encodes nonessential polypeptides. J. Virol. 40,387-395.

Moyer, R.W., Graves, R.L. and Rothe, C.T. (1980). The white pock (u) mutants of rabbit poxvirus. III. Terminal DNA sequence duplication and transposition in rabbit poxvirus. Cell 22,545-553.

Moyer, R.W., and Rothe, C.T. (1980). The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletion. Virology 102, 119-132.

Obom, K.M., Popple, S.W., Holland, J.G., Pogo, B.G.T. and Friend, C. (1986). Vaccinia virus DNA sequences in the nucleus of persistently infected Friend erythroleukemia cells. Virus Res. 5,221-234.

Paez, E., Dallo, S. and Esteban, M. (1985). Generation of a dominant 8-MDa deletion at the left terminus of vaccinia virus DNA. Proc. Natl. Acad. Sci. USA 82, 3365-3369.

Paez, E. and Esteban, M. (1988). Stablity of vaccinia virus DNA during persistent infections: Accumulation of left-end deletions and tandem repeats at both ends of the viral genome and prevention by interferon. Virology 163, 145-154.

Panicali, D., Davis, S.W., Mercer, S.R. and Paoletti, E. (1981). Two major DNA variants present in serially propagated stocks of the WR strain of vaccinia virus. J. Virol. 37, 1000-1010.

Perkus, M.E., Panicali, D.,Mercer, S. and Paoletti, E. (1986). Insertion and deletion mutants of vaccinia virus. Virology 152, 285-297.

Pogo, B.G.T. and Friend, C. (1982). Persistent infection of Friend erythroleukemia cells with vaccinia virus. Proc. Natl. Acad. Sci. USA 79, 4805-4809.

Pogo, B.G.T., Lai, A.C.K., Holland, J.G. and Friend, C. (1988) Differences in the susceptibility of hematopoietic human cell lines to vaccinia virus. Intervirology 29,11-20.

Rigby, P.W., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J. Mol. Biol. 113,237-251.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Stern, W. and Dales, S. (1974). Biogenesis of vaccinia: Concerning the origin of the enveloped phospholipids. Virology 62, 293-306.

Stern, W. and Dales, S. (1976). Biogenesis of vaccinia: Relationship of the envelope to the virus assembly. Virology 75, 242-255.

(Received 25 August 1988; revision received 7 Novermber 1988)

Fig. 1. Autoradiograph of ^{32}P-labeled vaccinia DNA restricted with the indicated enzymes. Both wild-type and Z viruses were grown in L cells. DNA was extracted from purified virions, digested with restriction enzymes, electrophoresed in 0.6% agarose gel and radioautographed as described in Material and Methods. Letter at the left indicated position of the BglI restriction fragments of the wild-type DNA and at the right of the HindIII fragments. Missing fragments are indicated by open triangles, extra fragments by closed triangles.

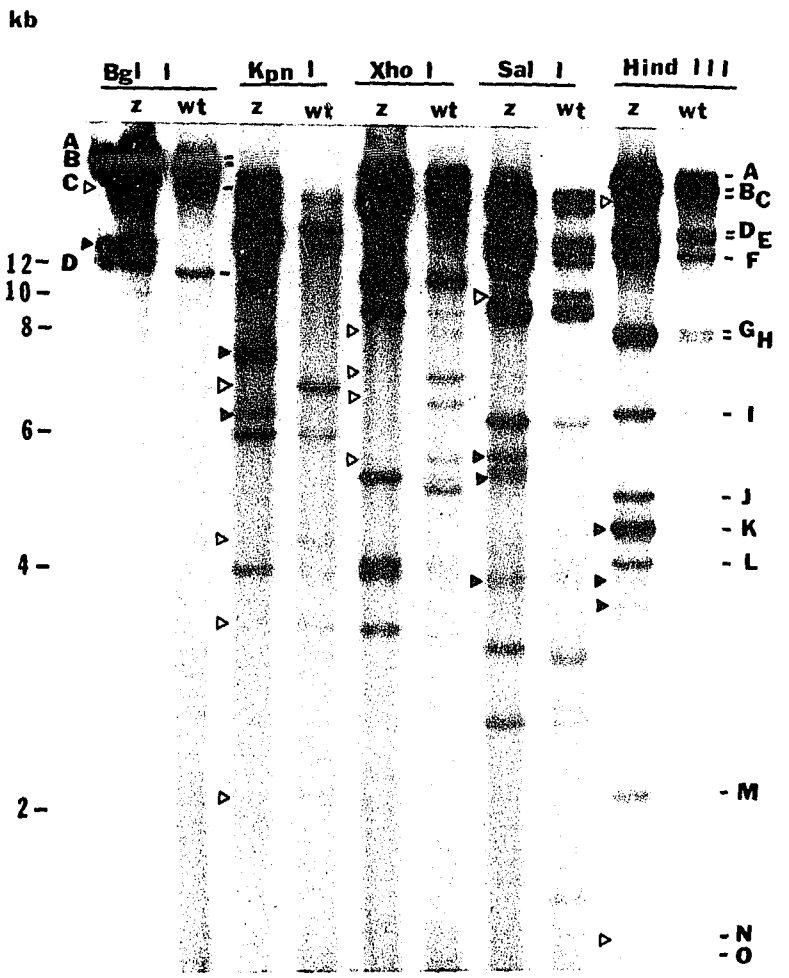

kb
Bgl I
Kpn I
Xho I
Sal I
Hind III
z
wt
A
B
C
D
12
10
8
6
4
2
A
B C
D E
F
G H
I
J
K
L
M
N
O

Fig.2. Southern blot and hybridization of viral DNAs. Panel A: ethidium bromide stain. Panel B: hybridization with pAG5, Panel C: hybridization with cloned HindIII fragment N; M: lambda DNA digested with HindIII; lane 1:wild-type DNA and lane 2:Z-4 DNA digested with HindIII; lane 3:wild-type DNA and lane 4:Z-4 DNA digested with BglI. The diagram shows the location of the probes in the genome and the approximate boundaries of the deletion in the HindIII and BglI maps (DeFilippes 1982). ITR=inverted terminal repeat.

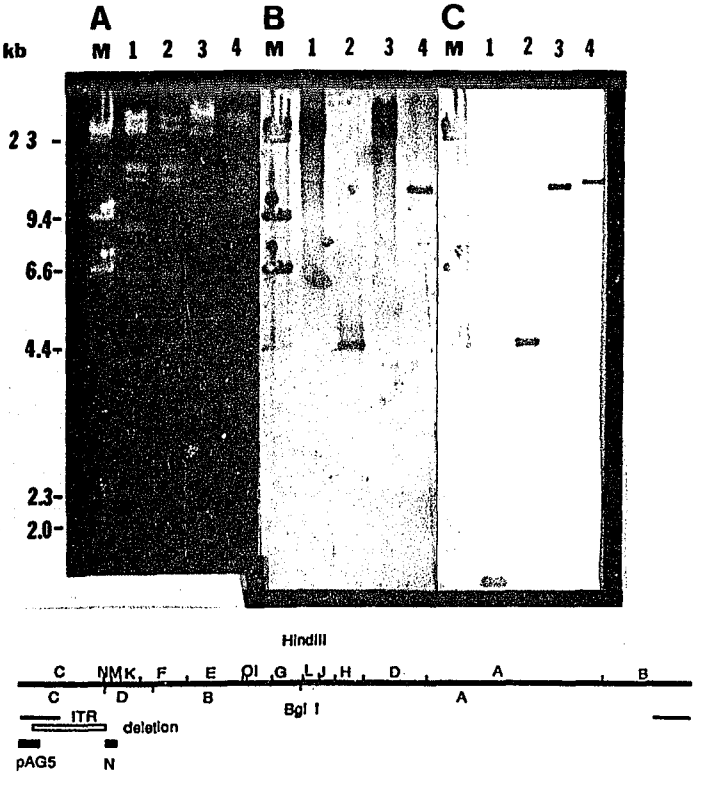

A
B
C
kb
M 1 2 3 4 M 1 2 3 4 M 1 2 3 4
23 -
9.4-
6.6-
4.4-
2.3-
2.0-
HindIII
C N M K F E O I G L J H D A B
C D B Bgl I A
ITR
deletion
pAG5
N

Fig. 3. Comparison of the restriction pattern of DNAs from wild-type and two deletion mutants digested with HindIII. Panel A: lane 1:wild-type; lane 2:Z-4; M: lambda DNA digested with HindIII. Panel B:lane 1:wild-type; lane 2 :Z-19. ITR= inverted terminal repeats. Fusion fragments designated K' are indicated by arrows.

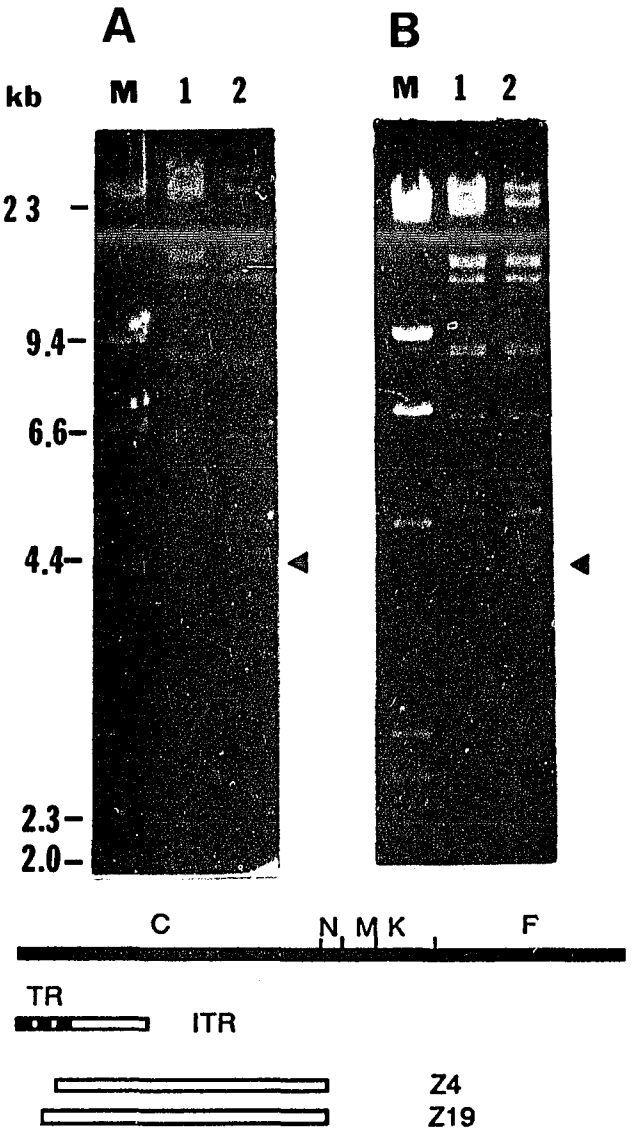

A
B
kb
M 1 2
M 1 2
23
9.4
6.6
4.4
2.3
2.0
C
N M K
F
TR
ITR
Z4
Z19

Fig. 4. Comparision of the HindIII B fragments of the wild-type and the mutant. Pane A: electrophoresis of HindIII digest of viral DNA in 0.2% agarose. M=lambda DNA digested with HindIII;lane 1: wild-type DNA; lane 2, Z-19 DNA. The position of fragment B is indicated by the arrow . Panel B: EcoRI digestion of fragments B electrophoresed in 0.7% agarose gel. M: 1 kb DNA ladder, lane 1:wild-type DNA; lane 2 Z-19 DNA. The EcoRI fragments of HindIII B fragment from the wild-type and the mutant are indicated by closed triangles and open triangles, respectively.

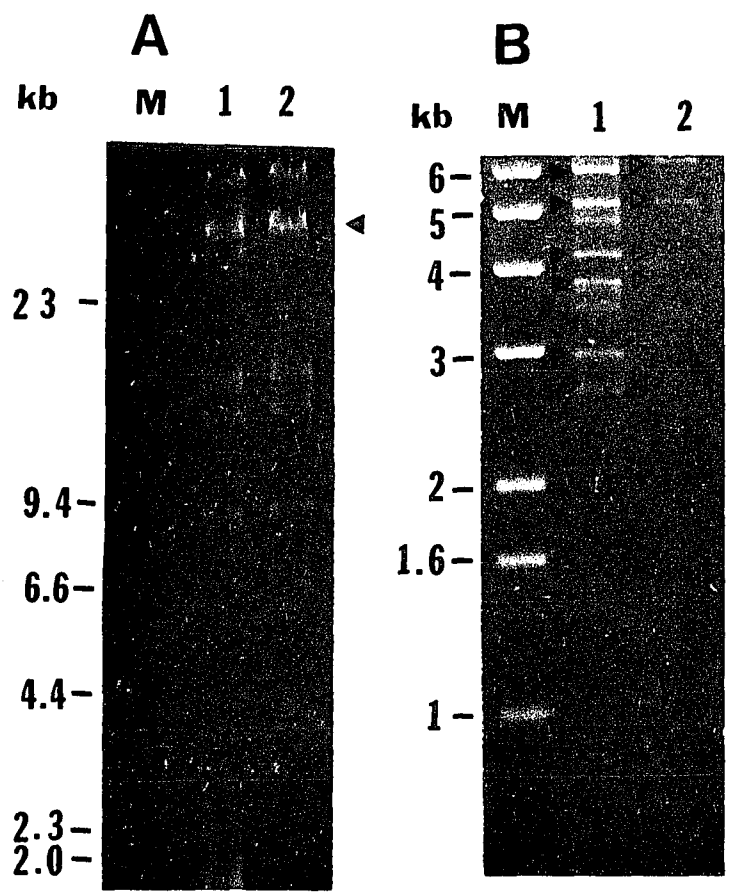

A
kb
M
1
2
23
9.4
6.6
4.4
2.3
2.0
B
kb
M
1
2
6
5
4
3
2
1.6
1

Fig. 5. Growth of vaccinia wild-type (open squares) and mutants Z-4 (closed squares) and Z-19 (closed diamonds) in various cell lines. 6A: L cells; 6B: HeLa cells; 6C chicken embryo fibroblast. Cells are infected with 5 PFU per cell and 24 and 48 h later the cells were harvested, sonicated, and virus multiplication was determined by plaque assay in L cells.

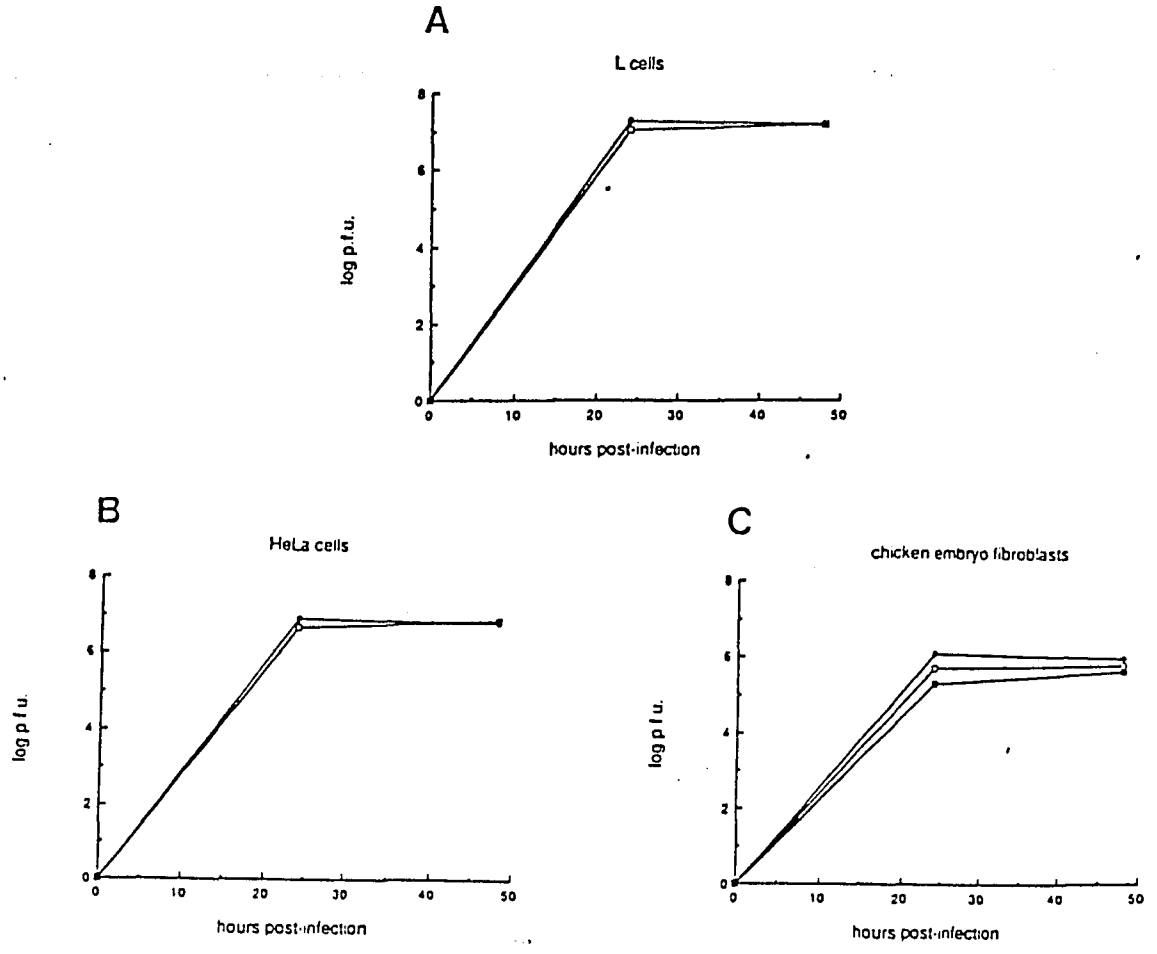
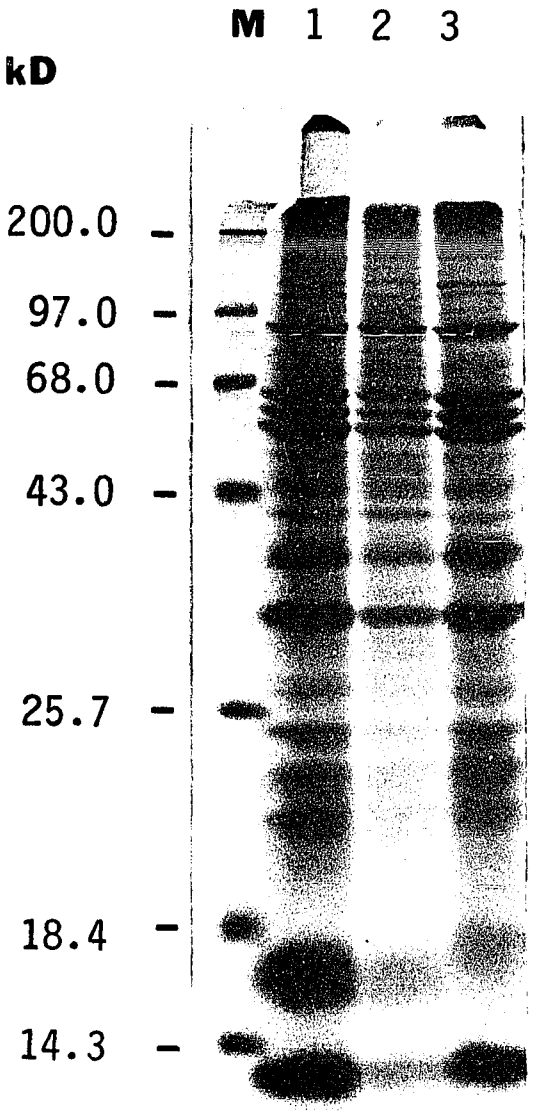

A
L cells
log p.f.u.
hours post-infection
B
HeLa cells
log p f u.
hours post-infection
C
chicken embryo fibroblasts
log p f u.
hours post-infection

Fig.6. Comparison of labeled virion proteins from wild-type vaccinia and mutants. Polyacrylamide gel electrophoresis and autoradiography of [^{35}S]methionine-labeled virions was carried out as described in Materials and Methods. Lane1:wild-type; lane 2: Z-4; lane 3: Z-19; M: molecular weight markers.


M 1 2 3
kD
200.0
97.0
68.0
43.0
25.7
18.4
14.3

Chapter 3

Attenuated deletion mutants of vaccinia virus lacking the vaccinia growth factor are defective in replication in vivo

Alexander C.-K. Lai* and Beatriz G.-T. Pogo**

*Department of Microbiology and the **Center for Experimental Cell Biology, Mount Sinai School of Medicine, City University of New York, New York, NY 10029, U.S.A.

Microbial Pathogenesis 1989;**6**: xxx-xxx

Address for correspondence: Dr. Beatriz G.-T. Pogo, Center for Experimental Cell Biology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

Abstract

Understanding the molecular basis of virulence in poxvirus is of great importance for the development of recombinant vaccines using vaccinia virus as a vector. We have previously described mutants of vaccinia virus with deletions ranging from 20 to 21 kb at the left terminus and with attenuated phenotype. The virulence of these mutants was studied, using different routes of inoculation, for protection from wild-type challenge in mice and for replication in vivo. Regardless of the route of inoculation, the LD_{50} of the deletion mutants is at least a 1000-fold higher than that of the wild-type. Results from protection experiments using viable and ultraviolet-inactivated viruses, and from determination of infectivity in different organs, indicated that the mutants were unable to replicate in vivo. Southern blot hybridization of viral DNA with pSC16, a plasmid containing the vaccinia growth factor (VGF) gene, revealed that in the IHD-W strain of vaccinia virus this gene is localized at the left terminus exclusively and that the mutants lack this gene. The results suggest that absence of the VGF gene is correlated with inability to replicate in vivo and decreased virulence.

Keywords: vaccinia virus;growth factor;replication in vivo;virulence;protective immunity.

Because of the high frequency of homologous recombination [1] and the ease with which foreign gene(s) can be inserted in and expressed, vaccinia virus has become a widely used eukaryotic expression vector[2,3]. Many recombinants have been constructed to date (for review, see references[4-6]), which are capable of eliciting appropiate immune response, making them potential candidates for vaccines. However, the recent case of disseminated vaccinia in a military recruit with the acquired immunodificiency syndrome [7] is a cogent reminder of the potential hazards of using recombinant vaccinia as a human vaccine and provides impetus to current research on the molecular basis of virulence of vaccinia virus.

At least two genes appears to be important for virulence, the thymidine kinase (TK) gene [8] and a gene or genes at the left terminus, since deletion at the left terminus have been shown to exhibit attenuated phenotypes[8,9] Recently, Buller et al. demonstrated that deletion of the vaccinia growth factor (VGF) gene decreased virulence [10]. We have previously reported the isolation of mutants with deletions at the left terminus with reduced virulence both in vitro and in vivo, in a persistently infected Friend erythroleukemia cell line.[11] In this communication, we present evidence that indicate that these mutants lack the VGF gene and are defective in replication in vivo, which may explain their attenuated phenotype.

Results

Determination of LD_{50} of wild-type virus and of deletion mutants

It has been reported that deletion at the left terminal fragments in vaccinia [8,9] and in rabbitpox [12] resulted in an attenuated phenotype. Since two mutants isolated in our laboratory, designated Z-19 and Z-4 [11], have been

shown to have deletion of around 21 kb involving the HindIII C and N fragments at the left terminus of the genome, the LD_{50} of these mutants was compared to the wild-type by different routes of inoculation. As shown in Table 1, the LD_{50} by intracranial (i.c.) inoculation of Z-19 mutant was $2.6x10^2$ pfu, whereas the wild-type was less than 10^1 pfu, indicating that the mutant has reduced neurovirulence. When inoculated intraperitoneally (i.p.) mice survive as much as 10^9 pfu of the mutant, as compared to LD_{50} of $1.2x10^6$ pfu of the wild-type. Also by intranasal inoculation, the mutant LD_{50} is 1000-fold higher than the wild-type (data not shown). Thus, regardless of the route of inoculation, the deletion mutant displayed an attenuated pheotype. Identical results were obtained with the mutant Z-4.

Determination of protective immunity by wild-type virus and by deletion mutants

To test if the mutants can elicit immunological protection, groups of three adult female mice were inoculated i.p. with increasing amounts of infectious or ultra-violet (uv) inactivated viruses and 3 to 4 weeks later they were challenged with 10 to 15 LD_{50} of the wild-type by i.p.route.

As shown in Table 2, 10^1 pfu of the wild-type elicit full protective immunity, whereas at least 10^5 pfu of the mutant (Z-19) are required for comparable protection. When uv-inactivated viruses were used, 10^5 pfu of the mutant and 10^4 of the wild-type were required for protection. Since uv-inactivated virus does not replicate and 10^5 pfu of the mutant was needed to elicit full protection in both cases, it seemed likely that the mutant is unable replicate in vivo, although its rate of growth in vitro is similar to that of the wild-type.

Growth of the wild-type and the mutant viruses in vivo

To further assess the ability of the mutant to replicate in vivo, the virus in different organs was titered after i.p. inoculation. Groups of three adult female mice were inoculated i.p. with 10^7 pfu of the wild-type or of the mutant (Z-19) and sacrificed at 2, 3 and 6 days post-inoculation. The livers and spleens, the major sites of replication, were removed and tested for infectivity. As shown in Fig. 1, virus infectivity was detected in both organs of the wild-type inoculated mice, reaching a peak titer at 2 to 3 days after inoculation. Although the mice in the surviving group were febrile and moribund, the virus titer decreased after 6 days of infection. By contrast, infectious virus was not detected in the livers of the group inoculated with the mutant viruses and only very low concentrations were detected in the spleens after 3 days, most probably due to residue virus from the inoculum. The mice in this group showed no sign of illness. In other experiments using higher and lower doses of virus, similar and reproducible results were obtained (data not shown). Mutant viruses also were not detected in other organs in which low titers of wild-type virus were documented.

Finally, intradermal scarification of the mouse tail with 10^7 pfu of wild-type resulted in the appearance of necrotic lesions in the site of inoculation whereas none were observed when 10^7 pfu of Z-19 was scarified, suggested again that the mutant failed to replicate in vivo. Collectively, these findings indicated a block in replication for the mutant viruses. Whether the block is in replication in the organs per se, in dissemination, or because the mutant is extremely sensitive to non-specific immunity in the peritoneal cavity has not been determined. The latter possibility, however, is unlikely since in

protection experiments using intravenous inoculation the results were similar to those in Table 2 (data not shown).

Determination of VGF gene in wild-type virus and in the mutants

The correlation of deletion in the left end of the genome and attenuated phenotypes clearly suggests that gene(s) within this region play a role in virulence.[8,9] The demonstration that a mutation in the VGF gene resulted in decreased virulence[10] prompted us to look for alteration of this gene in the mutants. DNA from wild-type and from the mutant (Z-4) was digested with HindIII, BglI, SalI and KpnI, electrophoresed in 0.5% agarose gel and blotted onto nitrocellulose filters. The transferred fragments were probed with pSC16, a plasmid containing the whole VGF gene [10]. As shown in Fig. 2(b), the probe hybridized only to the wild-type DNA (lanes 1, 3, 5, 7) indicating that the mutant lacks this gene. The complete absence of the VGF gene in the mutant was an unexpected finding. In the WR strain, the VGF gene is within the inverted terminal repeats (ITR) [13] and there are two copies of it, one in each of the terminal fragments. However, the parental strain IHD-W used in our laboratory has only one copy of the gene, since hybridization with the VGF gene is only seen in fragments HindIII C or BglI C [Fig.2(b)]. A deletion comprising these fragments results in total absence of the VGF gene as it is also shown in Fig.3(b) for the DNA of Z-19. Preliminary results using anti-epidermal growth factor (EGF) specific serum and immunoprecipitation of virus-infected cells indicated that the mutants do not produce secreted nor non-secreted VGF gene product (data not shown).

Discussion

Results from the experiments described here indicate a correlation between decreased virulence, lack of the VGF gene and failure to replicate in vivo. The VGF gene has been shown to play a role in virulence in vaccinia, although the mechanism involved has not been established.[10] As postulated by Brown et al. [14] and and Spriggs [15], secreted VGF might prepare surrounding un-infected cells for the replication of progeny viruses by stimulating growth or activating the metabolism of these cells. Higher metabolic state of host cells may facilitate productive virus infection. This interpretation could explain the results of Buller et al.[10] in which the yield of VGF- mutants is lower in resting Swiss 3T3 cells. The rate of growth in vitro of the Z mutants and of VGF- mutant of Buller et al., [10] is similar to that of their respective wild-type,but it is significantly lower when compared in vivo. This could be explained by the fact that the cells in vitro are metabolically more active than cells in the organs. Indeed, VGF has recently been shown to induce cell proliferation in vitro [16] and in vivo. [17] Another proposed mechanism is that membrane-bound VGF serves as a ligand for attachment to EGF receptors in un-infected cells. This seems unlikely, because although occupancy of the EGF receptors by EGF [18] or by monoclonal antibodies to EGF receptors [19] inhibit virus replication, vaccinia can infect NR-6 cells which lack detectable EGF receptors [20]. Moreover, if binding to the EGF receptors is indeed a mode of entry for the virus, it is probably not the only one.

The LD_{50} by i.p. inoculation of the wild-type (IHD-W strain) used in our laboratory is at least 100-fold less than that reported for the WR strain.[8] This could be due to the difference in the host mouse strain used. However, the finding that the VGF gene in the IHD-W strain is present only at the left

side of the genome,suggests that the difference in LD_{50} may be related to the amount of gene expressed. This assumption is supported by the recent finding the the LD_{50} of another orthopox virus used in our laboratory which possesses two copies of the growth factor gene is only 10^4 pfu (unpublished results).

Other genes located in the HindIII C and N fragments may also contribute to the attenuated phenotype. Two other secretory polypeptides are encoded by genes present at the left terminus.[21] One is related to the superfamily of complement control proteins and may be involved in helping to counteract host immune defenses.[21] Their role in virulence still remains to be proven. Experiments are now in progress to insert the VGF gene into the mutant and to test recovery of virulence and ability to grow in vivo.

In conclusion, the deletion mutants described here provides a good system to study virulence. First, the VGF- phenotype results from complete absence of the gene due to a naturally generated deletion and not to a partial replacement of the gene;[10] second, the genotype is stable as opposed to other deletion mutants in which the number of tandem repeats have been shown to undergo changes;[22] and, third, the size of the deletion (21 kb) which allows insertion of gene(s) whose effect on the phenotype can be tested.

Materials and methods

Cells and viruses

L cells were propagated as monolayers in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). Virus infectivity was monitored in L cells according to published procedures.[23] The IHD-W strain of

vaccinia was used when indicated. The isolation of the deletion mutants Z19 and Z-4 from persistently infected cells has been described.[11] Wild-type and mutant viruses were purified following published procedures.[24] For uv-inactivation, suspension of the wild-type or the mutant of know titers were spread in 60 mm sterile plates and exposed to uv-light (GE-IS) at a 30 cm distance with constant agitation at 4 C for 60 s. The titers of the irradiated viruses were found to be 3 to 4 logs lower than the original titers.

Southern hybridization

Viral DNA was extracted from purified virions as previously described [25] and digested with restriction enzymes according to the manufacturer's recommendations. Restricted fragments were separated by electrophoresis in 0.5% agarose gel. Southern blotting and hybridization were performed according to standard procedures.[26]. A plasmid that contained the entire VGF gene, pSC16, kindly provided by B. Moss from the NIAID, was labeled with [thio-^{35}S]dATP by nick-translation as described by Rigby.[27]

Mouse inoculation

For intraperitoneal inoculation, virus stock of known titer was thawed and sonicated for 60 s, then diluted with MEM. Aliquots of 0.2 ml containing appropiate pfu were inoculated into the peritoneal cavity using a 27-gauge needle and a tuberculin syringe. Groups of four to five female NIH-Swiss mice of age ranging from 6 to 8 weeks old randomly chosen from different siblings were used. They were monitored for signs of illness or mortality for at least 2 weeks. For intracerebral inoculation, 0.05 ml of the appropiate virus dilution was injected intracerebrally into 3-days old suckling mice using a 27 gauge

needle. They were monitored for at least 2 weeks. Results of three separate experiments were pooled and LD_{50} was calculated by the Reed-Meunch method.[28]

Determination of infectivity in vivo

6-8-week-old NIH-Swiss female mice were inoculated i.p. with 10^7 pfu of the wild-type or the mutants and sacrificed in groups of 3 at days 2, 3 and 6 post-infection. The spleens and livers were removed, weighed, and 100 mg of the samples were washed with cold saline solution, homogenized with a Dounce homogenizer under sterile conditions, sonicated, and assayed for infectivity (by plaque assay).

Supported by grants CA29262, CA13047 from the National Institute of Health and Chemotherapy Foundation.

We thank Dr Bernard Moss for supplying the pSC16 and to Ms Dolores Klaft for typing the manuscript.

References

1. Ball LA. High-Frequency homologous recombination in vaccinia virus DNA. J Virol 1987;61:1788-95.

2. Panicali D,Paoletti E. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad USA 1982;79:4927-31.

3. Mackett M, Smith GL, Moss B. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J Virol 1984;49:857-64.

4. Piccini A, Perkus ME, Paoletti E. Vaccinia virus as an expression vector. Methods Enzymol 1987;153:545-63.

5. Mackett M, Smith GL. Vaccinia virus expression vectors. J Gen Virol 1986;67:2067-82.

6. Moss B, Flexner C. Vaccinia expression vectors. Ann Rev Immunol 1987;5:305-24.

7. Redfield RR, Wright DC, James WD, Jones TS, Brown C, Burke DS. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N Eng J Med 1987;316:673-6.

8. Buller RML, Smith GL, Cremer K, Notkins AL, Moss B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinas-negative phenotype. Nature 1985;317:813-5.

9. Dallo S, Esteban M. Isolation and characterization of attenuated mutants of vaccinia virus. Virology 1987;159:498-22.

10. Buller RML, Chakrabarti S, Cooper JA, Twardzik DR, Moss B. Deletion of the vaccinia virus growth factor genes reduce virus virulence. J Virol 1988;62:866-74.

11. Lai ACK, Pogo BGT. Characterization of vaccinia virus deletion mutants isolated from persistently infected Friend erythroleukemia cells. Virus Res 1989;12:239-50.

12. Edwards KM, Andrews TC, Van Savage J, Palmer PS, Moyer RW.Poxvirus deletion mutants:virulence and immunogenicity. Microbial Pathogenesis 1988;4:325-33.

13. Moss B, Winters E, Cooper JA. Deletion of a 9000-base-pair segment of the vaccinia virus genome that encodes nonessential polypeptides. J Virol 1981;40:387-95.

14. Brown JP, twardzik DR, Marquardt H, Todaro GJ. Vaccinia virus encodes a polypeptide homologous to epidemal growth factor and transforming

growth factor. Nature 1985;313:491-2.

15. Spriggs DR. Vaccinia virus growth factor:A surprising catch. J Infect Dis 1986;153:382-3.

16. Twardzik DR, Brown JP, Ranchalis JE, Todaro GJ, Moss B. Vaccinia virus-infected cells release a novel polypeptide functionally related to transforming and epidermal growth factors. Proc Natl Acad Sci USA 1985;82:5300-4.

17. Buller RML, Chakrabarti S, Moss B, Fredrickson T. Cell proliferative response to vaccinia virus is mediated by VGF. Virology 1988;164:182-92.

18. Eppstein DA, Marsh YV, Schreiber AB, Newman SR, Todaro GJ, Nestor JJ Jr. Epidermal growth factor receptor occupancy inhibits vaccinia virus infection. Nature 1985;318:663-5.

19. Marsh YV, Eppstein DA. Vaccinia virus and the EGF receptor: A portal for infectivity. J Cell Biochem 1987;34:239-45.

20. Stroobant P, Rice AP, Gullick WJ, Cheng DJ, Kerr IM, Waterfield MD. Purification and characterization of vaccinia virus growth factor. Cell 1985;42:383-93.

21. Kotwal GJ, Moss B. Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 1988;335:176-8.

22. Paez E, Esteban M. Stability of vaccinia virus DNA during persistent infections: Accumulation of left-end deletions and of tandem repeats at both ends of the viral genome and prevention by interferon. Virology 1988;163:145-54.

23. Dales S. The uptake and development of vaccinia virus in L cells followed with labeled viral deoxyribonucleic acid. J cell Biol 1963;18:51-72.

24. Stern W, Dales S. Biogenesis of vaccinia:Concerning the origin of the envelope phospholipids. Virology 1974;62:293-306.

25. Obom KM, Popple SW, Holland JG, Pogo BGT, Friend C. Vaccinia virus DNA sequences in the nucleus of persistently infected Friend erythroleukemia cells. Virus Res 1986;5:221-34.

26. Maniatis T, Fritsh EF, Sambrook J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1982.

27. Rigby PW, Diekmann M, Rhodes C, Berg P. Labelling deoxyribonucleic acid to high specificity activity in vitro by nick translation with DNA polymerase I. J Mol Biol 1977;113:237-51.

28. Reed LJ, Meunch H. A simple method of estimating fifty endpoints. Am J Hygiene 1938;27:493-7.

TABLE 1

COMPARISON OF LD_{50} OF VACCINIA WILD-TYPE AND MUTANT (Z-19) IN SWISS-NIH MICE BY INTRAPERITONEAL AND INTRACEREBRAL INOCULATION

	PFU INOCULUM AT LD_{50}	
	IP*	IC**
WILD-TYPE	$1.2X10^6$	$<10^1$
Z19	$>10^9$	$2.6X10^2$

Groups of four, six- to eight-weeks-old female NIH-Swiss mice, were inoculated intraperitoneally (i.p.), and groups of three, three-days-old suckling mice, were injected intracerebrally (i.c.), as described in the Materials and methods section. Results of three separate experiments were pooled and LD_{50} was calculated by the reed-Meunch method.

TABLE 2

Determination of protective dose of live or uv-inactivated viruses against challenge with wild-type virus

A. LIVE VIRUSES:			B. UV-INACTIVATED VIRUSES:	
PFU INOCULUM		SURVIVAL/TOTAL	PFU INOCULUM	SURVIVAL/TOTAL
CONTROL		0/3	CONTROL	0/3
WILD-TYPE	10^1	3/3	10^1	0/3
	10^2	3/3	10^2	0/3
	10^3	3/3	10^3	1/3
	10^4	ND	10^4	3/3
	10^5	ND	10^5	3/3
	10^6	ND	10^6	3/3
Z19	10^1	ND	10^1	ND
	10^2	0/3	10^2	ND
	10^3	0/3	10^3	0/3
	10^4	0/3	10^4	0/3
	10^5	3/3	10^5	3/3
	10^6	ND	10^6	3/3

Groups of three adult NIH-Swiss female mice were inoculated i.p. with increasing amount of pfu of live (A) or uv-inactivated viruses (B). After 3-4 weeks, they were challenged with 10 to 15 LD_{50} of wild-type as described in the Materials and methods section.

ND: NOT DONE

Fig.1 Determination of viral replication in vivo. Mice were inoculated i.p. with 10^7 pfu wild-type or mutant (Z-19) viruses. Infectivity was determined in the organs by plaque assay. The results represent average of titers of three mice at the days indicated: (open squares), spleen; (closed squares), liver of wild-type inoculated mice; (open diamonds), spleen and (closed diamonds), liver of mutant inoculated mice.

In vivo replication: tissue titration of vaccinia after i.p. inoculation

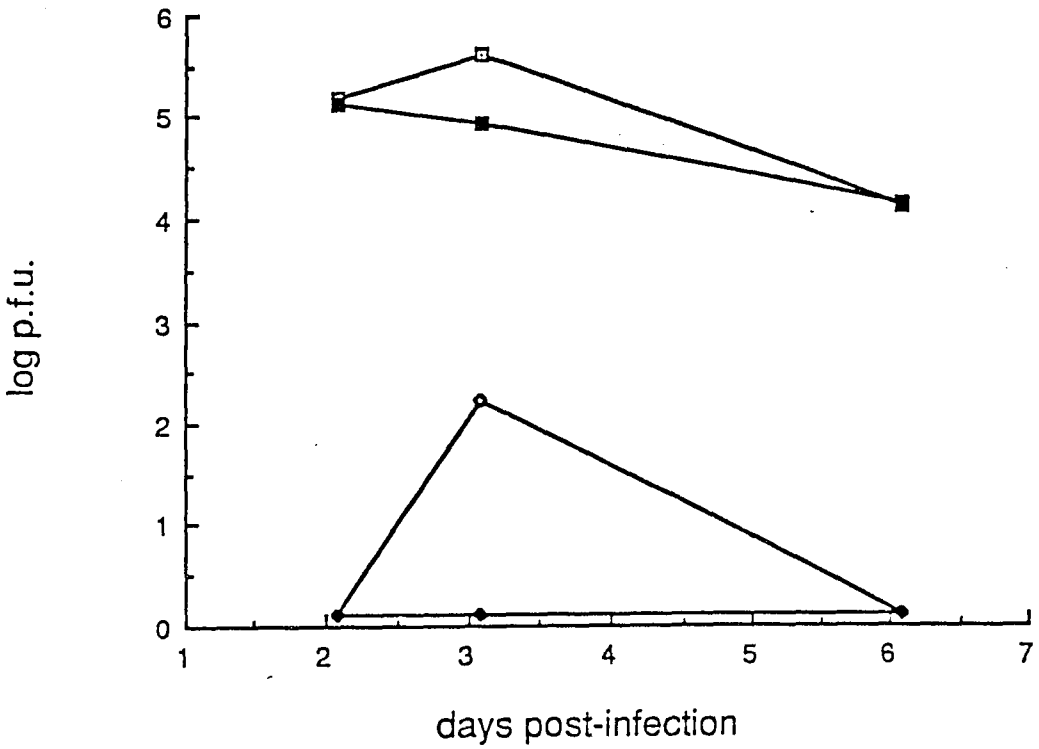


Fig.2. Southern hybridization of viral DNA with VGF. DNA from the wild-type (lanes 1, 3, 5, 7) or mutant (Z-4) (lanes 2, 4, 6, 8) was digested with HindIII (lanes 1, 2), BglI (lanes 3, 4), SalI (lanes 5, 6) and KpnI (lanes 7, 8) and separated in 0.5% agarose gel. (b) Southern blot of A hybridized with labeled-pSC16. Note that this probe hybridized only to the left terminus of the wild-type (HindIII C or BglI C fragments). M=lambda DNA digested with HindIII.

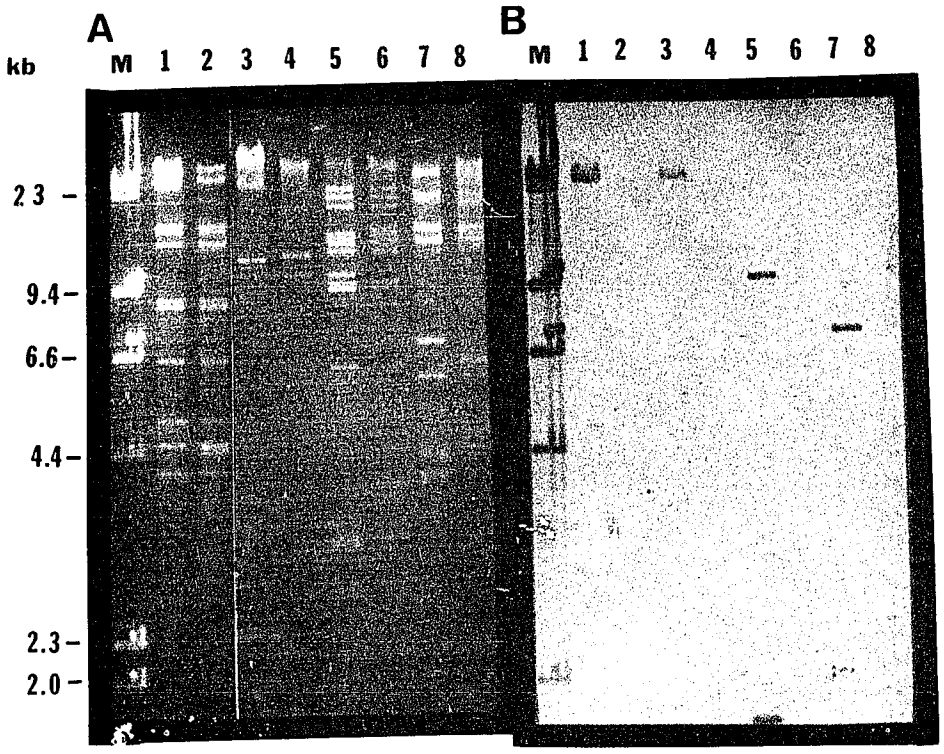
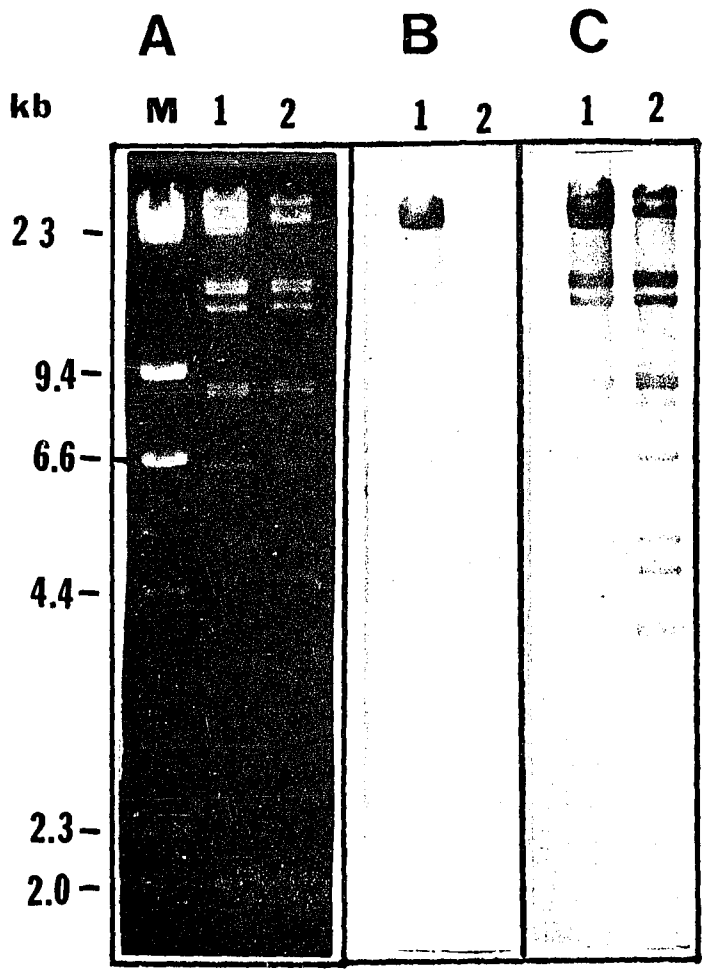

A
B
kb
M 1 2 3 4 5 6 7 8
M 1 2 3 4 5 6 7 8
23 –
9.4–
6.6–
4.4–
2.3–
2.0–

Fig. 3. Southern hybridization of HindIII restricted viral DNA. Conditions as in Fig.2. (a) Ethidium bromide stain; lane 1, wild-type; lane 2, mutant (Z-19). (b) Hybridization with labeled-pSC16. (c) Hybridzation with labeled wild-type total DNA. M=lambda DNA digested with HindIII.


A
B
C
kb
M
1
2
1
2
1
2
23
9.4
6.6
4.4
2.3
2.0

Chapter 4

Association of delayed-type hypersensitivity activity with protective immunity against vaccinia virus despite lack of cytotoxicity

Alexander C.-K. Lai*, Jerome L. Schulman*, Constantin C. Bona*, Thomas Moran* and Beatriz G.-T. Pogo **

*Department of Microbiology and ** Department of Neoplastic Diseases, Mount Sinai School of Medicine, City University of New York, New York.

Manuscript in preparation

Abstract

A better understanding of the mechanism of immunity to vaccinia infection is required before the utilization of vaccinia recombinant-based vaccines. We have isolated a deletion mutant which does not replicate in vivo and is defective in eliciting protection against lethal challenge with the wild-type. For the wild-type, since it can initiate a productive infection, immunization with 10^1 pfu is protective. However, 10^5 pfu of the mutant is required for protection for 3 days and 4 weeks immunization. When the duration of immunization was 7 days, 10^2 pfu of the mutant was protective. This protection was not due to cytotoxic T cells (T-CTL), since the mutant did not stimulate T-CTL. By contrast, delayed-type hypersensitivity (DTH) was elicited by both the wild-type and mutant viruses. The association of DTH with protective immunity despite the absence of T-CTL response suggested that protection against vaccinia may be mediated by T-DTH at 7 days post-infection.

Introduction

Vaccinia, the prototype of orthopoxvirus, is the largest of animal virus (for reveiw see (1)). Its genome consists of double-stranded DNA of 187 kb, which can code for more than 200 genes (2), of which more than 100 are structural proteins (3). The replication cycle occurs exclusively in the host cytoplasm (1). Because of the ease of insertion of foreign gene(s) by homologous recombination (4,5), vaccinia recombinats have been used as expression vectors (for review see 6-8). These vectors have been shown to induce appropiate immune response, even in the presence of pre-existing immunity (9). In addition, the relatively low production cost and the experience in mass vaccination gained during the smallpox eradication program coordinated by the World Health Organization (WHO) in the early 1970's, makes vaccinia recombinants good candidates for human vaccines. This is especially relevant in the Third World countries where communicable diseases are still great public health problems (10).

Despite the fact that vaccinia has been used as a human vaccine for a long time, its immunology is not well understood (11). Earlier work had shown that cellular immunity is important because people with thymic deficiency or other cellular defects have poor response to vaccination (12). Ultra-violet (uv) inactivated virus is less effective than live virus to induce protective immunity (13), again suggesting that cellular immunity is important. However, passive transfer of hyperimmune serum is protective, indicating that humoral immunity also plays a role. The case of generalized vaccinia in a military recruit with human immunodificiency disease (14) raised concern of safety in using vaccinia recombinant-based vaccines, and provided an impetus for better understanding of immune response during vaccinia infection.

We have previously described the isolation of several deletion mutants of vaccinia virus with attenuated phenotype (15). They are unable to replicate in vivo and are less efficient in eliciting protective immunity (16). Using one of these mutants, experiments were performed to elucidate which type of cellular immunity is important for protection. Evidence are presented in this communication that delayed-type hypersensitivity (T-DTH) response in the absence of cytotoxic T cell (T-CTL) response is sufficient to protect against a lethal challenge with the wild-type.

Materials and Methods

1) Mice

Swiss-NIH and DBA/2J female mice, age 6 to 8 weeks were inoculated as previously described and were maintained in pathogen-free conditions (16).

2) Viruses

The IHD-W strain of vaccinia virus and one of the deletion mutants, Z-19 previously described (15), were used. Viruses were propagated in L cells, purified, and infectivity determined by plaque assay as in previous publications (15,16). Uv-inactivation was carried out as described in (16).

3) Protection Experiments

Groups of 3, 6 to 8 weeks old female mice were inoculated intraperitoneally (i.p.) with increasing amount of either wild-type or mutant virus and later challenged with 10 to 15 LD_{50} of wild-type. Mortality was monitored for at least 2 weeks.

4) T-CTL assay

Immunized DBA/2J mice were sacrificed 7 days after inoculation. Spleen cells were prepared as described by Byrne et al (17) and resuspended in RPMI 1640 medium supplemented with 10% bovine serum (FBS) and

penicillin/streptomycin. P815, the target cells for the assay were prepared according to Braciale et al (18) with some modifications. Briefly, the cells were infected with 1 pfu per cell either the wild-type or the mutant for 18 h at 37 C, then washed once with PBS, and counted with trypan blue to determined viability. The target cells were then labeled with 250 mCi ^{51}Cr for 1 h at 37 C, washed twice with Hank's salt solution, followed by addition to the effector (spleen) cells at a ratio of 100 or 50 effector to target cells and incubated in a 96 round-bottom plate for 4 h at 37 C. The radioactivity in the supernatant was measured and the percent specific cytotoxicity was calculated by the formula : [(experimental release - spontaneously release)/(total release - spontaneously release)] x100%.

5) T-Helper assay

$1x10^5$, $5x10^5$ and 10^6 spleen cells from immunized mice were dispersed in 96 flat-bottom plate, 5 pfu per cell of uv-inactivated viruse were added and incubated for 5 days at 37 C. Cell proliferation was measured by adding 0.1 uCi of [3H]thymidine and further incubated for 18 h at 37 C, cells were then harvested using a cell harvestor and radioactivity determined with Beckman LS scintillation counter.

6) T-DTH assay

Groups of 4, of age 6 to 8 weeks female NIH-Swiss mice were inoculated i.p. with 10^2 and 10^6 pfu wild-type or mutant virus, after 7 days, uv-inactivated wild-type was injected into the left footpads while the right footpads were injected with the equivalent volume of Minimum Essential Medium (MEM). Footpad thickness was measured after 24 and 48 h with a circular calliper. The results were expressed as %incresed thickness of the left footpad over the right. The mice were lightly anaesthetized with ether during

all manipulations.

Results

1) Protection in vivo

Table 1 shows the results of protection experiments with different durations (3, 7, 10 and 28 days) and dose of immunization. With the wild-type, 10^1 to 10^2 pfu were already sufficient to elicit full protection, regardless of the duration of immunization. However, when mice were challenged with a lethal dose of wild-type virus after immunization with the mutant for 4 weeks, 10^5 pfu was required to be protective. When immunization was only 7 and 10 days long, 10^2 pfu was sufficient to protect. Larger dose was required (10^5 pfu) when the time of immunization was shortened to 3 days. Thus, the mutant is less efficient to elicit immunity in long term (4 weeks) or in short term (3 days) immunization. But after 7 to 10 days of infection, coincident with the activation of cellular mediated immunity (CMI) (19), less amount of the mutant is required.

2) T-CTL and T-helper assays in vitro

To investigate if the mutant elicits CMI, T-CTL and T-helper assays were performed. Fig. 1 shows that the wild-type at 10^3 and 10^6 pfu elicited significant cytotoxicity. By contrast, the mutant did not induce T-CTL response, even though the immunization doses were protective as shown in Table 1. Similar results were obtained when the target cells were infected with the mutant (data not shown), therefore, both the wild-type and the mutant infected target cells were recognized by the T-CTLs in the wild-type immunized mice, but no T-CTL was induced by the mutant. The T-CTLs were specific for vaccinia virus infected target cells, since influenza virus A/PR/8 infected target cells were not lysed (data not shown).

To see if the lack of T-CTL induction was due to a defect in T-helper cells induction, T-helper assays were performed. As shown in Fig.2, both the wild-type and the mutant elicit T-helper cells after 5 weeks of immunization. T-helper assays were also performed after 3 and 7 days of immunization. Although the controls wells showed high incorporation, probably due to the general proliferative state of the the spleen cells and not due to specific response to the antigens, there were no significant differences between the wild-type and the mutant immunized group (data not shown).

3) Footpad swelling assay

To delineate what cell type of the CMI was induced by the mutant after 7 and 10 days of infection, DTH assays, by measuring specific footpad swelling, were performed. As shown in Table 2, in all immunized mice, the mean thickness of the left footpads at 24 and 48 h after injection with uv-inactivated virus were significantly larger than the right footpads, where medium was injected. The swelling was specific since no increase in thickness was observed in the control mice. The swelling subsided quickily and was not detectable by 96 h. Therefore, all immunized groups elicited T-DTH.

Discussion

The results presented here indicated that primary immunization with the mutant for 7 days, even though no T-CTL was detected, rendered the mice resistant to lethal challenge with the wild-type. This protection is unlikely to be humoral since antibodies will not appear until late in the infection. Rather, detection of T-DTH response suggests a role for these cells in protection.

The requirement of 10^5 pfu of the mutant to protect after 4 weeks of immunization suggested that there is a 'threshold' dose. For the wild-type, since it can initiate productive infection, even at 10^1 pfu,it would eventually

reach that threshold. The protection induced by 10^2 pfu of the mutant after 7 and 10 days of infection was unexpected. It was not due to T-CTL, and this agrees with the general notion that non-replicating virus, for example, uv-inactivated or subunit vaccines, do not induce T-CTL. Although normal T-helper response was observed, this assay cannot differentiate T-helper from T-DTH responses (20). Finally, an in vivo footpad swelling test indeed indicated that the mutant, even at 10^2 pfu, induce significant T-DTH. This association of T-DTH with protection suggested that after 7 and 10 days of infection, even though T-CTL response was observed in the wild-type immunized mice, it is probably not responsible for the protection in vivo.

Lysis of viral infected target cells by T-CTL is major histocompatibility (MHC) restricted (21). With ectromelia virus infected mice, Gardner et al (22,23) detected specific cytotoxic cells as early as 2 days post-infection, reaching a peak at day 6. Similar kinetics of cytotoxicity in vaccinia virus infected mice was observed by Novembre et al (personal communication, 24). The induction of T-CTL by the wild-type vaccinia virus (described here) after 7 days of immunization agrees with the previous observations. The inability to elicit T-CTL by the mutant is not due to the lack of co-expression of viral antigen with H-2 histocompatibility antigen, since mutant virus infected target cells were lysed by wild-type immunized spleen cells (data not shown). The mutant does not replicate in vivo (16), however, whether there are some early gene expression has not been determined. It will be interesting to see whether complete virus replication, or just at some stage during the replication cycle is required to induce T-CTL in the host.

Blanden (19) showed that delayed-type hypersensitivity could be transferred with spleen cells of ectromelia virus infected mice as early as day

6. Hutt (26) detected T-DTH activity in mice immunized with vaccinia for more than 60 days. The requirement for 10^5 pfu of the mutant to protect against the wild-type after 4 weeks (Table 1) suggested that even though T-DTH can be detected, these cells may not be protective. The loss of protection may be due to the activity of suppressor T (Ts) cells which appears concurrently with humoral response (25).

Unlike the vaccinia mutants described by Esteban et al (27,28,29) which have alterations of their structural polypeptides, the deletion mutant used in this study does not differ from the wild-type (15), rendering it to be less suitable for identification of neutralizing epitopes. However, the recent findings that vaccinia virus secretes a polypeptide highly homologous to the human complement C4b binding protein (30) and encodes two polypeptides homologous to plasma serine protease inhibitors superfamily (31) suggest that non-structural proteins may play a role in evading host immune responses, and that the failure of the mutant to produce such inhibitors may render it susceptible to mechanisms such as complement mediated cytotoxicity. The mutant, Z-19, which has a deletion spaning the open reading frames (ORF) of these secretory polypeptides, provides a suitable system to study the role of these proteins in the immune response.

Further experiments are required to define the specificity and mechanism of the protective immune response elicited by the mutant 7 and 10 days after immunization. If the immune response is specific, adoptive transfer experiments in which different populations of lymphocytes are separated and transferred to naive animals would help to define which subpopulation of T cells are responsible for protection in vivo. These experiments are in progress.

References

1. Dales S, Pogo BGT. In: Biology of Poxviruses.(Kingsbury DW, Zur Hausen H ed) Virology Monographs 1981;vol. 18. Springer-Verlag, New York.

2. Moss B. Replication of poxviruses, 685-703 In: Virology (Fields BN. ed) 1986,Raven Press, New York.

3. Essani K, Dales S. Biogenesis of vaccinia virus:Evidence for more than 100 polypeptides. Virology 1979;95:385-94.

4. Panicali D, Paoletti E. Construction of poxviruses as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad USA 1982;79:4927-31.

5. Mackett M, Smith GL, Moss B. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J Virol 1984;49:857-64.

6. Piccini A, Perkus ME, Paoletti E. Vaccinia virus as an expression vector. Methods Enzymol 1987;153:545-63.

7. Mackett M, Smith GL. Vaccinia virus expression vectors. J Gen Virol 1986;67:2067-82.

8. Moss B, Flexner C. Vaccinia expression vectors. Ann Rev Immunol 1987;5:305-24.

9. Flexner C, Murphy BR, Rooney JF, Wohlenberg C, Yuferov V, Notkins AL, Moss B. Successful vaccination with a polyvalent live vector despite existing immunity to an expressed antigen. Nature 1988;335:259-62.

10. Assaad F. World Health Organization Programme for Vaccine Development. In: Vaccine 1987. Cold Spring Harbor Laboratory, p.445-7.

11. Hutt LM. The immune response to infection with vaccinia virus in mice. I. Infection and the production of antibody neutralizing cell-associated and cell-free virus. J.Hyg.,Camb. 1975;74:301-14.

12. Fulginiti VA, Kempe CH, Hathaway WE, et al. Progressive vaccinia in immunological deficient individuals. In: (Bergsma D, Good RA eds.) Immunological Deficient Diseases in Man. New York, National Foundation, 1968.

13. Boulter EA, Zwartouw HT, Titmuss DHJ, Maber HB. The nature of the immune state produced by inactivated vaccinia virus in rabbits. American J Epidemiology 1971;94:612-20.

14. Redfield RR, Wright DC, James WD, Jones TS, Brown C, Burke DS. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N Eng J Med 1987;316:673-6.

15. Lai ACK, Pogo BGT. Characterization of vaccinia virus deletion mutants isolated from persistently infected Friend erythroleukemia cells. Virus Res 1989;12:239-50.

16. Lai ACK, Pogo BGT. Attenuated deletion mutants of vaccinia virus lacking the vaccinia growth factor are defective in replication in vivo. Microbial Pathogenesis 1989;6:(in press)

17. Byrne JA, Soloski M, Holowczak JA. Immune responses of DBA/2J mice bearing melanoma tumors: Cell-mediated immune responses after challenged with vaccinia virus. Cancer Immunology and Immunotherapy 1983;16:81-7.

18. Braciale BM, Henkel TJ, Lukacher A, Braciale VL. Fine specific and antigen receptor expression among influenza virus-specific cytotoxic T lymphocyte clones. J Immunology 1986;137:995-1002.

19. Blanden RV. Mechanisms of recovery from a generalized viral infection: Mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells.J Exp Med 1971;133:1074-89.

20. Greene MI, Schatten S,Bromberg JS. Delayed Hypersensitivity.In: Fundamental Immunology (Paul WE ed) Raven Press, New York,1984. p.685-96.

21. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomenigitis within a syngeneic or semiallogeneic system. Nature 1974;248:701-2.

22. Gardner I, Bowern NA, Blanden RV. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. Eur J Immun 1974;4:63-7.

23. Gardner I, Bowern NA, Blanden RV. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. II. Identification of effector cells and analysis of mechanisms. Eur J Immun 1974;4:68-72.

24. Novembre F, Natuk R, Holowczak JA. The immune response to infection with vaccinia virus in mice. Meeting abstract, 6th workshop on poxvirus/iridovirus, 1986, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

25. Hutt LM. The immune response to infection with vaccinia virus in mice. II. Appearance of hypersensitivity, production of macrophage migration inhibitory factor and transformation of spleen cells in response to virus antigens. J Hyg.,Camb. 1975;74:315-27.

26. Ramshaw I, Bretscher PA, Parish CR. Regulation of the immune response. I. Suppression of delayed-type hypersensitivity by T cells from mice expressing humoral immunity. Eur J Immun 1976;6:674-9.

27. Paez E, Dallo S, Esteban M. Virus attenuation and identification of structural proteins of vaccinia virus that are selectively modified during virus persistence. J Virol 1987,61:2642-2647.

28. Maa J-S, Esteban M. Structural and functional studies of a 39,000-Mr immunodominant protein of vaccinia virus. J Virol 1987;61:3910-9.

29. Rodriguez JF, Paez E, Esteban M. A 14,000-Mr envelpoe protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. J Virol 1987;61:395-404.

30. Kotwal GJ, Moss B. Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 1988;335:176-8.

31. Kotwal GJ, Moss B. Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitors superfamily. J Virol 1989;63:600-6.

Table 1

Determination of protective dose of vaccinia wild-type and Z-19 mutant in primary immunization by i.p. inoculation

		Duration of Immunization			
		3 days	7 days	10 days	4 weeks
		Survival/Total			
Control		0/3	0/3	0/3	0/3
Wild-type	10^1	2/3	2/3	3/3	2/3
	10^2	3/3	3/3	3/3	3/3
	10^3	3/3	3/3	3/3	3/3
	10^4	ND	ND	ND	ND
	10^5	ND	ND	ND	ND
	10^6	ND	ND	ND	ND
Z-19	10^1	ND	ND	ND	ND
	10^2	0/3	3/3	3/3	0/3
	10^3	0/3	3/3	3/3	0/3
	10^4	0/3	3/3	3/3	0/3
	10^5	3/3	3/3	3/3	3/3
	10^6	ND	3/3	3/3	ND

ND: not done
Groups of 3 Swiss-NIH female mice, age 6 to 8 weeks, were inoculated with the wild-type or with Z-19 as described in Materials and Methods. At times indicated post-infection, they were challenged with 10 to 15 LD_{50} of wild-type and mortality monitored for 2 weeks.

Fig.1. T-CTL assay of primary immunized DBA/2J female mice. Six to 8 weeks old female DBA/2J mice were inoculated i.p. with 10^3 or 10^6 of the wild-type or Z19. At 7 days post-infection, groups of 3 mice were sacrificed and spleen cells were prepared and mixed with P815 target cells. They were incubated for 4 h at 37 C, and %cytotoxicity was calculated as described in Materials and Methods.

T-CTL ASSAY OF PRIMARY IMMUNIZED DBA/2J FEMALE MICE

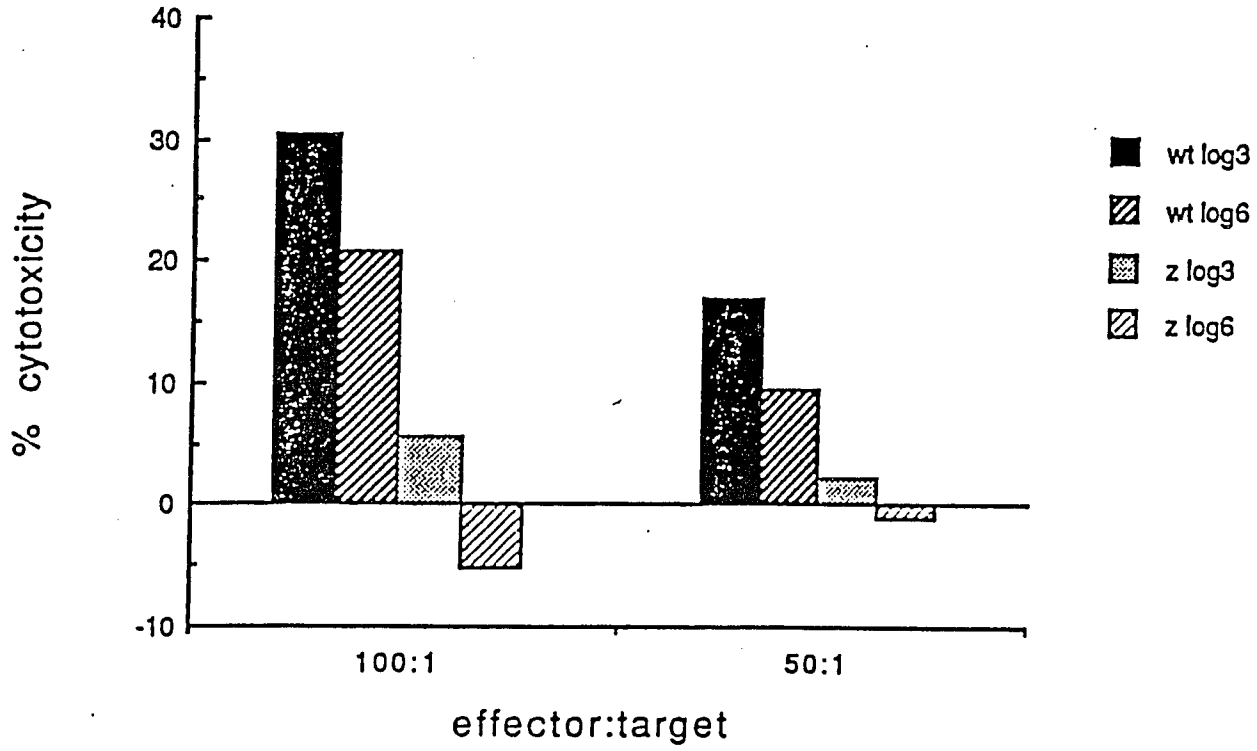


Fig.2. T-helper assay of primary immunization. Six to 8 weeks old NIH-Swiss female mice were inoculated i.p. with 10^6 PFU of the wild-type or Z19. After 5 weeks, the mice were sacrificed and spleen cells were prepared. 10^4 to 10^5 cells were cultivated in 96 wells microtiter plate. The cells were stimulated by uv-inactivated viruses at m.o.i. of 5 for 5 days and stimulation was monitored by [^{3}H]thymidine uptake. Z19/wt: immunization with Z19 and stimulated by uv-inactivated wild-type; Z19/Z19: immunized with Z19 and stimulated by Z19; wt/wt: immunized with wild-type and stimulated by wild-type; wt/Z19: immunized with wild-type and stimulated by Z19.

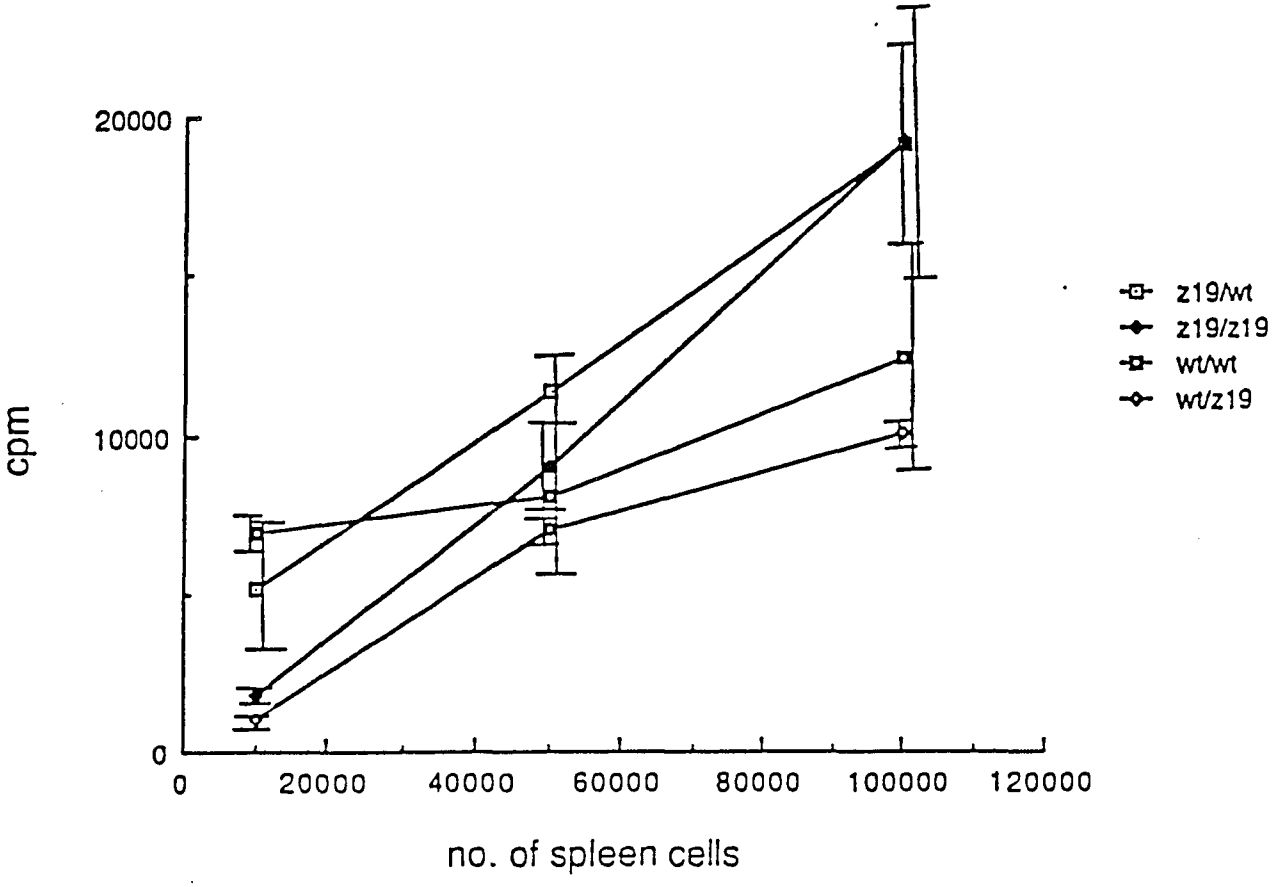

20000
10000
0
cpm
0
20000
40000
60000
80000
100000
120000
no. of spleen cells
z19/wt
z19/z19
wt/wt
wt/z19

Table 2

Footpad swelling assay in primary immunization with vaccinia wild-type and Z-19 mutant

		% increase in thickness +/- s.d.	
		24 h	48 h
control		3.1+/-2.7	0.0+/-5.0
wild-type	10^2 pfu	34.5+/-50.8	7.5+/-6.1
	10^6 pfu	28.1+/-12.0	12.2+/-10.2
Z-19	10^2 pfu	27.2+/-19.1	12.8+/-5.7
	10^6 pfu	17.9+/-9.8	14.1+/-4.1

Groups of 4, of age 6-8 weeks old NIH-Swiss female mice were injected with the viruses i.p.. 7 days later, 0.05 ml of uv-inactivated wild-type virus was injected into the left footpads and equivalent volume of medium injected into the right footpads. Footpad thickness was measured 24 and 48 h later, and increased thickness of the left footpad was expressed as the % thickness of the right footpad. s.d.=standard deviation.

Chapter 5

Unpublished results

I. Introduction

This chapter will describe experimental data that were not included in the previous chapters. They were addressed to answer the following questions: A) To compare the in vivo growth characteristics of the wild-type and the mutant viruses; B) to find out whether the mutants interfere replication of the wild-type; C) to determine whether the mutants produce any growth factors; D) to characterize another orthopox virus, Indiana, regarding the presence of VGF gene and virulence; and finally, E) to re-insert the VGF gene into the mutants, so that a better understanding of the mechanism of virulence can be attained.

II. Results and Discussion

A. Comparison of in vivo growth

i. Animal inoculation

It was previously shown (chapter 3) that the deletion mutants did not replicate in the livers and spleens of inoculated mice. It was therefore of interest to investigate their growth in other tissues and in younger mice. It has been shown that vaccinia virus induced pox in young suckling mice after intradermal (i.d.) inoculation. To find out whether the mutants also induce pox in suckling mice, groups of 3, 3 to 4 days old suckling mice were inoculated i.d. with 10^2 and 10^6 pfu of the wild-type or the mutant Z-19. Two out of 3 mice injected with 10^2 pfu of the wild-type died; one at 8 days p.i. and the other at 9 days p.i.. All mice died in the group injected with 10^6 pfu of the wild-type (at 5 and 6 days p.i.), and typical pox appeared in these mice as shown in Fig.1. All the mice injected with the mutant (at both inocula) remained healthy.

Scarification of adult mouse tails with 10^6 pfu of the wild-type resulted in characteristic necrotic lesions, as shown in Fig.2A, and in some mice, the part of the tail was so necrotic that the tip of the tail fell off as shown in Fig. 2B. None of these effects were observed in mice inoculated with mutant viruses.

ii. Sensitivity to interferon

Vaccinia virus has been shown to be resistant to interferon (Youngner et al 1972, Esteban 1986). However, the possibility that the mutant was susceptible to interferon, thus affecting its replication in vivo was considered. To test this hypothesis, in vitro interferon sensitivity assays were performed. As shown in Fig. 3A and 3B, the mutant replicate to the same extent in the absence or presence of interferon. Similar results were obtained even at interferon concentrations as high as 2000 units. Therefore, even though it has such a large deletion, it remains resistant to interferon. These results suggest that the gene(s) responsible for interferon resistance in vaccinia virus does not reside in this region of the genome. This also rule out the possibility that the mutants do not replicate in vivo because of sensitivity to interferon.

Taken together, the results described in section A indicate that the inability of the mutants to replicate in vivo is not due to particular susceptibility to host immunity, but rather to a block in replication per se. What stage of the viral replication cycle is blocked, remains to be determined.

B. Interference assays

Defective interfering (DI) mutants are common in many viruses, mainly negative stranded RNA viruses. To test if the deletion mutants were able to interfere with replication of the wild-type, L cells were coinfected with both

the wild-type and the mutant. Fig.4, shows that the replication curve is the same as either of the viruses. Although no effort was made to identify the progeny viruses, it was assummed that both the wild-type and the mutant were present. Similar results were obtained at different m.o.i. with either the wild-type or the mutant viruses.

To investigate if interference takes place in vivo, coinoculation experiments were performed. Coinjection of 10^6 pfu of the mutant Z-19 with 10^6 pfu of the wild-type appears to interfere the virulence of the wild-type, since the LD_{50} of the wild-type is 10^6 pfu , half of the mice were expected to be killed. However, all 4 mice survived, as indicated in Table 1. The interference seemed to take place in the spleen, since no protection occurred in the splenectomized mice, although these mice were not more susceptible to the wild-type.

Interpretation of the results on inteference in vivo should be made with caution since the number of mice used in these experiments was small. However, together with the results of in vitro interference, they suggested that the mutant probably was not interfering replication of the wild-type per se, but rather inducing stronger immune response in the host. In chapters 2 and 3, it has been shown that the mutants do not replicate in vivo, however, protective immunity was elicited after 7 to 10 days of primary immunization. After inoculation with 10^6 pfu of the wild-type, the majority of inoculated mice died after 7 to 10 days later. The protection observed by coinoculation may be due to immunity elicited by the mutant. This may be similar to what occurs with other viruses, for example, Dimmock et al (1986) had shown that protection by DI mutants of influenza virus was not due to interference of the replication of the wild-type, but rather to modulation of the immune

response.

C. Detection of VGF gene expression by immunoprecipitation

Experiments were also carried out to find out if the mutant produce secreted or non-secreted VGF gene product.

When the non-structural polypeptides isolated from the cytoplasm of wild-type or mutant (Z-19) infected L cells shortly after infection were compared by electrophoresis in SDS-polyacrylamide gels, specific viral peptides appeared and increased to about the same level as shown in Fig.5. Since VGF is an early gene and has been shown to be translated and secreted early (Stroobant et al 1985) , immunoprecipitation of the same cytoplasmic extract using rabbit anti-epidermal growth factor (EGF) polyclonal antiserum was performed. As shown in Fig.6B (lane 3), a specific band of MW 25 kDa, which corresponds to the membrane-associated VGF (Chang et al 1988) was precipitated in the wild-type infected cells extract at 5 h p.i., whereas none was observed in the mutant infected cells. It is concluded that the mutant does not produce any VGF gene product, confirming experiments in which the cloned VGF gene did not hybridized to the mutant DNA.

D. Characterization of Indiana

Studies were extended to another orthopoxvirus used in our laboratory, designated Indiana, which was originally isolated from a tumor in a rabbit (Chan and Hodes 1973). It has been found to be highly cytolytic in vitro, however, it does not produce cytopathic effects (CPE) nor prominent plaques in L cells. Hybridization of Indiana DNA with labeled VGF gene (pSC16) revealed that it has two copies of the VGF gene, as the WR strain of

vaccinia, since the probe hybridized to both HindIII B and C fragments (Fig.7). In addition, the hybridization signal was much stronger than the wild-type of IHD-W strain although similar quantities of DNA were employed. These results suggest that Indiana is more related to the WR strain of vaccinia than to the IHD-W strain.

To investigate how the presence of VGF gene affects the biological activity of the virus, the LD_{50} in NIH-Swiss female mice was determined. As shown in Table 2, Indiana was found to be highly virulent. In fact, its LD_{50} is significantly lower than that of the IHD-W wild-type for both i.p. or intranasal (i.n.) inoculation.

The finding that Indiana contains two copies of the VGF gene was interesting. Although Indiana was found to be tumorigenic in rabbits, it is highly cytolytic for several cell lines in culture, but do not produce CPE nor distinct plaques in L cells (Pogo et al 1982). However, careful examination of plaque assays carried out in L cells indicated that very tiny plaques were produced. One interpretation of this finding, as proposed by Buller et al (1988), is that the VGF activates surrounding uninfected cells, thus the 'plaques' become smaller because uninfected cells divide and occupied the area previously occupied by the lysed infected cells. The observed induction of subcutaneous tumors in rabbits after inoculation with Indiana could also be explained by the expression of the VGF gene.

E. Rescue experiments

The absence of VGF, inability to replicate in vivo and attenuated phenotype of the mutants as shown in chapter 3 was only a correlation. To have a better understanding of the mechanism of virulence and to find out if

other gene(s) play a role, rescue experiments in which DNA fragments from the wild-type were transfected into L cells infected with the mutants were performed. Attempts were also made to clone the mutant terminal fragment so that it can be used as a specific insertion vector for cloned VGF gene or other genes.

i.Marker rescue with wild-type HindIII C fragment

Since VGF resides in HindIII C fragment of the wild-type, this fragment was electroeluted from agarose gel and after phenol extraction, was used in rescue experiments. After selection of recombinants in mice (described in Materials and Methods), two recombinants, designated Z19HC2M1 and Z19N6M1 were obtained.

Fig.8 shows the results of southern blot hybridization of DNA extracted from the recombinants with the labeled VGF gene. The probe hybridized only to Z19HC2M1 DNA (Fig 8B, lanes 1 and 2). The restriction pattern of this DNA suggests a mixed population, although retaining the restriction pattern of Z19, the hybridization pattern resembles that of the wild-type Fig.1, chapter 3). Nevertheless, inoculation of 10^8 pfu of this recombinant into mice resulted in death, suggesting that virulence was partially recovered.

ii. Cloning of terminal K' fragment

It has been shown that in the HindIII C fragment other viral genes are present, therefore, the results of rescue experiments should be interpreted with caution. To provide specific insertion of test gene and facilitate site specific homologous recombination, cloning of the K' fragment of Z-19 mutant was attempted.

The protocol in initial attempts to clone the HindIII K' fragment of Z19 was essentially that of Pickup et al (1983). Briefly, the K' fragment was

electroeluted from agarose gel after electrophoresis, it was then treated with S1 nuclease, flushed blunt-end with T4 polymerase and ligated to pUC19 linearized with SmaI. However, no clones with inserted fragments was recovered.

Later experiments employing the protocol of Campbell et al (1989) with slight modification, as described in Materials and Methods, resulted in isolation of several clones. DNA extracted from four of such clones were transferred to nitrocellulose filter and probed with labeled wild-type DNA. As shown in Fig.9, lanes 1 to 8 contain DNAs from the four clones, digested with EcoRI or BglII. Lanes 9 to 12 contain DNAs from pSC16 and pAG5 used as positive controls. Only the positive control DNAs (pSC16 and pAG5) were hybridized to wild-type DNA. The absence of hybridization of labeled wild-type DNA to the 4 clones suggested no viral DNA were present in these plasmids.

Results of rescue experiments using HindIII C fragment prove to be promising. However, as discussed earlier, the presence of other genes complicates the interpretation. Cloning of the K' fragment which would facilitate insertion of the VGF gene into the mutant has been so far unsuccessful. One of the reasons why cloning was unsuccessful could be due to peculiar properties of this terminal fragment. For example, the structure of the terminal hairpin loop may be different from the wild-type, and may require different conditions for enzymatic treatments. It is worth mentioning that these deletion mutants differ from other deletion mutants because: 1) they are the largest spontaneously generated deletion mutants described to date; 2) the size of the deletion remains stable after serial passages; 3) there are additional alterations in the right terminal fragment. Cloning of the K'

fragment will help to have a better understanding of genomic structure of this mutant, as well as allowing re-insertion of genes.

In conclusion, results with Indiana and from rescue experiments strongly suggested that VGF is important for virulence in vivo. Further experiments are necessary to delineate the mechanism of this gene and virulence.

III. **Materials and Methods**

A.Materials

Procedures used to grow L cells, vaccinia virus wild-type and two deletion mutants were described in chapter 2. Mouse interferon alpha and beta ($4.2X10^5$ International Units /mg protein) were purchased from Sigma and rabbit anti-EGF polyclonal antiserum were from Collaborative Research. S1 and Micrococcus Nuclease were purchased from Boehringer Mannheim. Mungbean nuclease was purchased from BioLabs, New England. Protein A-Sepharose beads were obtained from Pharmacia. pUC19, DH5-alpha cells, T4 polymerase and T4 ligase were obtained from BRL. Swiss-NIH mice were bred in our laboratory and maintained in pathogen free condition. Two plasmids, pSC16 and pAG5, containing the entire VGF gene and the 3.5 kb termini fragment of the wild-type (WR strain), respectively were gifts from Dr. B. Moss of NIAID.

B. Methods

i. Preparation of labeled cytoplasmic extract

Monolayers of infected L cells were incubated with 5 uCi/ml of [^{35}S]methionine for 60 min. at 37 C. At the times indicated, cells were scrapped with a rubber policemen,centrifuged, washed once with phosphate

buffer, resuspended in 0.5 ml lysis buffer (1% Triton in 10 Tris-HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA with 1 uM PMSF) kept in ice for 20 min., and then homogenized with a Dounce homogenizer. The nuclear fraction was separated by centrifugation.

ii. Immunoprecipitation

Aliquots containing 100 ul of cytoplasmic extracts, prepared as described above, were incubated with 10 ul pre-immune serum for 18 h at 0 C. Then 50 ul of a protein A-Sepharose bead suspension in 10% Tris-HCl pH 7.8 was added, the mixture further incubated for 1 h at 4 C and then micro-centrifuged for 15 min. and 10 ul of the antiserum was added to the supernatant and incubated for 2 h at 4 C. Immune complexes were then precipitated by adding 50 ul of protein A-Sepharose suspension, centrifuged, and the precipitates washed 3 times with a solution containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% Triton, 0.5% Na deoxycholate and 0.1% SDS. Finally, the precipitates were resuspended in 50 mM Tris-HCl, pH7.5 with 1% SDS and 0.5% beta-mercaptoethanol, boiled for 5 min. at 100 C before loading in 12% polyacrylamide gel. Electrophoresis was carried out at 100 V for 3 h.

iii. Marker rescue

Approximately 10 ug of the wild-type HindIII C fragment was electroeluted from agarose gels and phenol extracted. Marker rescue experiments were carried out as described by Panicali et al (1982) Although there are no selectable markers for these recombinants, previous results indicated that only the wild-type virus which contains the VGF gene can replicate in the livers of inoculated mice. Therefore, to select for recombinants that acquired the VGF gene and express the wild-type

phenotype, recombinants were selected in vivo as illustrated in Fig.10. Recombinants were then screened by a rapid screening protocol described below.

iv.Rapid screening of recombinants

Confluent monolayers of L cell plates were infected with the recombinant at m.o.i. of 5. After overnight incubation at 37 C, the cells were scrapped, centrifuged, washed once with PBS, resuspended in 450 ul hypotonic buffer (10 mM Tris-HCl. pH 7.8 and 12 mM KCl) for 20 min at 0 C, homogenized with a Dounce homogenizer, and transferred to a 1.5 ml Eppendorf tube. 50 ul of 10X reaction buffer (50 mM Tris-HCl,pH 7.9, 10 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.1% NP-40 and 50% glycerol), 0.5 ul of 2.0 M $CaCl_2$ and 10 ul of Micrococus Nuclease (15 units per ul) were added. The reaction was carried out for 1 h at 25 C and stopped by adding 2 ul of 0.5 M EGTA.

Viral DNA was extracted by adding 50 ul of 5% Sarkosyl and 10 ul of proteinase K (1mg/ml) and incubated for 1 h at 25 C, followed by treatment with phenol and ethanol precipitation. About 2 to 5 ug of viral DNA from a 50 mm infected L cells plate were recovered.

This procedure utilize the digestion of cellular DNA in situ. Because viral DNA is protected by the virion polypeptides, it can be extracted after the enzymatic treatment with the Micrococus Nuclease.

v. Cloning of HindIII K' fragment

The cloning strategy of Campbell et al (1989) to clone the terminal fragments was followed with few modifications. About 10 ug DNA from the mutant Z19 was first treated with S1 nuclease, or in some cases, with mungbean nuclease. Incubation was for 30 min. at 50 C as recommended by

the manufacturer, followed by phenol extraction and finally digestion with HindIII for 2 h at 37 C. 1 ug of the vector, pUC19, was digested with HindIII and HincII for 2 h at 37 C. After restriction, both the mutant and vector DNAs were phenol extracted. They were then recombined in a 20 ul ligation reaction mixture containing 2 units of T4 ligase and incubated for 16 h at 15 C.

vi. Minipreparation for plasmid screening

A slight modification of the alkaline lysis method of plasmid DNA extraction described by Birnboin and Dolly (1977) was performed. Briefly, 1.5 ml of a bacterial culture grown overnight was microcentrifuged for 20 s, then resuspended in 100 ul alkaline lysis solution I (50 mM glucose, 25 mM Tris-HCl,pH 8.0 and 10 mM EDTA). 200 ul of alkaline solution II (made from 20 ul 10 N NaOH and 50 ul 20% SDS in 1.0 ml H_2O) was added and the mixture incubated for 5 min. at 0 C. Then 150 ul of 5 M potassium acetate, pH 6.0 was added, mixed by inversion and incubated for 5 min. at 0 C. The mixture was then microcentrifuged for 3 min., 250 ul phenol was added to the supernatant and mixed, followed by addition of 200 ul of chloroform. The aqueous phase was saved after centrifugation for 1 min., and the DNA precipitated with 0.9 ml absolute ethanol. About 5 to 10 ug DNA were recovered from 1.5 ml of bacterial culture.

Reference

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Res. 7:1513-1523.

Buller RML, Chakrabarti S, Moss B, Fredrickson T (1988) Cell proliferative response to vaccinia virus is mediated by VGF. Virology 164:182-92.

Campbell JIA, Binns MM, Tomley FM. Boursnell MEG (1989) Tandem repeat sequences within the terminal region of the fowlpox virus genome. J Gen Virol 70:145-154.

Chan JC, Hodes ME (1973) Mechanism of Shope fibroma virus-induces suppression of host deoxyribonucleic acid synthesis. Infection and Immunity 7:532-538.

Chang W, Lim J G, Hellstrom I, Gentry L E (1988) Characterization of vaccinia virus growth factor biosynthetic pathway with an antipeptide antiserum. J Virol 62:1080-1083.

Dimmock NJ, Beck S, McLain L (1986)Protection of mice from lethal influenza: evidence that defective interfering virus modulates the immune response and not virus multiplication. J Gen Virol. 67:839-850.

Esteban M, Benavente J, Paez E (1986) Mode of sensitivity and resistance of vaccinia virus replication to interferon. J Gen Virol 67:801-808.

Panicali D, Paoletti E (1982) Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci USA 79:4927-4931.

Pickup DJ, Bastia D Joklik WK (1983) Cloning of the terminal loop of vaccinia virus DNA. Virology 124:215-217.

Pogo BGT, Freimuth P Stein A (1982) Shope fibroma virus. I. Biological and molecular properties of a cytocidal and non-cytocidal strain. J Virol. 41:97-103.

Stroobant P, Rice AP, Gullick WJ, Cheng DJ, Kerr IM, Waterfield MD (1985) Purification and characterization of vaccinia virus growth factor. Cell 42:383-93.

Youngner J, Thacore H, Kelly M (1972) Sensitivity of ribonucleic acid and deoxyribonucleic acid viruses to different species of interferon in cell culture. J Virol 10:171-181.

Fig.1. Induction of pox in young suckling mice by vaccinia virus. The mouse was inoculated at the right footpad with 10^6 pfu wild-type, it died 5 days post-infection. Arrow indicates the site of pox.

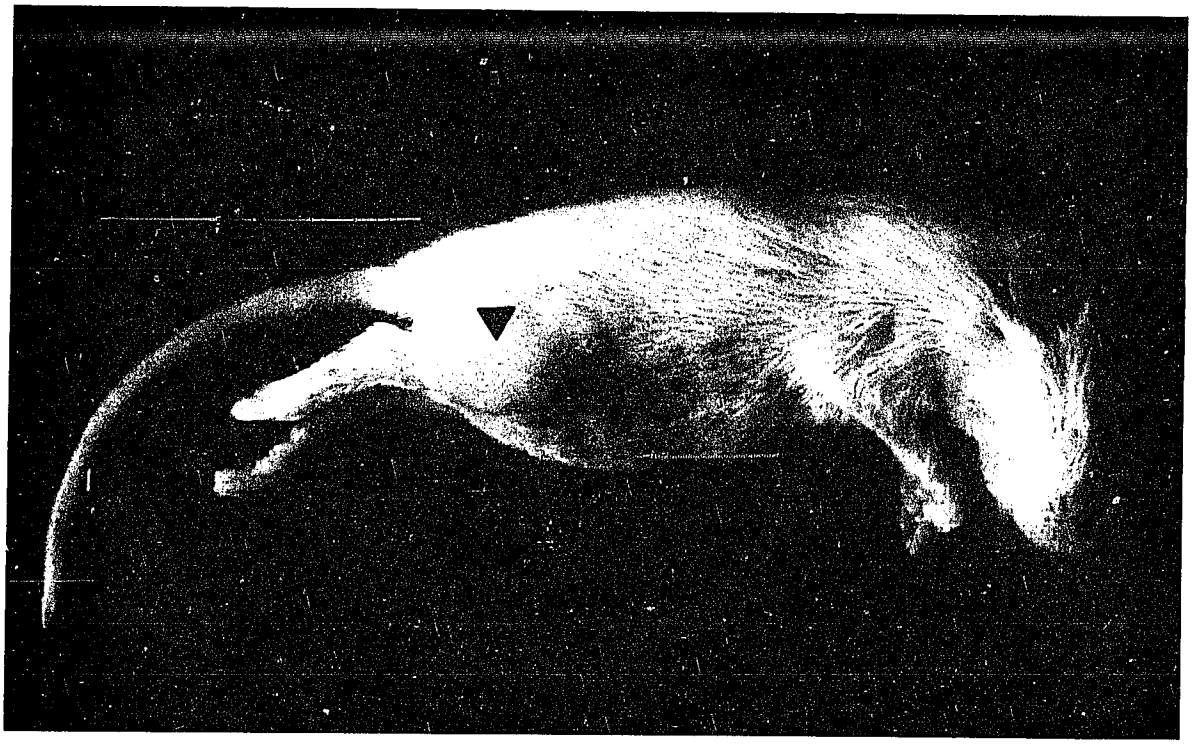


Fig.2. Necrotic lesion formation after scarification of mouse tails. A) post-infection 4 days: 1, control; 2, inoculated with 10^6 pfu of Z19; 3, inoculated with 10^6 pfu wild-type. Arrow indicates the lesion in the wild-type inoculated tail. B) post-infection 28 days: tail scarified with 10^6 pfu wild-type.

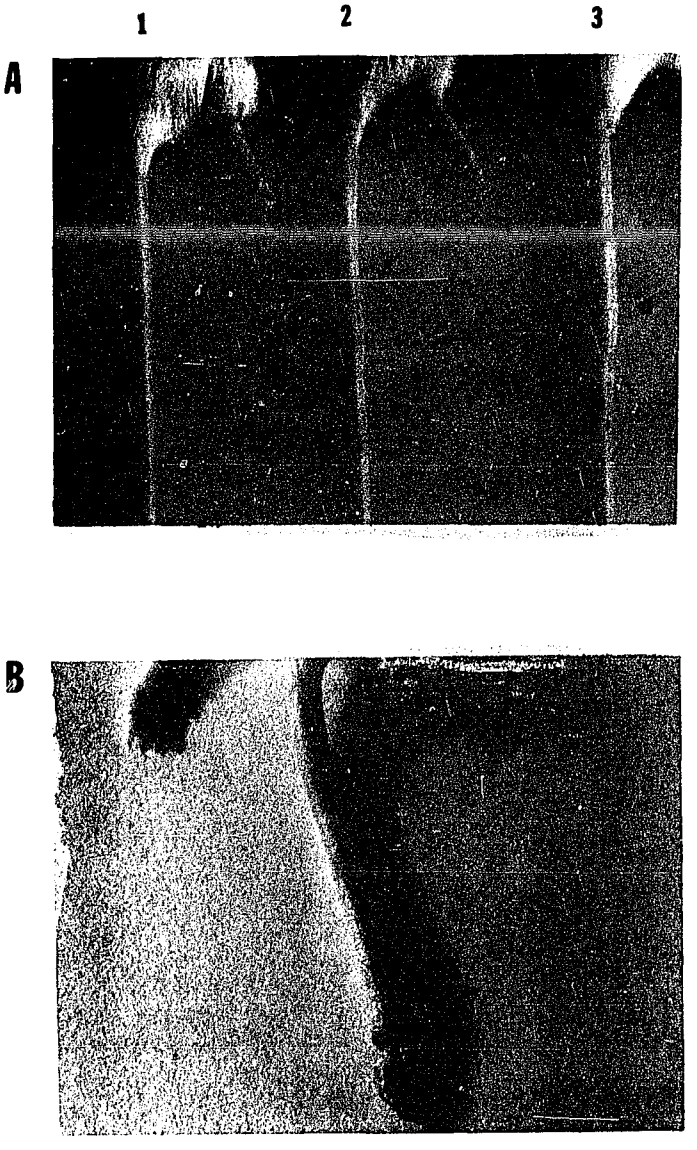
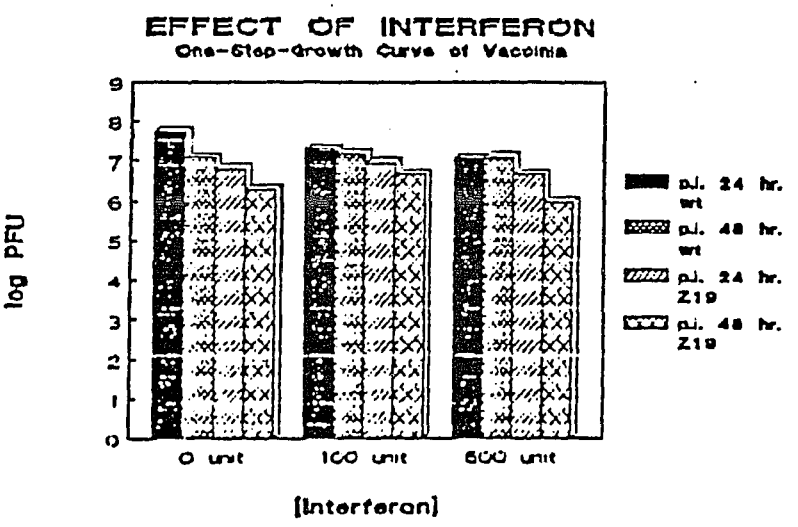

1
2
3
A
B

Fig.3. Effect of Interferon alpha and beta on replication of vaccinia virus in vitro. L cells were infected with either the wild-type of with Z19 at m.o.i. of 5. A) Interferon added at 0 h post-infection; B) Interferon added to L cells 24 h before infection.

A


EFFECT OF INTERFERON
One-Step-Growth Curve of Vaccinia
log PFU
0
1
2
3
4
5
6
7
8
9
0 unit
100 unit
500 unit
[Interferon]
p.i. 24 hr. wt
p.i. 48 hr. wt
p.i. 24 hr. Z19
p.i. 48 hr. Z19

B

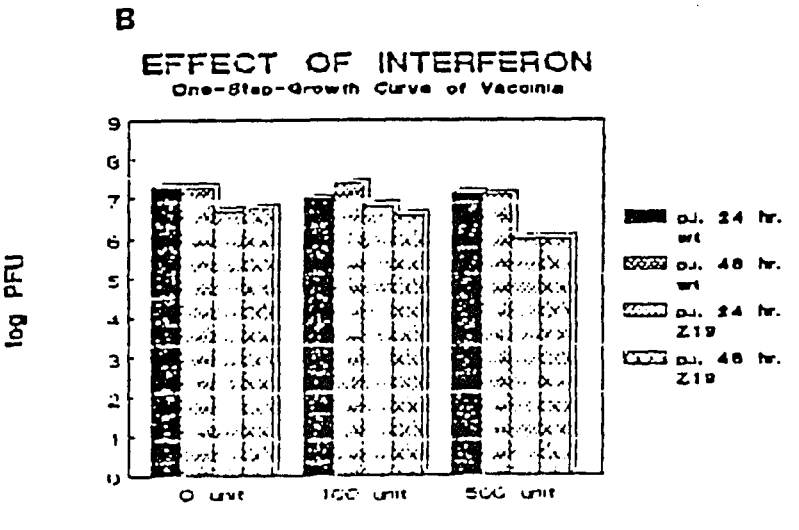

EFFECT OF INTERFERON
One-Step-Growth Curve of Vaccinia
log PFU
0
1
2
3
4
5
6
7
8
9
0 unit
100 unit
500 unit
[Interferon] pre-treated 24 hr.
p.i. 24 hr. wt
p.i. 48 hr. wt
p.i. 24 hr. Z19
p.i. 48 hr. Z19

Fig.4. Effect of co-infection of vaccinia viruses in L cells. Cells were infected with either wild-type at m.o.i. of 5, or co-infected with Z19, also at m.o.i. of 5.

In vitro interference: one-step growth curve in L cells

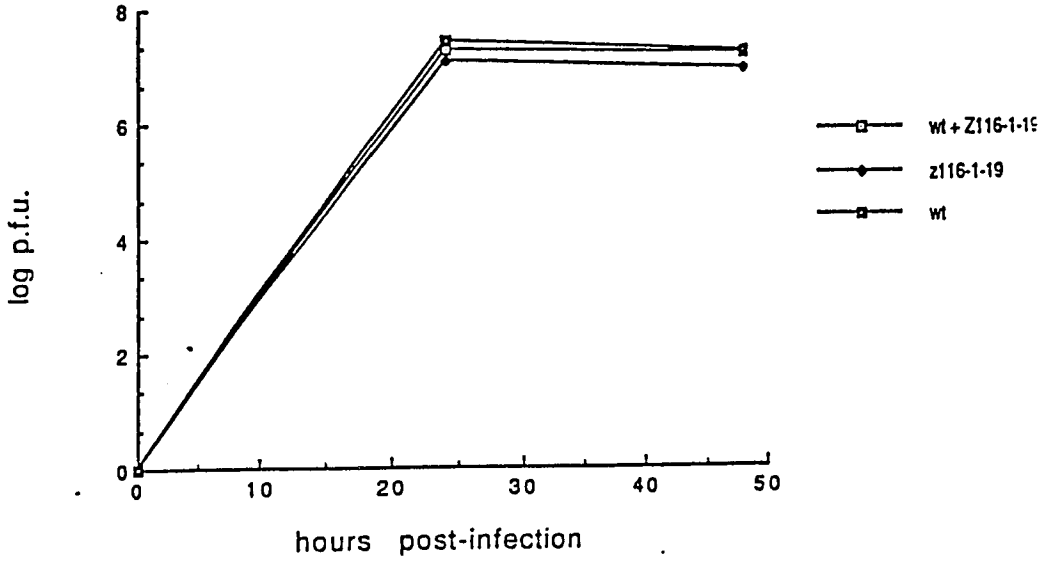


Table 1

Effect of co-inoculation intraperitoneally of vaccinia virus in vivo.

PFU inoculum		Survival/total
wild-type	10^5	4/4
	10^6	2/4
	10^7	1/4
Z19	10^6	4/4
wild-type 10^5 + Z19 10^6		4/4
wild-type 10^6 + Z19 10^6		4/4
wild-type 10^7 + Z19 10^6		0/4
wild-type 10^7 + Z19 10^7		0/4
Splenectomized mice:		
wild-type 10^6 + Z19 10^6		2/4

Fig.5. SDS-PAGE of cytoplasmic extract of vaccinia virus infected L cells. L cells were infected with the wild-type or Z19 at m.o.i. of 5, and cytoplasmic extraction were performed as described in Materials and Methods. Lanes 1 to 3, control; lane 4 to 6, wild-type and lanes 7 to 9, Z-19 infected cells. Lanes 1, 4 and 7, at 2 h p.i.; lanes 2, 5 and 8, at 4 h p.i.; lanes 3, 7 and 9, at 8 h p.i.. Arrows indicate viral specific polypeptides.

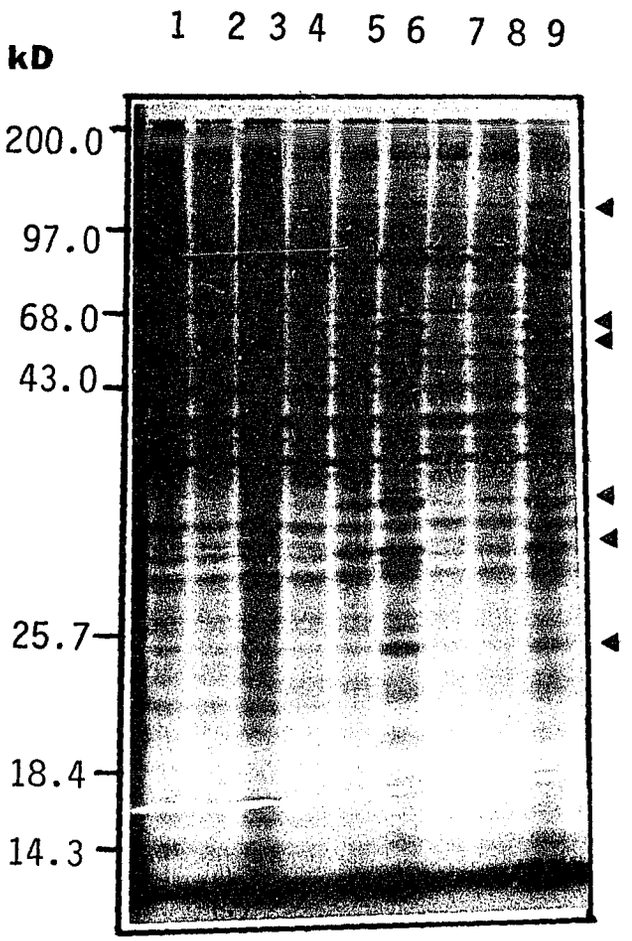

1 2 3 4 5 6 7 8 9
kD
200.0
97.0
68.0
43.0
25.7
18.4
14.3

Fig.6. Immunoprecipitation of cytoplasmic extract from infected L cells with anti-EGF polyclonal antiserum. A) Without immunoprecipitation. Lane 1, control; lane 2 and 3, wild-type infected cells; lane 4 and 5, Z19 infected cells. Lanes 2 and 4, post-infection 2 h; lanes 1, 3 and 5, p.i. 5.5 h. B) Immunoprecipitation of A.

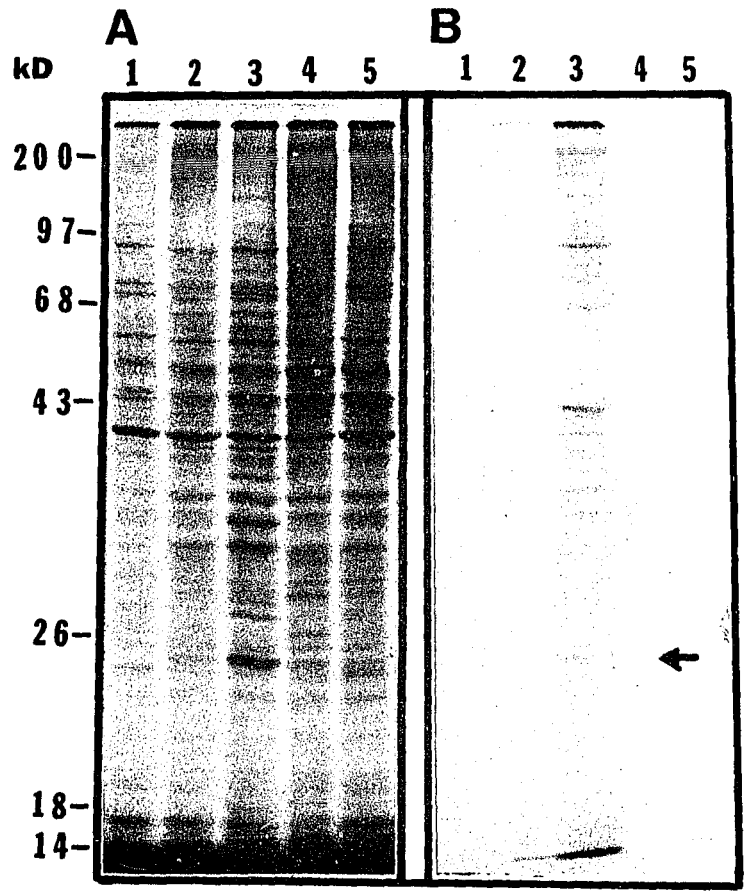

A
B
kD
1 2 3 4 5
1 2 3 4 5
200
97
68
43
26
18
14

Fig.7. Southern hybridization of Indiana DNA with VGF. A) Ethidium bromide stain. M: lambda digested with HindIII. lane 1 to 3, viral DNA digested with HindIII; lane 4 to 6, digested with PstI. IHD-W wild-type: lane 1 and 4; Indiana: Z19: lane 2 and 5; Indiana: lane 3 and 6. B) Hybridization of A with pSC16.

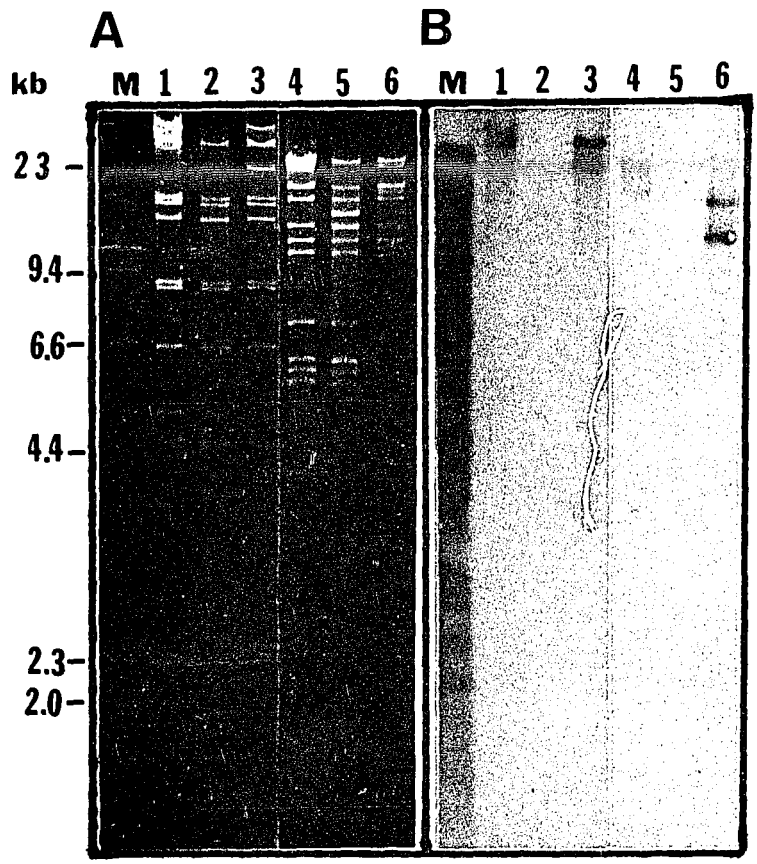

A
B
kb
M 1 2 3 4 5 6
M 1 2 3 4 5 6
23
9.4
6.6
4.4
2.3
2.0

Table 2

Comparison of LD_{50} of vaccinia wild-type (IHD-W), mutant z19 and Indiana in NIH-Swiss female mice

virus	PFU inoculum at LD_{50}		
	i.p.	i.n.	i.c.
WT	1.2×10^6	3.2×10^4	$<10^1$
z19	$>10^9$	$>10^6$	5.6×10^2
Indiana	1.8×10^4	1.6×10^3	$<10^1$

Fig.8. Southern hybridization of recombinants from rescue experiments with VGF. A) Ethidium bromide stain. M: 1 kb DNA ladder; lanes 1 and 2, Z19HC2M1; lanes 3 and 4, Z19N6. Lanes 1 and 3, viral DNA digested with SalI; lanes 2 and 4, viral DNA digested with KpnI. B) Southern blot hybridization of A with labeled pSC16.

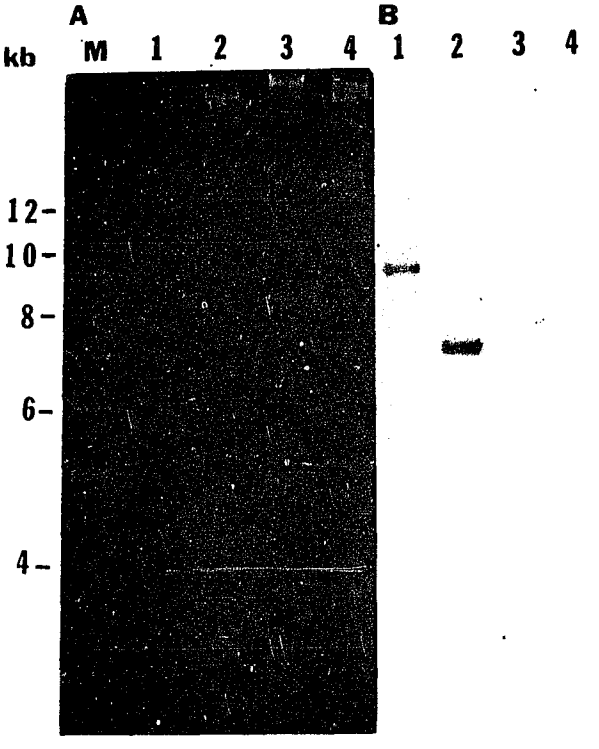

A
B
kb
M
1
2
3
4
1
2
3
4
12-
10-
8-
6-
4-

Fig.9. Southern hybridization of plasmids from cloning of Z19 K' fragment. A) Ethidium bromide stain. M: 1 kb DNA ladder. Lanes 1 and 2, clone #1; lanes 3 and 4, clone #2; lanes 5 and 6, clone #4; lanes 7 and 8, clone #5; lanes 9 and 10, pSC16; lanes 11 and 12, pAG5. Plasmid DNAs were digested with EcoRI (lanes 1, 3, 5, 7, 9, 11) or with BglII (lanes 2, 4, 6, 8, 10, 12) and separated by electrophoresis in a 0.6% agarose gel. B) Southern blot hybridization of A with labeled wild-type DNA.

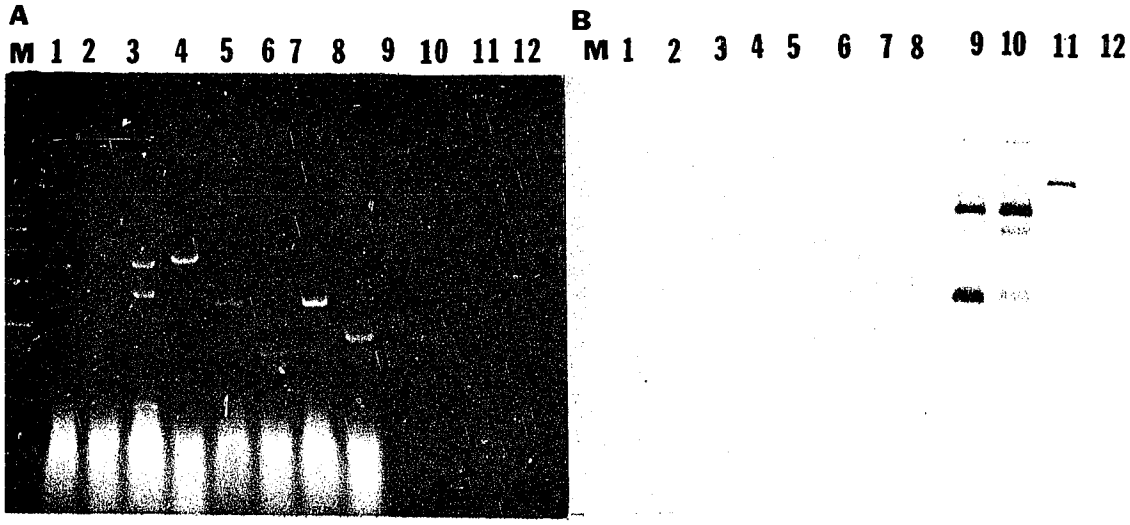
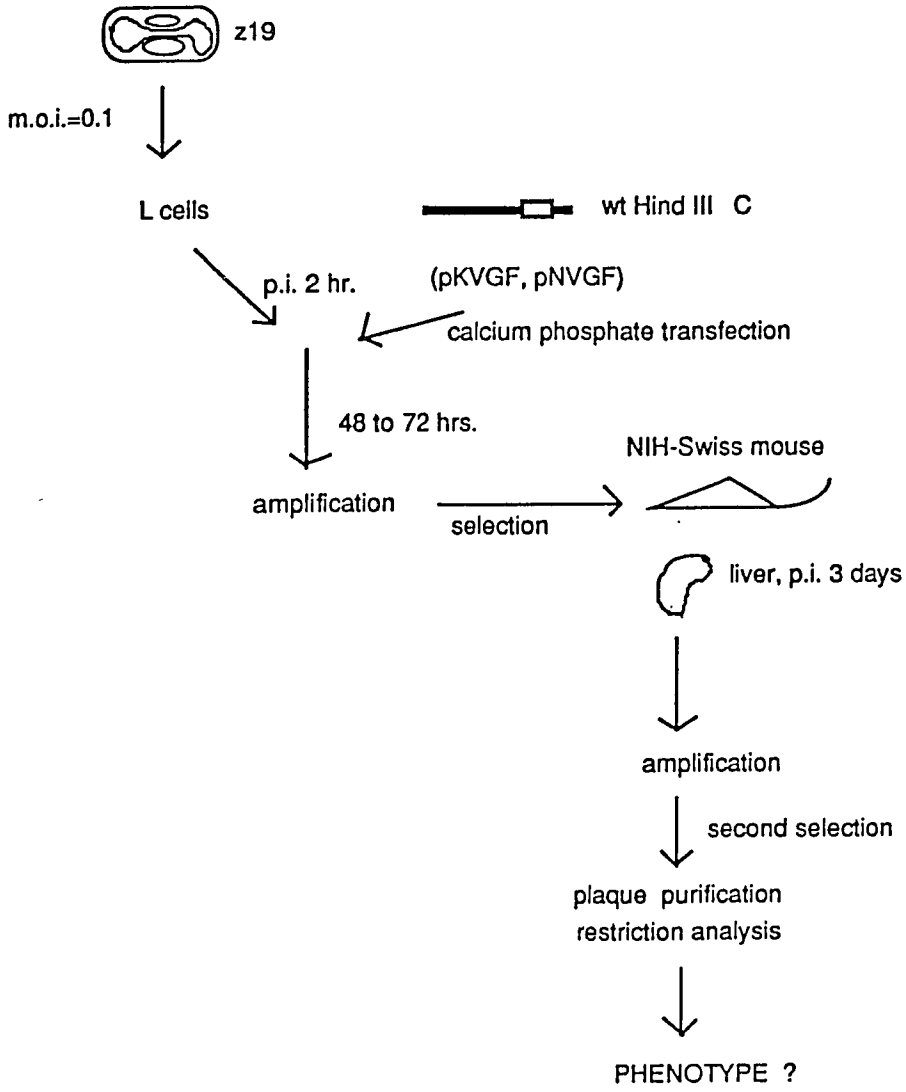

A
M 1 2 3 4 5 6 7 8 9 10 11 12
B
M 1 2 3 4 5 6 7 8 9 10 11 12

Fig.10. General scheme of isolation of recombinants in rescue experiments.

INSERTION OF VGF INTO THE DELETION MUTANTS



Chapter 6

Discussion

The results of the studies on the deletion mutants described here provide an insight into :A) the evolution of deletion mutants of vaccinia virus and the effect of the deletion in virulence; B) the role of the VGF gene in viral replication in vivo; C) the immunological response during infection and D) the effect of rescue of the deletion mutants with the restriction fragment containing the VGF gene.

As shown in chapter 2, a deletion of 12% of the genome at the left side had no major effect on the in vitro properties of the mutant viruses. However, it affected their in vivo properties significantly, as indicated by lack of replication and pathogenicity in mice. A comparison of published deletion mutants generated spontaneously is summarized in Fig. 1. The correlation between deletion and virulence suggested that gene(s) located in the genome where the deletion occurred are involved in virulence. Indeed, one of the genes, the VGF gene, has recently been shown to play a role in virulence (chapter 3, Buller et al 1988). Other genes, which are located at the left terminus, such as a complement C4b binding protein (Kotwal and Moss 1988a) and two plasma serine protease inhibitors (Kotwal and Moss 1989) may also play a role in virulence.

Deletion mutants are not uncommon in poxviruses. Fenner noticed that after inoculation of cowpox or rabbitpox viruses into chorioallantoic membrane (CAM) of eggs, white pock variants occurred frequently and might represent up to 1% of the cowpox virus progeny (Fenner 1958). These white pock variants had been examined recently and found to have deletions and duplications in their genome (Moyer et al 1980, Pickup et al 1984). The 9 kb deletion mutant described by Moss et al (1981), which was originally thought to be a simple deletion, has recently been found to have a duplication as well

(Kotwal and Moss 1988b).

The mechanism by which deletions are generated is not understood at the moment. Several models have been proposed. Moyer et al (1980) suggested that deletions resulted from a single nonhomologous recombination, and duplication was due to unprecise unit length deletion of head to head or tail to tail concatameric replicative intermediates. Pickup et al (1984) proposed a model of nonreciprocal transfer of DNA between the terminal fragments. Intramolecular exchange would result in deletions and duplications, while intermolecular exchange would also form simple deletions. The view of Panicali et al (1981) that the unique sequences in the L variant was from cellular DNA was provocative at that time. However, recent findings of sequence homology of genes located in this region with cellular genes may shed light on that interpretation.

The Z mutants described here were generated in a Friend erythroleukemia cell line (FEL) persistently infected with vaccinia virus (Pogo and Friend 1982). Although vaccinia DNA was found in the nucleus of the persistently infected host cell (Obom et al 1986), there was no evidence for interactions between the host genome and the viral DNA. It was concluded that viral DNA was simply 'trapped' in the nucleus due to rapid host cell division.

Paez et al (1985) reported that generation of a 8 MDa deletion mutant of vaccinia virus in a similar persistently infected FEL cell line, was suppressed by interferon (IFN) (Paez and Esteban 1985). They proposed that either IFN had direct effects on the mechanism generating the deletion, or IFN selected against the deletion mutants. The latter possibility is unlikely. Results from experiments conducted in our laboratory indicated that the

deletion mutants were not sensitive to interferon (chapter 5), implying that the gene(s) responsible for resistance to interferon in vaccinia virus is unlikely to be located in the region spaning the deletion. This agrees with the fact that IFN did not 'cure' the persistent infection of FEL cells by the deletion mutant (Paez and Esteban 1985)

The Z mutants were isolated form the persistently infected cell line that had been serially passaged in vitro. In another subclone of the original persistently infected cell line, a mutant with a smaller deletion was isolated at an earlier passage. However, after serial passages in L cells, the restriction pattern of this mutant became similar to that of the wild-type. Whether it was a true revertant or the mutant population was originally contaminated by the wild-type is not clear. Mutants with different size deletions could be isolated from different subclones of the persistently infected cell line. Larger deletion mutants were isolated at later passages suggesting that evolution of deletion mutants may be a continous process. Experiments with cloned persistently infected cell line followed by constant monitoring of the restriction pattern of vaccinia virus isolated would help to understand the mechanism of generation of these deletion mutants.

The finding that the lack of the VGF gene correlated with inability to grow in vivo and hence the attenuated phenotype (chapter 3) implied that comparison of properties in vitro may not necessary correlated with those observed in vivo. To show that the lack of VGF gene in the mutant affects the growth in vitro, the use of tissue culture medium without addition of growth factors is required. Growth factors are usually present in the bovine calf serum that supplement the medium. However, a minimum amount of serum is necessary for the wild-type to replicate, suggesting that the expression of

the VGF gene alone is not sufficient to support growth of the virus.

Although vaccinia had been used as a human vaccine for almost two centuries, the immune response to vaccination is not fully understood. The mutants were found to be less efficient to elicit protective immunity after 3 days and 4 weeks of primary immunization. However, after 7 and 10 days of immunization, 10^2 pfu of Z19 induced protective immunity (chapter 4). Results from in vitro T-CTL assays (chapter 4) indicated that T-CTLs were elicited by the wild-type, and that they were specific for vaccinia virus, since they did not lyse A/PR/8 influenza virus infected target cells. No T-CTL was elicited by the mutant Z19 at both low (10^3 pfu) or high (10^6 pfu) inocula, even though protective immunity was induced. The lack of T-CTL induction by Z19 can be explained by the inability of this mutant to replicate in vivo. However, delayed-type hypersensitivity (DTH) was readily demonstrated by both the wild-type and the mutant, suggesting that T-DTH may be associated with protection.

Hypersensitivity was first observed by Jenner himself (Fenner 1979). The association of T-DTH with in vivo protection described in chapter 4 agrees with the results of others (Bladden 1971, Hutt 1975, Aoyama et al 1986) that suggested the activation of T-DTH by vaccinia virus may be important for protection. T-DTH was elicited by the Z mutants even though they were unable to replicate in vivo, implying that T-DTH induction may not require productive viral infection, as oppose to T-CTL induction. Also, these results suggested that T-CTL plays a minor, if any, role in protection.

At what stage the replication of the mutants is blocked in vivo has not yet been determined. Whether there is early transcription of the Z mutants in vivo, or the viral structural proteins are able to elicit T-DTH, remains to be

determined. The answer to these questions will be helpful in the design of vaccinia-based expression vectors. It is important to determine whether the foreign antigen has to be placed under an early or a late transcriptional control element to be effective in inducing immunity.

Several attempts to engineer safe vaccinia recombinant vaccines have been reported. Shida et al (1988) used the LC16mO strain, an attenuated strain of vaccinia virus isolated from the parental Lister (Elstree) strain (LO) with temperature-sensitivity and lower neurovirulence. Rodriguez et al (1989) employed the attenuated deletion mutants isolated from a persistently infected FEL cell line as discussed earlier. Ramshaw et al (1987) and Flexner et al (1987) incorporated murine and human interleukin-2 (IL-2) gene, respectively, in vaccinia virus to modulate the immune response of the host. The activation of host cellular immunity by this approach resulted in protection. Taylor et al (personal communication) used fowlpox virus recombinants to vaccinate non-avian species. Protective immunity was elicited. Fowlpox virus do not replicate in species other than avian, therefore, the results indicate that first, no virus replication is required to induce immunity, and second, this approach would circumvent the obstacles of vaccinia-based vectors, that is, virulence and spread of the virus from vaccinees.

The interference effect observed by coinoculation with the Z mutant and the wild-type (chapter 5) suggested another approach to reduce the virulence of vaccinia. That is, by coinjection of the Z mutants with recombinant vectors, the immune response specific for vaccinia is enhanced, reducing the virulence of the virus while the induction of immunity to the heterologous antigen should not be affected.

Finally, the high virulence displayed by in Indiana virus, which is

probably due to higher expression of the VGF gene, and the results of the rescue experiments, in which virulence was conferred to the deletion mutants, indicated that the VGF gene plays an important role in virulence. The finding of a growth factor gene in a cytolytic virus was 'a surprising catch' as expressed by Spriggs (1986). Similar genes had been found in Molluscum contagiousum (Porter and Archard 1987), myxoma (Upton et al 1987), Shope fibroma (Chang et al 1987), and fowlpox (Campbell et al 1989). Growth factors have been detected in other viral infections such as those produced by cytomegalovirus (Gonczol and Plotkin 1984), and simian sarcoma virus (Klein and Thiel 1988). EGF-like domains were also identified in the DNA sequence of the surface protein of the sporozoite of Plasmodium falciparum, the causative agent of human malaria (Kaslow et al 1988). Therefore, it seems that growth factors are widely spread among pathogens, and that understanding their mode of action could lead to better ways of controlling pathogenicity.

Because of the extensive genomic relatedness between vaccinia and variola (Esposito and Knight 1985), a similar VGF gene is expected to be present in variola. However, experiments to find out if variola virus also encode and express the VGF gene may not be possible. At present, only two W.H.O. Collaborating Centers still have the smallpox virus, one is the Center for Disease Control (CDC) in the United States, and the other is the Research Institute of Viral Preparations in Moscow, Soviet Union. Since there were suggestive evidence indicating smallpox was used as a biological weapon in the past (Poupard et al 1989), the destruction of the remaining stocks of variola virus seems justified. However, this will deprive any future study of the virus.

Reference

Aoyama A, Yoshioka T, Sato S, Mizushima Y, Ogata M, Ueda S, Kato S, Fujiwara H, Hamaoka T. (1986) Role of L3T4 antigen in the activation of various functions of Lyt-1+2- T cells against vaccinia virus. Microbiol Immunol 30:799-810.

Blanden RV.(1971) Mechanism of recovery from a generalized viral infection: Mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. J Exp Med 133:1074-1089.

Buller RM, Chakrabarti S, Cooper JA, Twardzik DR,Moss B. (1988) Deletion of the vaccinia virus growth factor gene reduces virus virulence. J Virol 62:866-874.

Campbell JIA, Binns MM, Tomley FM. Boursnell MEG (1989) Tandem repeat sequences within the terminal region of the fowlpox virus genome. J Gen Virol 70:145-154.

Chang W,Upton C, Hu SL, Purchio AF, McFadden G.(1987) The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and transforming growth factor alpha Mol Cell Biol 7:535-540.

Esposito JJ, Knight JC.(1985) Orthopoxvirus DNA: a comparison of restriction profiles and maps. Virology 143:230-251.

Fenner F.(1958) The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. Virology 5:502-529.

Fenner F.(1979) Portraits of viruses: The poxviruses. Intervirology 11:135-157.

Flexner C, Hugin A, Moss B. (1987) Prevention of vaccinia virus infection in immunodificient mice by vector-directed IL-2 expression. Nature 330:259-262.

Gonczol E, Plotkin SA.(1984) Cells infected with human cytomegalovirus release a factor(s) that stimulates cell DNA synthesis. J Gen Virol 65:1833- 1837.

Hutt LM.(1975) The immune response to infection with vaccinia virus in mice.II. Appearance of hypersensitvity, production of macrophage migration inhibitory factor and transformation of spleen cells in response to virus antigens. J Hyg, Camb 74:315-327.

Kaslow DC, Quakyi IA, Syin C, Raum MG, Keister DB, Coligan JE, McCutchan TF, Miller LH.(1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. Nature 333:74-76.

Klein R, Thiel H-J.(1988) Highly glycosylated PDGF-like molecule secreted by simian sarcoma virus-transformed cells. Virology 164:403-410.

Kotwal GJ, Moss B. (1988a) Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 335:176-8.

Kotwal GJ, Moss B. (1988b) Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. Virology 167:524-537.

Kotwal GJ, Moss B.(1989) Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitors superfamily. J Virol 63:600-6.

Moss B, Winters E, Cooper JA. (1981) Deletion of a 9000-base-pair segment of the vaccinia virus genome that encodes nonessential polypeptides. J Virol 40:387-395.

Moyer RW, Graves RL, Rothe CT. (1980) The white pock (u)mutants of rabbit poxvirus. III. Terminal DNA sequence duplication and transposition in rabbit poxvirus. Cell 22:545-553.

Obom KM, Popple SW, Holland JG, Pogo BGT. (1986) Vaccinia virus DNA sequences in the nucleus of persistently infected Friend erythroleukemia cells. Virus Res 5:221-234.

Paez E, Dallo S, Esteban M. (1985) Generation of a dominant 8-MDa deletion at the left terminus of vaccinia virus DNA. Proc Natl Acad Sci USA 82:3365-69.

Paez E, Esteban M. (1985) Interferon prevents the generation of spontaneous deletions at the left terminus of vaccinia virus DNA. J Virol 56:75-84.

Panicali D, Davis SW, Mercer SR, Paoletti E. (1981) Two major DNA variants presented in serially propagated stocks of the WR strain of vaccinia virus. J Virol 37:1000-1010.

Pickup DJ, Ink BS, Parsons BL, Hu W, Joklik WK. (1984) Spontaneous deletions and duplications of sequences in the genome of cowpox virus. Proc Natl Acad Sci USA 81:6817-6821.

Pogo BGT, Friend C. (1982) Persistent infection of Friend erythroleukemia cells with vaccinia virus. Proc Natl Acad Sci USA 79:4805-4809.

Porter CD, Archard LC (1987) Characterization and physical mapping of Molluscum contagiosum virus DNA and location of a sequence capable of encoding a conserved domain of epidermal growth factor J Gen Virol 68:673-682.

Poupard JA, Miller LA, Granshaw L. (1989) The use of smallpox as a biological weapon in the French and Indian War of 1763:Smallpox may have been spread deliberately to an Indian population known to be vulnerable to its ravages. ASM News 55:122-124.

Ramshaw IA, Andrew ME, Phillips SM, Boyle DB, Coupar BEH. (1987) Recovery

of immunodificient mice from a vaccinia virus/IL-2 recombinant infection. Nature 329:545-546.

Rodriguez D, Rodriguez J-R, Rodriguez JF, Trauber D, Esteban M. (1989) High attenuated vaccinia virus mutants for the generation of safe recombinant viruses. Proc. Natl. Acad. Sci. USA 86:1287-1291.

Shida H, Hinuma Y, Hatanaka M, Morita M, Kidokoro M, Suzuki K, Maruyama T, Takahashi-Nishimaki F, Sugimoto M, Kitamura R, Miyazawa T, Hayami M. (1988) Effects and virulence of recombinant vaccinia viruses derived from attenuated strains that expressed the human T-cell leukemia virus type I envelope gene. J Virol 62:4474-4480.

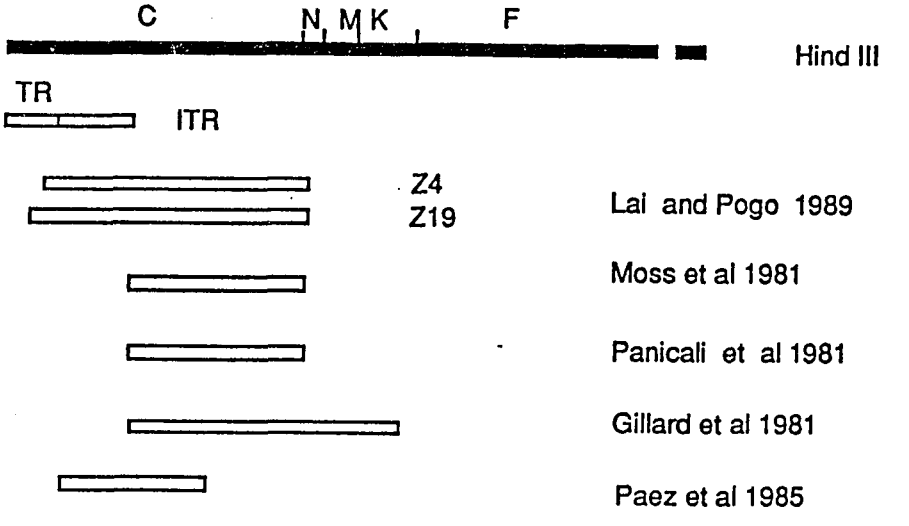
Spriggs DR.(1986) Vaccinia virus growth factor:A surprising catch. J Infect Dis 153:382-383.

Taylor J, Weinberg R, Languet B, Desmettre P, Paoletti E. (1988) Use of fowlpox virus recombinant to vaccinate non-avian species. Meeting abstract, 7th international meeting on poxvirus/iridovirus, Heidelberg, West Germany, 1988.

Upton C, Macen JL, McFadden G.(1987) Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. J Virol 61:1271-1275.

Fig.1. A comparison of deletion mutants of vaccinia virus. Only the left terminal portion is shown.

Deletion mutants of vaccinia



	Size/ kb
Lai and Pogo 1989	21
Moss et al 1981	9
Panicali et al 1981	9
Gillard et al 1981	18
Paez et al 1985	12