JOURNAL OF VIROLOGY, Mar. 1994, p. 1737–1749
0022-538X/94/$04.00+0

Vol. 68, No. 3

Multigenic Evasion of Inflammation by Poxviruses

GREGORY J. PALUMBO,[1]* R. MARK BULLER,[1] AND WAYNE C. GLASGOW[2]

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892,[1] and Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709[2]

Received 23 September 1993/Accepted 6 December 1993

Analyses of different cowpox virus (Brighton Red strain [CPV-BR]) mutants indicate that there is a minimum of three genes encoded by CPV-BR that are nonessential for virus replication in tissue culture but are involved in inhibiting the generation of an inflammatory response in the chicken embryo chorioallantoic membrane (CAM) model. The CPV-BR-encoded anti-inflammatory genes include the gene encoding the 38-kDa protein (also called 38K, crmA, SPI-2, or VV-WR-ORF-B13R), a tumor necrosis factor receptor homolog, and an unidentified gene that maps to the right end of the CPV genome. The kinetics of triggering of an inflammatory response at the site of virus infection as well as the magnitude of the response is dependent on the virus-encoded inhibitor that is deleted. Virus yields recovered from pocks decreased in proportion to the magnitude of the inflammatory response. The deletion of these identified inhibitors of inflammation was associated with attenuation of the mutant viruses in mice. These data confirm the existence of multiple poxvirus-encoded host defense modifiers whose function is to block the generation of an inflammatory response at the site of virus infection, which allows enhanced virus replication and potentially facilitates virus transmission.

The first virus-encoded gene product shown to inhibit the generation of an inflammatory response was the 38-kDa protein (also termed 38K, SPI-2, crmA, and VV-WR-ORF-B13R) of cowpox virus (strain Brighton Red [CPV-BR]) (40). The loss of this single open reading frame (ORF) of CPV changed the wild-type red lesions (pocks) which develop on the chorioallantoic membranes (CAMs) of 12-day-old chicken embryos to white lesions because of a massive inflammatory response (heterophilic and monocytic infiltrate) (23, 40). Because chicken embryos lack a functional immune system at this stage of development (10–12, 18), analyses of virus-encoded genes which may be directly involved in the inhibition of the generation of an inflammatory response are possible. Importantly, the inflammatory cell infiltrate is associated with a severe reduction in virus replication in the CAM (40).

The mechanism by which the 38-kDa protein inhibited the generation of an inflammatory response was suggested by two recent studies which indicated that the 38-kDa protein may possess two functional activities. It is associated with inhibition of dihydroxyeicosatetraenoic acid (diHETE) (a leukotriene) formation from the lipoxygenase pathway of arachidonic acid metabolism (39) and with inhibition of the interleukin 1 (IL-1)-converting enzyme (44). Both pathways are important components of the inflammatory cascade. Moreover, this former biochemical pathway is also an essential cellular component of poxvirus replication (38), and elevation of this pathway following poxvirus infection occurs rapidly (39).

Other poxviruses often give pock morphologies that are intermediate between those of CPV-BR and CPV-BR.D1 (which lacks the gene encoding the 38-kDa protein) on the CAMs of the 12-day-old chicken embryos. One example is the WR strain of vaccinia virus (VV), which induces large muddy white pocks on chicken embryo CAMs. The morphology of the WR-induced pocks is due to ectodermal and endodermal cell proliferation induced by the epidermal growth factor homolog, VV growth factor (VGF) (6). The loss of VGF from the WR strain of VV, however, still induces small whitish pocks on the CAMs of chicken embryos, suggesting that VV lacks an inhibitor or possesses an elicitor of inflammation. In total, these observations suggest that orthopoxviruses such as VV may lack a functional 38-kDa protein or a gene product(s) whose function is inhibition of the generation of an inflammatory response at the site of virus infection, unlike CPV-BR, which induces no inflammation and possesses a full complement of anti-inflammatory genes for the chicken embryo model.

Several poxvirus-encoded genes which possess homology to mammalian proteins that could be expected to inhibit host defense mechanisms have been discovered. To date, poxvirus ORFs with homology to IL-1 type 2 receptor (50), tumor necrosis factor receptor (TNF-R) (27, 28, 49), gamma interferon receptor (53), and the C4-binding protein (34) have been reported. The functional significance and mode of action of these genes in vivo is poorly understood, but there is some evidence to suggest that the loss of these molecules influences the pathogenicity of poxviruses in vivo (2, 29, 51, 52). Cytokines such as TNF-alpha and IL-1 serve as inflammatory mediators through the induction of other inflammatory molecules such as leukotrienes (diHETEs) by the lipoxygenase pathway (44, 47); thus, it is possible that this pathway is the target of poxvirus-encoded inhibitors at the site of virus infection.

The ability to monitor orthopoxviruses' effects on arachidonate metabolism, and specifically to measure the level of diHETE formed after infection as a means of determining whether there is a functional 38-kDa protein (39), allows the identification of poxviruses which give rise to whitish pocks on the CAMs of chicken embryos but which induce an inflammatory response through a 38-kDa-protein-independent mechanism. The objectives of this study were, therefore, to determine the status of the 38-kDa protein in different orthopoxviruses, to determine whether there was an inflammatory response

* Corresponding author. Mailing address: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 4, Room 137, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 496-1370. Fax: (301) 480-1147.

induced by viruses with a functional 38-kDa protein, and to identify 38-kDa-protein-independent inhibitors of inflammation.

MATERIALS AND METHODS

Viruses. Wild-type CPV-BR and the derivation of CPV-BR.D1 (a mutant lacking the gene for the 38-kDa protein), CPV-BR.DC2 (a mutant lacking the gene for the 38-kDa protein and the selection marker cassette), CPV-BR.R2, and CPV-BR.R4 (a virus with reinsertion and reversion of the gene for the 38-kDa protein) have been described previously (40). Characterization of CPV-BR/A500 and CPV-BR/W2, a TNF-R homolog-minus mutant, has been reported (28, 41). The thymidine kinase (TK) deletion mutant of CPV-BR (CPV-BR/A460) was constructed with pSC11 (40a). The reinsertion recombinants containing the gene encoding the 38-kDa protein, CPV-BR/W2/A361 (G-series recombinants), and the 5.2-kbp *Eco*RI fragment that has the gene encoding the 38-kDa protein deleted into CPV-BR/W2, CPV-BR/W2/A304 (E-series recombinants), were constructed with a *Hin*dIII J-fragment insertion vector of strain WR of VV (41). The WR strain of VV and the VGF-minus mutant VV-WR/SC20 have been described previously (5, 6). The Copenhagen VV strain was kindly provided by R. Drillien (Institut de Virologie de la Faculte de Medicine de Strasbourg, Strasbourg, France) while the parent, the Koppe VV strain, was kindly provided by J. Esposito (Centers for Disease Control and Prevention, Atlanta, Ga.). The CVA (Ankara) strain of VV and its egg-passaged, attenuated vaccine strain, MVA, were kindly provided by G. Sutter (National Institute of Allergy and Infectious Diseases). The CPV-BR complement-control protein-minus mutant VSIGK5 was kindly provided by Stuart Isaacs (National Institute of Allergy and Infectious Diseases) and Girish Kotwal (University of Louisville School of Medicine, Louisville). The ectromelia virus utilized in these studies was a Moscow strain (13). Poxviruses purified on sucrose gradients were utilized in all experiments (17). Human parainfluenza virus 3 and herpes simplex virus type 1 (KOS) were kindly provided by B. Murphy and M. Challberg, respectively (National Institute of Allergy and Infectious Diseases).

Cells and culture. BS-C-1 cells, an African green monkey kidney cell line, were used for analysis of arachidonate metabolism, while HeLa cells were used for virus propagation (American Type Culture Collection, Rockville, Md.). Monolayer cultures of BS-C-1 cells were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, Md.). HeLa cells used for large-scale virus propagation were grown in Spinner's medium (Quality Biological Inc., Gaithersburg, Md.) containing 5% horse serum (Hazleton Biologics Inc., Lenexa, Kans.).

Chemicals and arachidonate metabolites. [^{14}C]linoleic acid was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.) (specific activities of 50 to 60 mCi/mmol) and was used at a final concentration of 10 μM. The calcium ionophore A23187 (Sigma Chemical Co., St. Louis, Mo.) was used at a final concentration of 5 μM.

Addition of [^{14}C]linoleic acid label to cells. The infection of cells and the labeling procedure were the same as previously described (39). Briefly, cultures of BS-C-1 cells (10×10^6 cells per sample) were infected with 5 PFU per cell for 1 h or remained uninfected. Three hours after the addition of virus, the cells were incubated for 1 h with [^{14}C]linoleic acid ([^{14}C] linoleic acid was utilized more efficiently by BS-C-1 cells than [^{14}C]arachidonic acid; 3 μCi; final concentration of 10 μM) and A23187 (5 μM). Extraction of soluble fatty acids was done by a procedure identical to those previously published (39).

RP-HPLC. Reverse-phase high-pressure liquid chromatography (RP-HPLC) analyses were conducted with a C_{18} Ultrasphere column (5 μm; 4.6 by 250 mm; Altex Scientific) equipped with a Waters model U6K injector and a model 6000A pump (Waters). Separation of eicosanoids was achieved by elution with a stepwise methanol gradient (55 to 100%; pH 5.05) at a flow rate of 1.1 ml/min as described previously (39). Eluted radioactivity was monitored by using a Flow-One radioactivity detector (Radiomatic Instruments) equipped with a Qume computer for data processing. Peaks were normalized on the basis of recovery of prostaglandin B_2 added as an internal standard and detected by UV monitoring at 275 nm.

UV-spectral analysis. The diHETE fraction eluting at 53 min in the RP-HPLC system described above was collected and subjected to a second RP-HPLC analysis. The sample was injected on an Altex C_{18} Ultrasphere column and eluted with an isocratic mobile phase composed of methanol-water-acetic acid (70/30/0.01; vol/vol/vol) at a flow rate of 1.0 ml/min. The effluent was monitored with a Waters model 900 photodiode array detector. One UV-absorbing, radiolabeled peak was detected at 55 min, and its UV spectrum was recorded.

Chicken embryo inoculation and harvest. Eleven-day-old embryonated hens' eggs, obtained from Truslow Farms, Inc. (Chestertown, Md.), were incubated at 37 to 38.5°C in approximately 50% humidity, without rocking or exogenous CO_2, for 24 h before inoculation. Viruses were diluted in phosphate-buffered saline (PBS) (pH 7.2), and a 0.1-ml aliquot containing approximately 10 to 50 pock-forming units was inoculated onto the dropped CAMs of the chicken embryos (40). At the times indicated, the chicken embryos were killed by hypothermia and each CAM was removed, rinsed three times with PBS, and spread on a petri plate for further manipulation.

Virus infectivity assays. Individual virus pocks, excised from the CAMs as pieces of approximately 4 mm^2, were suspended in PBS containing 0.1% bovine serum albumin (BSA) and stored at −20°C. For virus assays, the pocks were ground in a tissue grinder (Bellco Inc., Vineland, N.J.), dispersed by two 30-s periods of sonication in a chilled cup attached to a model W-385 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.), and analyzed as described before (40).

Identification of activated inflammatory cells by measurement of oxygen intermediates. The CAM was removed from each egg, washed three times with PBS, and incubated for 1 h at 37°C in a 0.15% solution of nitroblue tetrazolium (NBT) (Sigma Chemical Co.) in PBS. The NBT was removed, and the CAM was washed three times with PBS and stored at 4°C prior to photography. The CAMs were coded and scored in a blind manner for NBT deposition.

Intracranial 50% lethal dose (LD_{50}) assay. Female BALB/cNCR mice obtained through the National Cancer Institute were used at ages of 21 to 28 days. The assay utilized procedures identical to those previously published (8).

RESULTS

Arachidonate metabolism in BS-C-1 cells. The expression of a functional 38-kDa protein in CPV-BR can be determined by monitoring the metabolism of arachidonic acid within infected chicken embryo cells, where the deletion of the gene encoding the 38-kDa protein in CPV resulted in elevated levels of 14*R*,15*S*-diHETE (39). To determine whether the spectrum of arachidonate metabolites produced by BS-C-1 cells following infection by poxviruses was similar to that detected in chicken embryo cells, BS-C-1 cells were infected with CPV-BR or

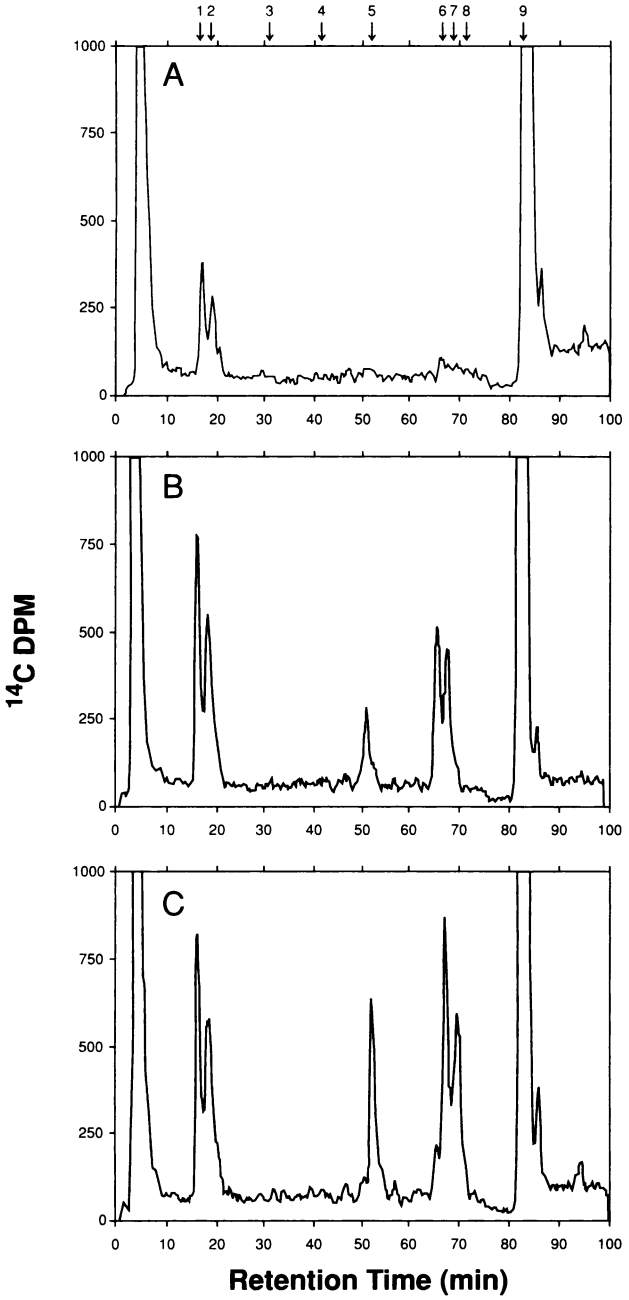


FIG. 1. RP-HPLC radiochromatograms of products formed during incubation of [^{14}C]linoleic acid and A23187 with BS-C-1 cells or infected cells. The radiochromatograms shown are from cells that were uninfected (A) or cells infected with wild-type CPV-BR (B) or the 38-kDa-protein-minus mutant, CPV-BR.D1 (C). The retention times of authentic ^{3}H standards are indicated with arrows on chromatogram A. The standards are numbered as follows: prostaglandin E_2, 1; prostaglandin $F_{2\alpha}$, 2; leukotriene C_4, 3; leukotriene B_4, 4; 14,15-diHETE, 5; 15-HETE, 6; 12-HETE, 7; 5-HETE, 8; and arachidonic acid, 9. DPM, disintegrations per minute.

CPV-BR.D1 (38-kDa-protein-minus mutant virus) or were mock infected, and 3 h after virus addition the calcium ionophore A23187 and [^{14}C]linoleic acid were added. The spectrum of arachidonic acid metabolites was analyzed by

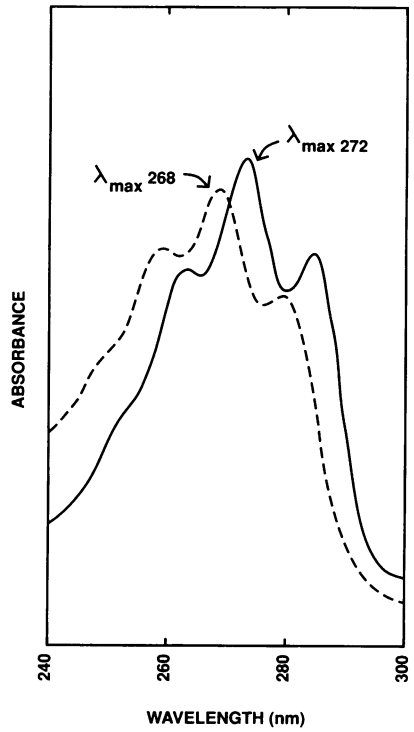


FIG. 2. UV spectrum of diHETE product formed in CPV-BR.D1-infected BS-C-1 cells. The diHETE fraction from the first RP-HPLC run was isolated and subjected to a second RP-HPLC analysis as described in Materials and Methods. One UV-absorbing and radiolabeled peak was detected at 55 min, and its UV spectrum was recorded. The UV spectrum of the biologic sample (——) was identical to that of authentic 14,15-diHETE. The UV spectrum of 8,15-diHETE is shown for comparison (- - - - -).

RP-HPLC. Infection of BS-C-1 cells with CPV-BR (Fig. 1B) stimulated an increase in eicosanoids derived from both lipoxygenase and cyclooxygenase pathways in comparison to mock-infected cells (Fig. 1A). The prominent lipoxygenase products were 15-hydroxyeicosatetraenoic acid (15-HETE) and 12-HETE, and the major cyclooxygenase products detected were prostaglandin E_2 and prostaglandin $F_{2\alpha}$. Infection of BS-C-1 cells with CPV-BR.D1 resulted in a spectrum of arachidonate metabolites similar to those found in CPV-BR-infected BS-C-1 cells and a major increase in one peak which eluted at 53 min in the region of 15-lipoxygenase-series diHETE (Fig. 1C). These results indicated that BS-C-1 cells infected with the 38-kDa-protein-minus CPV-BR were capable of forming a diHETE product, as was found for chicken embryo cells (39).

Identification of the diHETE metabolite. The elution profile of the diHETE in the RP-HPLC system described above indicated that the diHETE formed following poxvirus infection of BS-C-1 cells could be either a 8,15- or a 14,15-diHETE, the former diHETE having reported chemoattractant properties (48). Structural identification of the diHETE material was done by additional RP-HPLC and UV analysis as described previously (39). The BS-C-1-derived diHETE product co-eluted with authentic 14*R*,15*S*-dihydroxy-5(Z),8(Z),10(E), 12(E)eicosatetraenoic acid standard and displayed a highly characteristic triene chromophore with maximum absorbance at 272 nm. As shown in Fig. 2, this UV spectrum is clearly distinguishable from spectra of other 15-series diHETE regioisomers.

TABLE 1. Virus-induced alteration of arachidonate metabolism in BS-C-1 cells[a]

Virus	Pock color[b]	Arachidonate metabolite (dpm)				Genotype (VV-WR-ORF)					
		PGE_2	$PGF_{2\alpha}$	15-HETE	14,15-diHETE	38K/crmA (B13R)	TNF-R (C22L)	IL-1R (B15R)	IL-1R (B18R)	SPI-1 (B24R)	TK (J2R)
None	NA[c]	2,570	2,100	ND[d]	ND	NA	NA	NA	NA	NA	NA
CPV-BR	R	11,880	9,170	12,380	2,230	+	+	+	+[e]	+[f]	+
CPV-BR.D1	W	12,120	9,310	14,850	9,140	−	+	+	+	+	+
CPV-BR.DC1	W	11,870	9,220	14,540	9,010	−	+	+	+	+	+
CPV-BR.DC2	W	11,760	9,120	14,660	9,120	−	+	+	+	+	+
CPV-BR.R2	R	11,760	9,160	12,070	2,120	+	+	+	+	+	+
CPV-BR.R4	R	11,710	9,100	11,990	2,140	+	+	+	+	+	+
CPV-BR/W2	W	11,410	8,710	12,630	9,510	−	+	−	−	−	+
CPV-BR/W2/A361	P	11,640	8,970	12,760	2,340	+	+	−	−	−	−
CPV-BR/W2/A304	W	11,300	8,590	12,330	9,405	−	+	+	−	−	−
CPV-BR/A460	R	11,770	8,990	12,010	2,260	+	+	+	+	+	−
CPV-BR/A500	R	11,290	8,800	11,700	2,150	+	−	+	+	+	+
VV-WR	MW	10,690	8,340	10,890	1,980	+	−	+	+	+	+
VV-WR/SC20	W	10,740	8,390	10,630	1,940	+	−	+	+	+	+
VV-Koppe	MW	11,970	8,510	12,054	3,190	?	?	?	?	?	+
VV-Copenhagen	MW	12,310	9,660	12,530	3,540	±[g]	−	+	+	+	+
VV-CVA	MW	10,850	8,890	10,780	1,960	?	?	?	?	?	+
VV-MVA	W	10,630	8,820	11,100	2,350	?	?	?	?	?	+
Ectromelia-Moscow	±	11,880	8,990	11,880	2,530	?	?	−[h]	?	+[i]	+
HSV-1	NA	3,360	2,300	710	ND	−	−	−	−	−	−
PIV-3	NA	3,180	2,290	750	ND	−	−	−	−	−	−

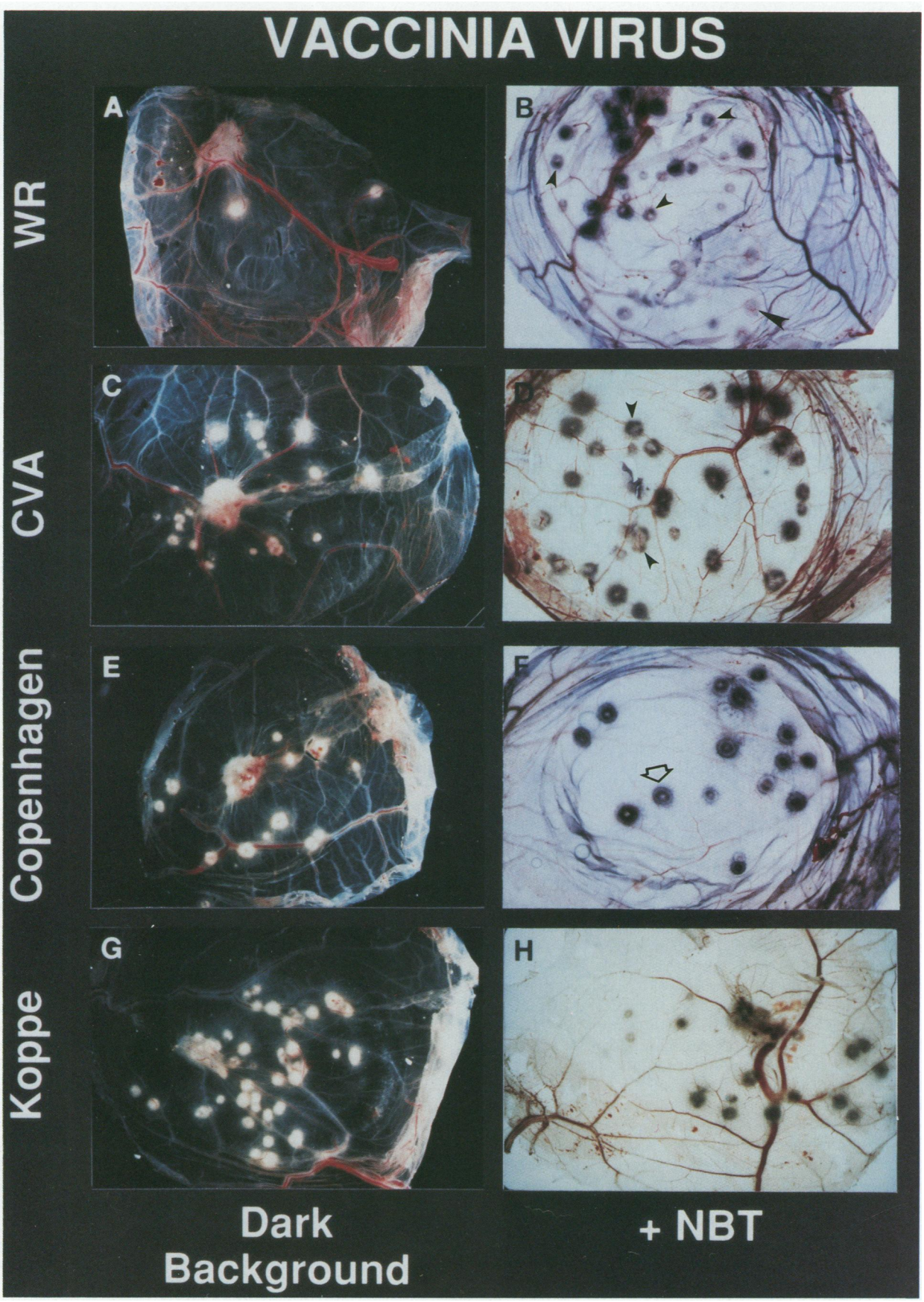
[a] Label was [^{14}C]linoleic acid and A23187. Values were determined by RP-HPLC for prostaglandin E_2, prostaglandin $F_{2\alpha}$, 15-HETE, and 14,15-diHETE, and they represent integrated disintegrations per minute normalized to recovery of an internal prostaglandin B_2 standard.
[b] R, red; W, white; MW, muddy white; P, pink.
[c] NA, not applicable.
[d] ND, not detected.
[e] Restriction endonuclease analyses suggests that this gene is present in CPV-BR.
[f] Reference 37c.
[g] The 38K/crmA gene is divided into two ORFs, B13R and B14R (25).
[h] Reference 4a.
[i] Reference 47a.

Arachidonate metabolism in BS-C-1 cells infected with different viruses. Together, the above results established that the absence of 38-kDa-protein expression by CPV-BR resulted in elevated levels of 14*R*,15*S*-diHETE in BS-C-1 cells and indicated that screening arachidonate metabolism in BS-C-1 cells after infection with a number of different viruses was a way of determining whether the gene encoding the 38-kDa protein was functional. The viruses examined for alteration of arachidonate metabolism after infection included some poxviruses that were known to express a 38-kDa protein, some in which the gene encoding the 38-kDa protein was deleted or fragmented and some for which the status of the gene encoding the 38-kDa protein was unknown (Table 1). All of the poxviruses examined upregulated arachidonate metabolism after infection of BS-C-1 cells, which was measured as an increase in both cyclooxygenase and lipoxygenase pathway-derived metabolites (Table 1). In contrast, neither herpes simplex virus type 1 nor parainfluenza virus 3 upregulated arachidonate metabolism after infection, which indicated that the upregulation by poxviruses in this system was due to a poxvirus-induced event and not to virus infection in general.

There was a correlation with the expression of a 38-kDa-protein-gene product by poxviruses and a decrease in 14*R*,15*S*-diHETE formation (Table 1). Conversely, poxviruses that lacked the gene encoding the 38-kDa protein or that had the gene interrupted (e.g., the Copenhagen strain of VV has the gene divided into two ORFs [25]) exhibited a marked increase in 14*R*,15*S*-diHETE levels (Table 1). Importantly, many of the poxviruses whose arachidonate metabolite profiles indicated that there was a functional 38-kDa protein expressed after infection of BS-C-1 cells form white or muddy white pocks on the CAMs of 12-day-old chicken embryos (Table 1), in contrast to CPV-BR, which forms red pocks that lack inflammatory cells (40).

VV strains that express a functional 38-kDa protein elicit an inflammatory response on CAMs. To determine whether the muddy, whitish pocks induced following infection by different VVs (Fig. 3A, C, E, and G) possessed an inflammatory component or were due solely to proliferation of ectodermal and endodermal cells induced by VGF (6), 12-day-old chicken embryo CAMs were infected and assessed for the presence of activated inflammatory cells through staining with NBT (23). All four VVs examined induced lesions that contained an area of inflammation that stained with NBT (Fig. 3B, D, F, and H), with the earliest inflammatory infiltrates detected 2 days after infection (data not shown). The triggering of inflammation by

FIG. 3. Pocks that developed on the CAMs of 12-day-old chicken embryos 2 to 3 days after inoculation with the WR (A and B), CVA (C and D), Koppe (E and F), and Copenhagen (G and H) strains of VV. The four left panels are photographs of infected CAMs that were left untreated. Activated inflammatory cells were detected by the reduction of NBT to formazan in pocks 3 days after infection (B, D, F, and H). Small arrowheads mark pockets of NBT deposition. Large arrowhead marks a lesion lacking deposition of NBT (B). Lesions with clear centers in panel F (open arrow) are due to loss of tissue from necrosis. Magnifications, approximately ×0.6.


VACCINIA VIRUS
WR
CVA
Copenhagen
Koppe
A
B
C
D
E
F
G
H
Dark Background
+ NBT

the CVA and WR strains of VV was somewhat stochastic, and thus 3 days after inoculation of the CAMs there were lesions which had no inflammation, some which had pockets of inflammation, and others which had inflammation throughout (Fig. 3B and D). Because the CVA- and WR-induced lesions continued to increase in size between days 2 and 3 after inoculation, and because once triggered the inflammation increased in intensity, those lesions that elicited an inflammatory cell infiltrate the earliest could have inflammation throughout. Of note, the CVA and WR strains of VV which expressed a functional 38-kDa protein induced lesions that initially lacked pockets of activated inflammatory cells in their centers (Fig. 3B and D) as is characteristic of lesions induced by CPV-BR (see Fig. 5B). In contrast, pocks induced by the Koppe and Copenhagen strains of VV, which yielded an intermediate level of diHETE formation, uniformly have inflammation throughout the lesion at this time (Fig. 3F and H). Poxviruses that induced very small lesions on the CAMs of 12-day-old chicken embryos such as VV-WR/SC20 (VGF-minus mutant of the WR strain of VV) or the highly attenuated MVA strain which was derived from CVA (36) also had lesions which exhibited NBT deposition (data not shown). The observation that VVs that express a 38-kDa protein can still trigger an inflammatory response indicated that VV lacks an additional biologic response modifier(s) which inhibits the triggering of an inflammatory response at the site of virus infection.

CPV-encoded genes with anti-inflammatory activity. CPV-BR fails to induce an inflammatory cell infiltrate on the CAMs of 12-day-old chicken embryos (40), and this suggests that CPV-BR must contain a full complement of anti-inflammatory genes for this model, unlike the VV strains. There are a number of poxvirus-encoded genes, in addition to the gene encoding the 38-kDa protein of CPV, whose gene products might be expected to inhibit the triggering of an inflammatory response at the site of virus infection, including the IL-1 receptor (IL-1R) homologs identified in VV (WR-ORF-B15R and -B18R [50]); the TNF-R homologs identified in myxoma virus (ORF-T2 [27, 49]), VV (WR-ORF-A53R and WR-ORF-C22L [25, 27]), and CPV (homolog of VV-ORF-C22L [28]); and the VV complement control protein (WR-ORF-C21L [34]). Some of these genes are located on the right terminal 30 kbp of CPV genomic DNA. In order to determine whether any of these CPV-encoded gene products had anti-inflammatory activity, we utilized several viruses that contained either a large or a single ORF deletion (Fig. 4; Table 1). The loss of a CPV-encoded gene that inhibits the triggering of an inflammatory response should elicit an inflammatory response after infection of 12-day-old chicken embryo CAMs, assuming that there is no species specificity for the molecule.

Shown in Fig. 5 are lesions that typify the two extremes in pock morphology induced after infection with either CPV-BR, which elicited no inflammatory response (Fig. 5A and B), or CPV-BR.D1 (38-kDa-protein-minus mutant), which elicited a massive inflammatory response 30 to 36 h after infection (Fig. 5C and D). Other viruses with a deletion in the gene encoding the 38-kDa protein such as CPV-BR/W2 or CPV-BR/W2/A304 also triggered a massive inflammatory response indistinguishable from that induced by CPV-BR.D1, with the center of the lesion filled with activated inflammatory cells (data not shown). The presence of a functional 38-kDa protein expressed by CPVs, as determined by inhibition of 14*R*,15*S*-diHETE formation, correlated with an absence of activated inflammatory cells in the center of the lesion (Fig. 5E through H), as was noted for the VVs.

In contrast to these two extremes in pock morphology, the loss of the TNF-R homologs (VV-WR-ORF-C22L homolog) located in the inverted terminal repeats of CPV (CPV-BR/A500) elicited an inflammatory response that was intermediate in intensity (Fig. 5G and H). The inflammatory response triggered after infection with CPV-BR/A500 arose as a small local response at the periphery of the lesion, which may or may not grow to surround the site of infection (Fig. 5H). Of note, the inflammatory response elicited by CPV-BR/A500 was very similar to those which were observed after infection of the CAMs with WR and CVA strains of VV (Fig. 3B and D), the former of which has a frameshift mutation in this particular ORF. The insertion of one gene copy of the CPV-BR TNF-R homolog (VV-WR-ORF-C22L homolog) in the WR strain of VV, however, failed to diminish the inflammatory response at the site of infection (39a), suggesting that there was another gene encoded by poxviruses other than the gene encoding the 38-kDa protein and the TNF-R homolog that was capable of inhibiting the triggering of an inflammatory response.

Examination of the pocks that formed after infection with CPV-BR/W2/A361 also demonstrated that inflammation arose at the periphery of the lesion, but the inflammation often surrounded the site of virus infection and the inflammatory cells were more diffuse (Fig. 5E and F). This result indicated that there was another gene encoded on the right end of the CPV genome that interfered with triggering of an inflammatory response. Finally, the loss of the complement control protein gave rise to pocks which possessed a well-demarcated zone of inflammatory cells surrounding the lesions on the CAMs but was restricted to a very few (<5%) of the chicken embryos examined (37b).

A summary of the types of lesions which arose after infection of the 12-day-old chicken embryo CAMs with various CPVs indicated that the loss of these 38-kDa-protein-independent anti-inflammatory genes in CPV-BR/A500 and CPV-BR/W2/A361 elicited an inflammatory response of intermediate intensity but that inflammation was not uniformly present in all lesions (Fig. 6). Only a small percentage of these virus-induced lesions contained an inflammatory cell infiltrate several days after infection. The loss of the TK gene in CPV-BR/A460, which served as a control for the reinsertion of the *Nco*I-*Hae*III fragment containing the gene encoding the 38-kDa protein into CPV-BR/W2 in construction of CPV-BR/W2/A361 (41), did not change the wild-type pock morphology. In contrast, almost 100% of the lesions induced by 38-kDa-protein-minus mutants had a massive infiltration of inflammatory cells by 30 to 36 h after infection. Taken together, these results suggest that there are at minimum two types of CPV-induced lesions which exhibit an influx of inflammatory cells, but the mechanisms by which inflammation is triggered probably differ, as do the intensities of the responses.

Kinetics of inflammatory cell influx into CPV-induced lesions. To determine the kinetics of the influx of inflammatory cells into pocks induced by CPV-BR/A500, 12-day-old chicken embryo CAMs were inoculated with either CPV-BR.D1, CPV-BR/A500, or wild-type CPV-BR, and 3 through 5 days after infection the CAMs were isolated, stained with NBT, and scored for the presence of inflammatory cells. The pocks that formed after infection with either CPV-BR or CPV-BR/A500 were equivalent in size and lacked localized, activated inflammatory cells through 3 days after inoculation (Fig. 7). Lesions induced by CPV-BR had only a slight increase from 3 through 5 days after infection in the number of pocks containing pockets of activated inflammatory cells (Fig. 7). In contrast, by 4 days after infection there was a sharp increase in the number of lesions containing activated inflammatory cells that were induced by CPV-BR/A500, and the percentage of pocks exhib-

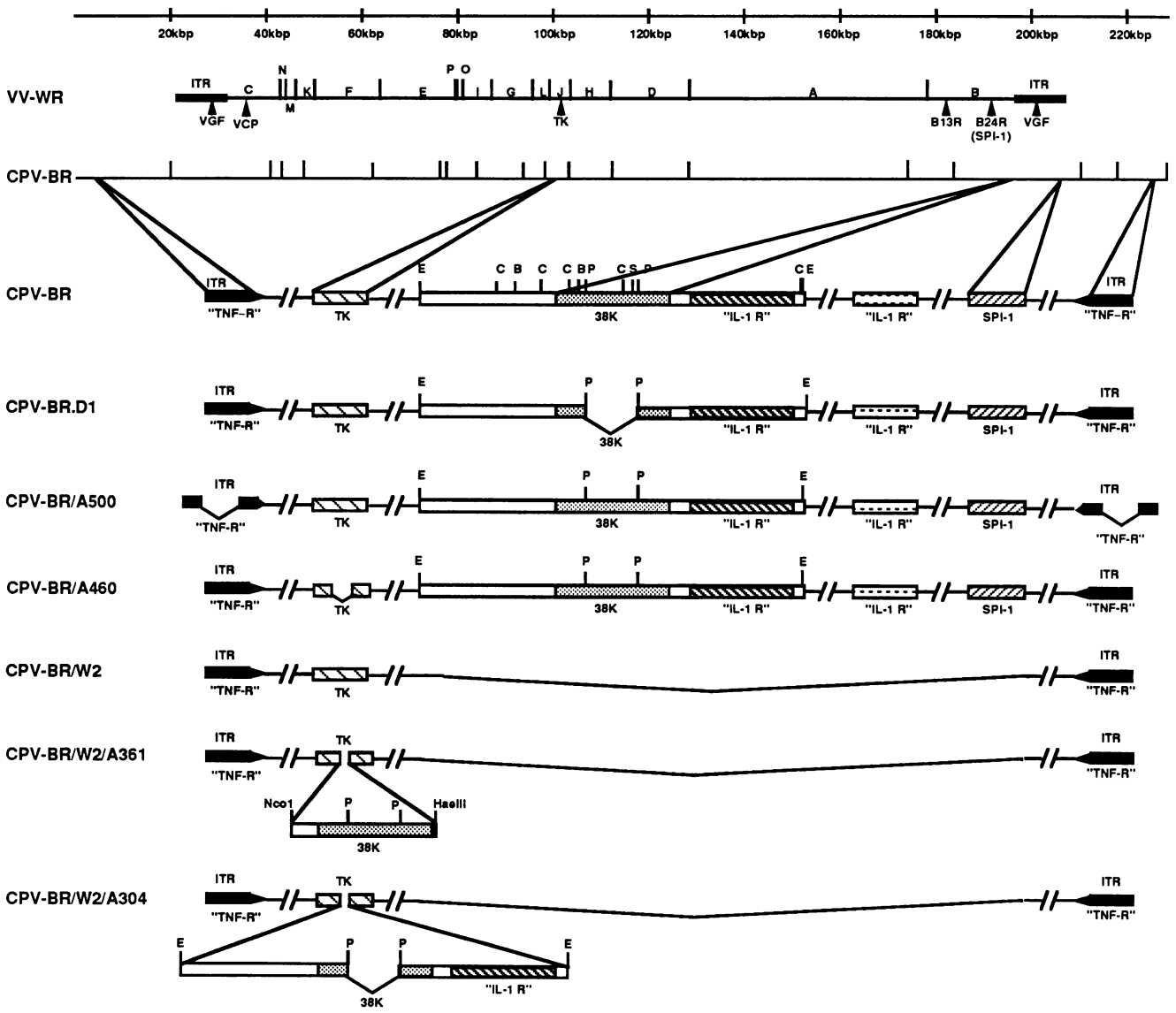
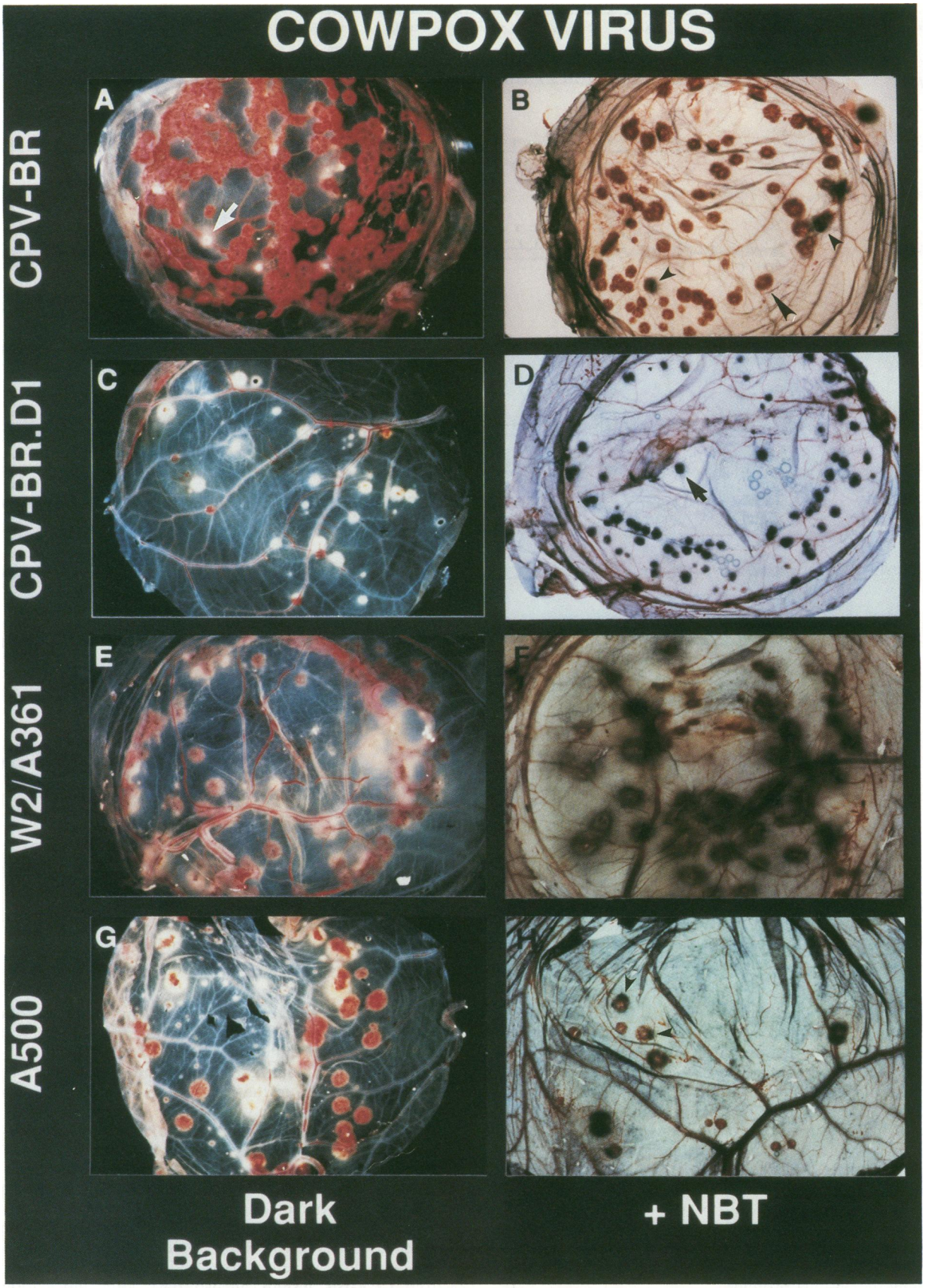


FIG. 4. *Hin*dIII digest restriction map of CPV genomic DNA (19) and location of potential anti-inflammatory genes based on sequenced DNA or the presence of conserved restriction sites in the ORF that are also present in VV. The ORFs identified include the TK gene (VV-WR-J2R), the gene encoding the 38-kDa protein (SPI-2, crmA, or VV-WR-B13R), the serine protease inhibitor 1 (SPI-1 or VV-WR-B24R) gene, the IL-1R homolog that binds IL-1 (VV-WR-B15R), the second IL-1R homolog with undetermined ligand specificity (VV-WR-B18R), and the TNF-R homolog (VV-WR-C22L) gene. Also depicted is the genotype of each CPV used in this study. ITR, inverted terminal repeat.

iting inflammation continued to increase through 5 days after infection (Fig. 7). For comparison, 90 to 98.5% of the lesions induced by the 38-kDa-protein-minus mutant, CPV-BR.D1, contained a massive inflammatory cell infiltrate 3 through 5 days after inoculation (data not shown). In addition, there could be variation in the time period from virus inoculation to influx of inflammatory cells into lesions induced by CPV-BR/A500, i.e., it could occur at earlier times or it could be delayed, yet the triggering of inflammation always occurred in greater numbers of those lesions induced by CPV-BR/A500 or CPV-BR/W2/A361 than by CPV-BR (data not shown). These kinetic results were consistent with a different mechanism of triggering an inflammatory response by these mutants in comparison to the trigger(s) caused by deletion of the gene encoding the 38-kDa protein.

Virus yields recovered from pocks are reduced following inflammation. A previous study indicated that the massive inflammatory cell influx into lesions caused by the 38-kDa-protein-minus mutant severely reduced the amount of infectivity recovered from the lesion (40). To determine whether there was a reduction in virus yields recovered from pocks that contained pockets of inflammation, chicken embryos were inoculated with CPV-BR, CPV-BR/A500, or CPV-BR.D1 and the pocks were excised from the CAMs at various times after infection. Virus infectivity levels in CPV-BR-induced pocks that lacked visible inflammatory cell infiltrates increased from days 2 through 4 postinoculation (Table 2). In contrast, infectivity levels in CPV-BR.D1-induced pocks having a massive inflammatory cell influx were lower than those recovered from CPV-BR lesions at two days after inoculation and


COWPOX VIRUS
CPV-BR
CPV-BR.D1
W2/A361
A500
A
B
C
D
E
F
G
Dark Background
+ NBT

decreased drastically through 4 days after inoculation (Table 2). Infectivity levels recovered from CPV-BR/A500-induced pocks that had pockets of inflammation or had inflammation surrounding the lesion were intermediate, with lower infectivity levels than those in CPV-BR lesions in two of three experiments by 4 days after inoculation (Table 2). In summary, a host inflammatory response to poxvirus infection reduced the level of infectivity at the sites of virus infection, proportional to the magnitude of the inflammatory response.

Loss of CPV-encoded anti-inflammatory genes reduces virulence. The intracranial LD_{50} assay for mice gives a good correlation between the ability of a poxvirus to replicate in vivo and the virulence of the virus, on the basis of the observations that poxviruses are severely attenuated upon deletion of genes such as TK or VGF (5, 8). Presumably poxvirus genes which are nonessential in cell culture for virus replication, and whose products inhibit host defense mechanisms, allow for enhanced virus replication and spread in the host because of neutralization of host defense mechanisms. To determine whether the anti-inflammatory genes encoded by CPV influenced virus virulence, a number of different CPV strains, including the wild type, specific deletion mutants, or spontaneous mutants, were assessed for virulence in a mouse intracranial LD_{50} assay (Table 3). Inoculation of mice with CPV-BR yielded an LD_{50} that was equivalent to that of the WR strain of VV. Deletion of the TK gene of CPV (CPV-BR/A460) attenuated CPV-BR approximately 5 orders of magnitude, which is similar to what has been reported for the WR strain of VV (8). The loss of any single ORF that inhibits the generation of an inflammatory response such as the genes encoding the 38-kDa protein or the TNF-R homolog attenuated CPV-BR 1 to 2 orders of magnitude in this assay. The deletion of the complement control protein, a C4-binding protein homolog, also was associated with attenuation at 1 to 2 orders of magnitude in this assay (37a). Of note, the construction of revertant viruses of CPV-BR.D1 on which the gene encoding the 38-kDa protein was restored and both pock morphology and inhibition of diHETE formation were like those of CPV-BR yielded separate virus isolates that were more attenuated than the wild-type CPV-BR. This suggested that the revertant viruses contained a mutation(s) elsewhere in the genome which attenuated the virus.

The loss of the right terminal 36 kbp of the CPV-BR genome in CPV-BR/W2 attenuated the virus 4 orders of magnitude. Interestingly, the loss of the TK gene in CPV-BR/W2 by reinsertion of a *Nco*I-*Hae*III genomic fragment that contained the gene encoding the 38-kDa protein (Fig. 4) in the creation of CPV-BR/W2/A361 attenuated the virus 2 orders of magnitude more than the separate loss of the TK locus (CPV-BR/A460) or the right end of the CPV genome including the gene encoding the 38-kDa protein (CPV-BR/W2) when these values are combined. This result suggests that there was a synergistic loss in virulence in a virus with a gene expressing the 38-kDa protein. A CPV-BR/W2 virus that had an insertion of the CPV genomic 5.2-kbp *Eco*RI fragment which contains the IL-1R homolog (VV-WR-ORF-B15R), but with a deletion in the gene encoding the 38-kDa protein, into the TK gene (CPV-BR/W2/A304) gave rise to a virus that was slightly more attenuated than either CPV-BR/A460 (TK^-) or CPV-BR/W2 (36-kbp deletion mutant) but was 2 orders of magnitude more virulent than CPV-BR/W2/A361. Together, these results suggested that complementation of virus-encoded genes and possibly a host response to infection were determinants of virus virulence.

DISCUSSION

The number of virus-encoded gene products that influence host defense mechanisms, as well as apparent viral strategies that evade these responses, continues to expand (3, 26). The strategies and effectiveness of evasion of host defenses employed by each virus family are likely influenced by genome size, which in turn limits the number of genes or regulatory elements dedicated to evasion that can be encoded. In addition, it is likely that viruses fall into a minimum of two categories of host defense evasion strategies based on the type of infection that occurs in the host, i.e., acute or persistent. For example, a virus that causes a persistent or extended infection may need to employ an evasion strategy (or strategies) such as interfering with recognition by cells of the immune system or downregulation of the immune system (e.g., adenoviruses, herpesviruses, arenaviruses, and retroviruses). In contrast, a virus that causes an acute infection needs to replicate to high levels in order to facilitate rapid transmission to a susceptible host (e.g., poxviruses, influenza viruses, and enteroviruses) and thus may have less need for evasion of an immune response.

Many poxviruses, including variola virus, cause an acute infection in their appropriate hosts (22), and therefore virus-encoded host defense modifiers that defeat early nonimmune defense mechanisms might enhance virus replication and dissemination. Previous studies of host responses to poxvirus infection and examination of patients with progressive VV indicated that for a primary infection the T-lymphocyte effector arm was required for virus clearance and that the plethora of other defense mechanisms were ineffective in clearing the infection and protecting the host (7, 24, 32, 37). One hypothesis is that these other host defense mechanisms fail to provide protection of the host from poxvirus infection because poxviruses encode gene products which neutralize their action. Several recent reports have identified poxvirus-encoded genes whose products likely interfere with host defense mechanisms, and thus elucidation has begun of the apparent paradox of how a poxvirus, such as variola virus, that was capable of causing pandemics with high mortality was also capable of eliciting a potent immune response that afforded lifelong protection for survivors of infection.

Consistent with the above-stated hypothesis is our previous demonstration that the 38-kDa protein of CPV-BR inhibits the triggering of inflammation (40), potentially through two mechanisms: inhibition of the production of leukotriene-like molecules from arachidonate metabolism (39) and the inhibition of IL-1-converting enzyme (42). Analysis of arachidonate metabolism in poxvirus-infected BS-C-1 cells demonstrated that all of the poxviruses examined upregulated both the cyclooxyge-

FIG. 5. Pocks that developed on the CAMs of the 12-day-old chicken embryos 3 to 4 days after inoculation with CPV-BR (A and B), the 38-kDa-protein-minus mutant CPV-BR.D1 (C and D), the spontaneous deletion mutant with a gene encoding the 38-kDa protein reinserted into the TK gene CPV-BR/W2/A361 (E and F), and the TNF-R homolog-minus mutant CPV-BR/A500 (G and H). The panels on the left and right sides are photographs of untreated and NBT-treated CAMs, respectively. A white arrow identifies a spontaneous white-pock mutant (A). Small arrowheads mark pockets of NBT deposition. Large arrowhead marks a lesion lacking deposition of NBT (B). Arrow marks a lesion with massive NBT deposition (D). Magnifications, ×0.6.

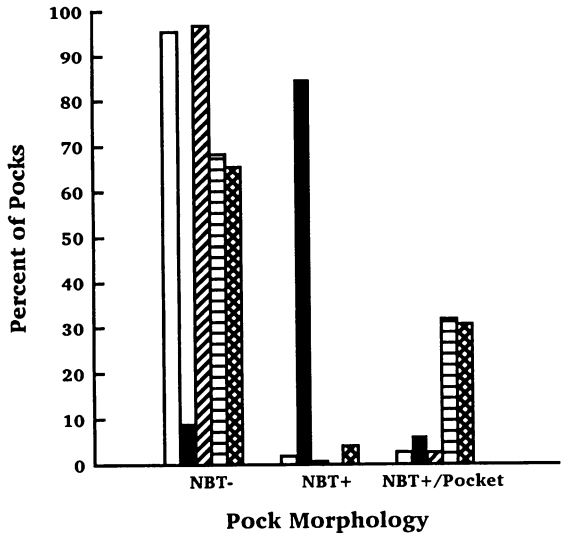


FIG. 6. Summary of pock morphologies present on the CAMs of 12-day-old chicken embryos 3 days after infection. CAMs were inoculated with either wild-type CPV-BR (□); CPV-BR.D1, which has a deletion of the gene encoding the 38-kDa protein (■); CPV-BR/A460, which has an interruption in the TK ORF (▨); CPV-BR/W2/A361, which is a spontaneous deletion mutant missing the right terminal 36 kbp of genomic DNA but has the gene encoding the 38-kDa protein reinserted into the TK ORF (▤); or CPV-BR/A500, which has both copies of the TNF-R homolog in the inverted terminal repeat interrupted (▩). CAMs were stained with NBT to aid in the visualization of pockets of inflammatory cells. Percentages were determined from categorizing the pocks present on 12 infected CAMs per virus (20 to 70 pocks per CAM) for absence of inflammation (NBT−), massive inflammation including the center of the lesion (NBT+), or pockets of inflammation with an absence of inflammatory cells in the center of the lesion (NBT+/pocket).

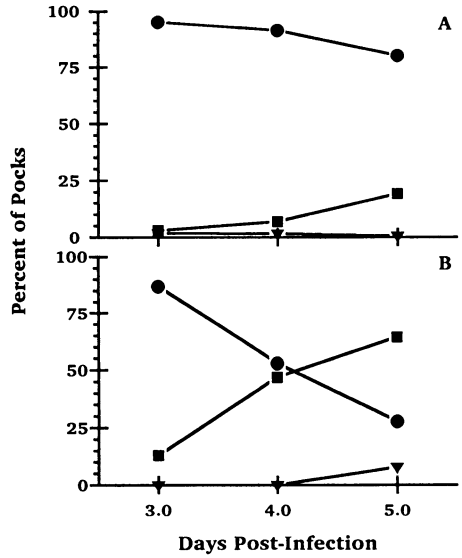


FIG. 7. Influx of inflammatory cells into the site of virus replication. Twelve-day-old chicken embryos were inoculated with CPV-BR (A) or CPV-BR/A500 (B) (7 to 10 eggs per virus per time point), and at the indicated times the CAMs were isolated and the pocks were assessed for the presence of inflammation (10 to 30 pocks per CAM) after staining with NBT. Percentages were determined from categorizing the pocks present on the infected CAMs for absence of inflammation (●), massive inflammation including the center of the lesion (▼), or pockets of inflammation with an absence of inflammatory cells in the center of the lesion (■).

nase and lipoxygenase pathways, and in the absence of the 38-kDa protein there was a major increase in diHETE product formed (Table 1). In addition, if the 38-kDa protein works in cells as an inhibitor of IL-1-converting enzyme, then the loss of this gene product can result in release of IL-1, which induces the production of IL-8, which in turn causes formation of leukotrienes (diHETEs) that are potent chemoattractants for leukocytes (47). These notions are consistent with the detection of a chemoattractant(s) in lesions which formed on the CAMs of chicken embryos after infection with white-pock variants of CPV-BR (15).

One consequence of the generation of an inflammatory response to poxvirus infection in chicken embryo CAMs was a decrease in infectious virus progeny recovered (40) (Table 2). The more intense the inflammatory response elicited was, the lower the virus yields recovered from the lesion were. In humans, smallpox virus lesions which developed on the mucous membranes of the nasal and oropharyngeal cavities, and to a lesser extent skin lesions, were a source of infectious virus which could be transmitted to susceptible individuals (21), and thus an inflammatory response that limited production of infectious virus progeny might be expected to lessen the probability of virus transmission to a susceptible host.

There are many different molecules which have inflammatory properties either directly or indirectly through induction of another mediator, including cytokines, chemokines, and hydroxy fatty acids. Thus, a poxvirus would require potentially a number of virus-encoded gene products in order to inhibit or limit the triggering of an inflammatory response at the site of infection. Consistent with this notion is the observation of 38-kDa-protein-dependent and -independent inhibition of inflammation in this study. We have used the chicken embryo CAM as a model for detecting virus-encoded inhibitors of inflammation, which allows analysis of host defense mechanisms that occur in the absence of specific immune responses (23). Using this model, we have identified at minimum the presence of three ORFs encoded by CPV-BR that interfere with the triggering of a nonimmune inflammatory response at the site of virus infection. In addition to the gene encoding the 38-kDa protein of CPV-BR, there is the gene encoding the TNF-R homolog (VV-WR-ORF-C22L homolog) and an ORF yet to be identified that maps to the right end of the CPV-BR genome which inhibit the triggering of inflammation. Poxviruses therefore employ a multigenic strategy of evasion of one host defense mechanism—the generation of an inflammatory response—presumably through neutralization of several different inflammatory mediators.

Poxviruses are not unique in employing a strategy of evading a host inflammatory response. Influenza virus A infections of humans are associated with an impairment of neutrophil function. Influenza virus has been shown to inhibit chemotaxis (1, 35, 43, 46), phagocytosis (35, 45), and intracellular killing by neutrophils (1), although the exact mechanisms have not yet been elucidated.

Previous studies demonstrated that the deletion of an ORF which promotes virus replication in vivo, such as the TK, the VGF, or the ribonucleotide reductase ORF, attenuated the WR strain of VV in vivo (5, 8, 14). Others found that spontaneous CPV white-pock deletion mutant variants (which presumably do not express a functional 38-kDa protein as well as other gene products and thus trigger inflammation) were

TABLE 2. Virus recovered from pocks[a]

Day p.i.[b]	Virus yield/pock (10^4 PFU)								
	Expt 1[c]			Expt 2[c]			Expt 3[d]		
	CPV-BR	CPV-BR.D1	CPV-BR/A500	CPV-BR	CPV-BR.D1	CPV-BR/A500	CPV-BR	CPV-BR.D1	CPV-BR/A500
2	10.2 ± 2.8	6.9 ± 2.4	7.1 ± 4.9	24.5 ± 8.2	7.9 ± 3.3	3.8 ± 0.2	ND	ND	ND
3	78 ± 38.8	5.1 ± 2.2	49.2 ± 7.9	72.1 ± 12	1.2 ± 1.0	123 ± 21	ND	ND	ND
4	59.3 ± 5.9	2.1 ± 0.3	5.9 ± 0.6	157 ± 19	0.7 ± 0.05	158 ± 84	117 ± 61	0.2 ± 0.07	25.5 ± 5.4

[a] CPV-BR pocks isolated from CAMs had no visible inflammatory cell infiltrate, while those isolated for CPV-BR.D1 (38-kDa-protein-minus mutant) had a massive inflammatory cell infiltrate and those induced by CPV-BR/A500 (TNF-R homolog-minus mutant) had some (pockets) inflammatory cell infiltrate. The lesions chosen were similar in size for each virus.
[b] p.i., postinfection.
[c] Six pocks per CAM were assessed for virus infectivity, with three CAMs per virus per time point. Shown are the means ± the standard errors of the means.
[d] Eight pocks per CAM were assessed for virus infectivity, with five CAMs per virus. Shown are the means ± the standard errors of the means.

less virulent than wild-type virus after inoculations of rabbits, mice, guinea pigs, nonhuman primates, and cows (20, 54). Together these results suggest that diminished virus replication, regardless of the mechanism, attenuates the virus in vivo. In this study, the deletion of CPV-BR-encoded anti-inflammatory genes such as those encoding the 38-kDa protein or the TNF-R homolog decreased the virulence of the mutants in comparison with wild-type CPV-BR in a mouse LD_{50} assay (Table 3). Other poxviruses which have deletions in genes which would be expected to inhibit the generation of an inflammatory response, such as the C4-binding protein of VV, the TNF-R homolog of myxoma virus, and the IL-1R homolog of VV also are attenuated in mice and rabbits (29, 51, 52).

One caveat of a mouse LD_{50} assay or assays testing virulence in other animals, and demonstrated potentially in these studies with revertant viruses that express the 38-kDa protein, is the possibility of other mutations arising in the single virus isolated during construction of the mutant or revertant viruses. The construction of the revertants (CPV-BR.R2 and CPV-BR.R4) from CPV-BR.D1 yielded viruses that had a wild-type pock morphology (data not shown) and inhibited diHETE formation (Table 1); both are characteristics associated with the expression of a functional 38-kDa protein. The phenotypic and genotypic reversion of a 38-kDa-protein-minus mutant to wild-type CPV-BR was not matched, however, by a reversion to wild-type virus virulence (Table 3). The LD_{50}s of the two revertants were similar to values obtained with a mutant virus with a single deletion in a host defense modifier, suggesting that in the process of isolation of revertant viruses, a spontaneous mutation(s) occurred in a gene(s) other than the gene encoding the 38-kDa protein and had no effect on virus growth in cell culture but attenuated the virus in vivo.

TABLE 3. Virulence of the CPV wild type and mutants[a]

Virus	LD_{50} in BALB/c mice		
	Expt 1	Expt 2	Expt 3
CPV-BR	≤2	9	17
CPV-BR.D1	≤200	238	224
CPV-BR.DC1	≤44	205	ND
CPV-BR.DC2	≤66	364	ND
CPV-BR.R2	≤17	71	≥4.3×10^3
CPV-BR.R4	≤15	397	≥280
CPV-BR/W2	1.7×10^4	3.3×10^4	ND
CPV-BR/W2/A361	>1.1×10^7	>1.0×10^7	ND
CPV-BR/W2/A304	1.8×10^5	1.5×10^5	ND
CPV-BR/A460	8×10^4	8.5×10^4	ND
CPV-BR/A500	55	519	ND
VV-WR	≤2	7	ND

[a] Groups of four to five female BALB/cNCR mice that were 4 weeks of age were inoculated intracranially with 10-fold dilutions of virus in a 30-μl volume. Cumulative mortality was noted through 2 weeks after inoculation. The average LD_{50} for the mice was calculated by the Spearman-Karber method. ND, not determined.

Virulence of a poxvirus is also apparently determined by host elements. For example, in this study, the loss of the 38-kDa-protein expression alone from virulent CPV-BR caused attenuation, yet the expression of the 38-kDa protein in another CPV mutant also caused attenuation, a caveat being that there were no other genetic alterations that occurred in the mutant viruses. Specifically, the insertion of the *Nco*I-*Hae*III CPV genomic DNA fragment that contains the gene encoding the 38-kDa protein into the TK gene of CPV-BR/W2 (Fig. 4) created a virus that was more attenuated than a TK-minus mutant that lacked the gene encoding the 38-kDa protein, CPV-BR/W2/A304 (Table 3). Thus, expression of a functional 38-kDa protein in this latter example with a virus that is already highly attenuated is associated with decreased virulence of the virus, presumably because of the absence of triggering of an inflammatory response that could cause nonspecific damage to uninfected cells. A second example is provided by examination of VV virulence following deletion of the IL-1R homolog (VV-WR-ORF-B15R). Spriggs and colleagues found that deletion of VV-WR-ORF-B15R attenuated the virus about 2 orders of magnitude in a mouse intracranial LD_{50} assay (51). In contrast, Alcami and Smith, using an intranasal route of virus inoculation, found no difference in the lethality of a mutant with a deletion of VV-WR-ORF-B15R (2). Thus, those studies may suggest that when different routes of infection which can influence virus replication in different tissues are used, host responses to infection differ and/or the effects of the virus gene products vary. Together, these results examining poxvirus virulence suggest that virulence is determined by a balance of all of the virus genes in total, including genes which directly influence virus replication in vivo and genes which help the virus evade host defenses. Moreover, it is the combination of the magnitude of virus replication and spread, virus gene products expressed, and the efficacy of host responses to infection that determine the pathological consequences of virus infection of an organ or tissue and the host.

Inflammation is only one nonimmune host defense system that is triggered shortly after poxvirus infection. A second host defense mechanism triggered following poxvirus infection is the production of interferon, which may play a role in inflammation but also induces an antiviral state (reviewed in reference 7). Interferons can be important inhibitors of poxvirus

replication in vitro and in vivo, although differences in efficacy have been reported (30, 31, 33). To date, three poxvirus ORFs that interfere with the interferons have been identified, including two which block antiviral activities induced by alpha/beta interferon (VV-ORF-K3L [4, 16] and VV-ORF-E3L [9]) and the gamma interferon receptor homolog of myxoma virus (M-ORF-T7 [53]). Thus, like inflammation, interferon production which limits virus replication in vivo probably decreases the eventual likelihood of successful virus transmission to a new host.

Establishment of viral infection in a host presumably requires evasion of primitive nonimmune host defense mechanisms such as interferons and inflammation. It is therefore not surprising that poxviruses which cause an acute infection, and which possess a large genome, encode many gene products which target the two different nonimmune host defense strategies for thwarting infection, inflammation and interferons. Together, these evasive mechanisms likely result in the generation of sufficient virus progeny to ensure transmission to another nonimmune host.

ACKNOWLEDGMENTS

We thank C. Duarte for technical assistance; D. Pickup for thoughtful discussions and for the generous gift of CPV-BR mutant viruses; and T. Eling, Guna Karupiah, Stuart Isaacs, and D. Pickup for critical review of the manuscript.

We also thank NIAID and NIEHS for support.

REFERENCES

1. **Abramson, J. S., G. S. Giebink, E. L. Mills, and P. G. Quie.** 1981. Polymorphonuclear leukocyte dysfunction during influenza virus infection in chinchillas. J. Infect. Dis. **143:**836–845.
2. **Alcami, A., and G. L. Smith.** 1992. A soluble receptor for interleukin-1β encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. Cell **71:**153–167.
3. **Barinaga, M.** 1992. Viruses launch their own star wars. Science **258:**1730–1731.
4. **Beattie, E., J. Taraglia, and E. Paoletti.** 1991. Vaccinia virus-encoded eIF-2α homolog abrogates the antiviral effect of interferon. Virology **183:**419–422.

4a. **Buller, R. M. L.** Unpublished data.

5. **Buller, R. M. L., S. Chakrabarti, J. A. Cooper, D. R. Twardzik, and B. Moss.** 1988. Deletion of the vaccinia virus growth factor gene reduces virus virulence. J. Virol. **62:**866–874.
6. **Buller, R. M. L., S. Chakrabarti, B. Moss, and T. Frederickson.** 1988. Cell proliferative response to vaccinia virus is mediated by VGF. Virology **164:**182–192.
7. **Buller, R. M. L., and G. J. Palumbo.** 1991. Poxvirus pathogenesis. Microbiol. Rev. **55:**80–122.
8. **Buller, R. M. L., G. L. Smith, K. Cremer, A. L. Notkins, and B. Moss.** 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature (London) **317:**813–815.
9. **Chang, H.-W., J. C. Watson, and B. L. Jacobs.** 1992. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. Proc. Natl. Acad. Sci. USA **89:**4825–4829.
10. **Chen, C.-L. H., L. L. Ager, G. L. Gartland, and M. D. Cooper.** 1986. Identification of a T3/T cell receptor complex in chickens. J. Exp. Med. **164:**375–380.
11. **Chen, C.-L. H., J. Cihak, U. Losch, and M. D. Cooper.** 1988. Differential expression of two T cell receptors, TcR1 and TcR2, on chicken lymphocytes. Eur. J. Immunol. **18:**539–543.
12. **Chen, C.-L. H., and M. D. Cooper.** 1987. Identification of cell surface molecules on chicken lymphocytes with monoclonal antibodies, p. 137–154. *In* A. Toivanen and P. Toivanen (ed.), Avian immunology: basis and practice. CRC Press, Boca Raton, Fla.
13. **Chen, W., R. Drillien, D. Spehner, and R. M. L. Buller.** 1992. Restricted replication of ectromelia virus in cell culture correlates with mutations in virus-encoded host range gene. Virology **187:** 433–442.
14. **Child, S. J., G. J. Palumbo, R. M. L. Buller, and D. E. Hruby.** 1990. Insertional inactivation of the large subunit of ribonucleotide reductase encoded by vaccinia virus is associated with reduced virulence *in vivo*. Virology **174:**625–629.
15. **Chua, T. P., C. E. Smith, R. W. Reith, and J. D. Williamson.** 1990. Inflammatory responses and the generation of chemoattractant activity in cowpox virus-infected tissues. Immunology **69:**202–208.
16. **Davies, M. V., O. Elroy-Stein, R. Jagus, B. Moss, and R. J. Kaufman.** 1992. The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2. J. Virol. **66:**1943–1950.
17. **Earl, P. L., and B. Moss.** 1990. Expression of proteins in mammalian cells using vaccinia, p. 16.17.6–16.17-8. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley and Sons, New York.
18. **Eerola, E., T. Veromaa, and P. Toivanen.** 1987. Special features in the structural organization of the avian lymphoid system, p. 9–21. *In* A. Toivanen and P. Toivanen (ed.), Avian immunology: basis and practice. CRC Press, Boca Raton, Fla.
19. **Esposito, J. J., and J. C. Knight.** 1985. Orthopoxvirus DNA: a comparison of restriction profiles and maps. Virology **143:**230–251.
20. **Fenner, F.** 1958. The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. Virology **5:**502–529.
21. **Fenner, F., D. A. Henderson, I. Arita, Z. Jezek, and I. D. Ladnyi.** 1988. The epidemiology of smallpox, p. 182–194. *In* F. Fenner et al. (ed.), Smallpox and its eradication. World Health Organization, Geneva.
22. **Fenner, F., R. Wittek, and K. R. Dumbell.** 1989. The pathogenesis, pathology, and immunology of orthopoxvirus infections, p. 85–141. *In* F. Fenner et al. (ed.), The orthopoxviruses. Academic Press, Inc., New York.
23. **Fredrickson, T. N., J. M. G. Sechler, G. J. Palumbo, J. Albert, L. H. Khairallah, and R. M. L. Buller.** 1992. Acute inflammatory response to cowpox virus infection of the chorioallantoic membrane of the chick embryo. Virology **187:**693–704.
24. **Fulginiti, V. A., C. H. Kempe, W. E. Hathaway, D. S. Pearlman, O. F. Serber, Jr., J. J. Eller, J. J. Joyner, Sr., and A. Robinson.** 1968. Progressive vaccinia in immunologically deficient individuals. Birth Defects Orig. Artic. Ser. **4:**129–145. National Foundation March of Dimes, New York.
25. **Goebel, S. J., G. P. Johnson, M. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete nucleotide sequence of vaccinia virus. Virology **179:**247–266.
26. **Gooding, L.** 1992. Virus proteins that counteract host immune defenses. Cell **71:**5–7.
27. **Howard, S. T., Y. S. Chan, and G. L. Smith.** 1991. Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. Virology **180:**633–647.
28. **Hu, F.-H., R. G. Goodwin, T. Davis, C. Smith, and D. J. Pickup.** Cowpox virus contains two copies of an early gene encoding a soluble form of the type 2 secreted tumor necrosis factor receptor. Submitted for publication.
29. **Isaacs, S. N., G. J. Kotwal, and B. Moss.** 1992. Vaccinia virus complement control protein prevents antibody dependent complement-enhanced neutralization of infectivity and contributes to virulence. Proc. Natl. Acad. Sci. USA **89:**628–632.
30. **Karupiah, G., T. N. Fredrickson, K. L. Holmes, L. H. Khairallah, and R. M. L. Buller.** 1993. Importance of interferons in recovery from mousepox. J. Virol. **67:**4214–4226.
31. **Karupiah, G., Q.-W. Xie, R. M. L. Buller, C. Nathan, C. Duarte, and J. D. MacMicking.** 1993. Inhibition of viral replication by interferon-γ-induced nitric oxide synthetase. Science **261:**1445–1448.
32. **Kempe, C. H.** 1960. Studies on smallpox and complications of smallpox vaccination. Pediatrics **20:**176–189.
33. **Kohonen-Corish, M. R. J., N. J. C. King, C. E. Woodhams, and I. A. Ramshaw.** 1990. Immunodeficient mice recover from infec-

tion with vaccinia virus expressing interferon-γ. Eur. J. Immunol. **20:**157–161.
34. **Kotwal, G. J., S. N. Isaacs, R. McKenzie, M. M. Frank, and B. Moss.** 1990. Inhibition of the complement cascade by the major secretory protein of vaccinia virus. Science **250:**827–830.
35. **Larson, H. E., and R. Blades.** 1976. Impairment of human polymorphonuclear leucocyte function by influenza virus. Lancet **i:**283.
36. **Meyer, H., G. Sutter, and A. Mayr.** 1989. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J. Gen. Virol. **72:**1031–1038.
37. **O'Connell, C. J., D. T. Karzon, A. L. Barron, M. E. Plaut, and V. M. Ali.** 1964. Progressive vaccinia with normal antibodies. A case possibly due to deficient cellular immunity. Ann. Intern. Med. **60:**282–289.
37a. **Palumbo, G., R. M. L. Buller, S. Isaacs, and G. Kotwal.** Unpublished data.
37b. **Palumbo, G., S. Isaacs, and G. Kotwal.** Unpublished data.
37c. **Palumbo, G. J.** Unpublished data.
38. **Palumbo, G. J., and R. M. L. Buller.** 1991. Inhibitors of the lipoxygenase pathway specifically block orthopoxvirus replication. Virology **180:**457–463.
39. **Palumbo, G. J., W. C. Glasgow, and R. M. L. Buller.** 1993. Poxvirus-induced alteration of arachidonic acid metabolism. Proc. Natl. Acad. Sci. USA **90:**2020–2024.
39a. **Palumbo, G. J., and D. Pickup.** Unpublished results.
40. **Palumbo, G. J., D. J. Pickup, T. N. Fredrickson, L. J. McIntyre, and R. M. L. Buller.** 1989. Inhibition of an inflammatory response is mediated by a 38-kDa protein of cowpox virus. Virology **172:**262–273.
40a. **Pickup, D.** Unpublished data.
41. **Pickup, D. J., B. S. Ink, W. Hu, C. A. Ray, and W. K. Joklik.** 1986. Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. Proc. Natl. Acad. Sci. USA **83:**7698–7702.
42. **Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath, G. S. Salvesen, and D. J. Pickup.** 1992. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. Cell **69:**597–604.
43. **Ruuta, P.** 1977. Depression of rat neutrophil exudation and motility by influenza viruses. Scand. J. Immunol. **6:**1113–1120.
44. **Samuelsson, B.** 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science **220:**568–575.
45. **Sawyer, W. D.** 1969. Interaction of influenza virus with leukocytes and its effect on phagocytosis. J. Infect. Dis. **119:**541–556.
46. **Schlesinger, J. J., C. Ernst, and L. Weinstein.** 1976. Inhibition of human neutrophil chemotaxis by influenza virus. Lancet **i:**650–651.
47. **Schröder, J. M.** 1989. The monocyte-derived neutrophil activating peptide (NAP-1/interleukin-8) stimulates human neutrophil arachidonate-5-lipoxygenase, but not the release of cellular arachidonate. J. Exp. Med. **170:**847–863.
47a. **Senkevich, T. G.** Unpublished data.
48. **Shak, S., H. D. Perez, and I. M. Goldstein.** 1983. A novel dioxygenation product of arachidonic acid possesses potent chemotactic activity for human polymorphonuclear leukocytes. J. Biol. Chem. **258:**14948–14953.
49. **Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Beckmann, R. Jerzy, S. K. Dower, D. Cosman, and R. G. Goodwin.** 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science **248:**1019–1023.
50. **Smith, G. L., and Y. S. Chan.** 1991. Two vaccinia virus proteins structurally related to the interleukin 1 receptor and the immunoglobulin superfamily. J. Gen. Virol. **72:**511–518.
51. **Spriggs, M. K., D. E. Hruby, C. R. Maliszewski, D. L. Pickup, J. E. Sims, R. M. L. Buller, and J. VanSlyke.** 1992. Vaccinia and cowpox viruses encode a novel secreted interleukin-1 binding protein. Cell **71:**145–152.
52. **Upton, C., J. L. Macen, M. Schreiber, and G. McFadden.** 1991. Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. Virology **184:**370–382.
53. **Upton, C., K. Mossman, and G. McFadden.** 1992. Encoding of a homolog of the IFN-γ receptor by myxoma virus. Science **258:** 1369–1372.
54. **van Tongeren, H. A. E.** 1952. Spontaneous mutation of cowpox virus by means of egg passage. Arch. Gesamte Virusforsch. **5:**35–52.