Monkeypox Virus: Responses of Infection in Chick Embryos[1] (36330)

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Our previous studies of monkeypox virus (MPV) more succinctly than theretofore defined the clinical, immunological and pathogenetical features of infection in Old World monkeys (1–3). Monkey pox is a contagious disease with characteristic cutaneous lesions, virological recruitment of various visceral organs, and specific antibody development. Monkeypox shares many clinical and pathological features of variola (4) occurring in human beings.

Tissues of the developing chick embryo have been used for cultivation of pox viruses, and provide advantages in separating specific characteristics of infection between pox virus members. In this study we have studied *in ovo* local pock development and the spread of MPV into the tissues of embryos.

Materials and Methods. viruses. MPV. Strain 10001 was provided by Dr. P. von Magnus, Stats Seruminstitut, Copenhagen (5). The stock used in this experiment had been used in our earlier studies (6). This virus stock had an infectivity titer of $1.6 \times 10^{6.0}$ plaque forming units (pfu) per milliliter in a continuous line of green African monkey (CV-1 cells) (7) cultured *in vitro*. *Vaccinia.* A strain isolated from the pharynx of a recently vaccinated child (1) had been passaged on CV-1 cells. The virus stock titered $3 \times 10^{6.0}$ pfu/ml on these cell cultures.

Chick embryos. Eleven-day-old white leghorn chick embryos were used. For studies of pock formation, chorioallantoic membranes (CAM) of chick embryos were dropped and inoculated with several passage levels of MP or vaccinia virus. For temperature dependency study, the CAMs were inoculated with MPV and incubated at either 33, 37, or 40°. Pock formation on CAMs were scored on the third day postinfection. In pathogenesis studies, groups of 11-day-old embryos were infected with graded doses of MPV inoculated into the yolk sac. The definitive studies were made using $3.2 \times 10^{5.0}$ pfu/embryo. With this infective dose embryonic death occurred within 6 or 7 days. Beginning on the 1st, and through the fifth day, six embryos were harvested daily; tissues from viable embryos were studied for virus content, pathologic lesions, and labeling with fluorescent antibody. Methods for collection of tissues, and preparation of tissue suspensions were similar to those used earlier (1). The concentrations of virus in various tissues were determined in CV-1 cell cultures.

Portions of tissues collected for virus isolation were fixed in 10% neural formalin; fixed tissues were stained with hematoxylin and eosin. Selected tissues were frozen and prepared for direct FA staining with fluorescein-conjugated-MPV antisera; the procedures for preparation and staining of tissues have been described (3).

Results. Pocks on the CAM. MPV produced discrete focal lesions on the CAM. Virus stocks derived from cultured cells produced the kinds of lesions illustrated in Fig. 1B, C. Pocks produced by MPV were always smaller than the pocks produced by our strain of vaccinia virus (Fig. 1 D).

In previous studies blood from experimentally infected monkeys caused thickening and opacity of the inoculated CAM; minute

[1] This investigation was aided in part by U.S. Public Health Service, NIH Grants AI-06263 and 5SO 1 RR OS373.

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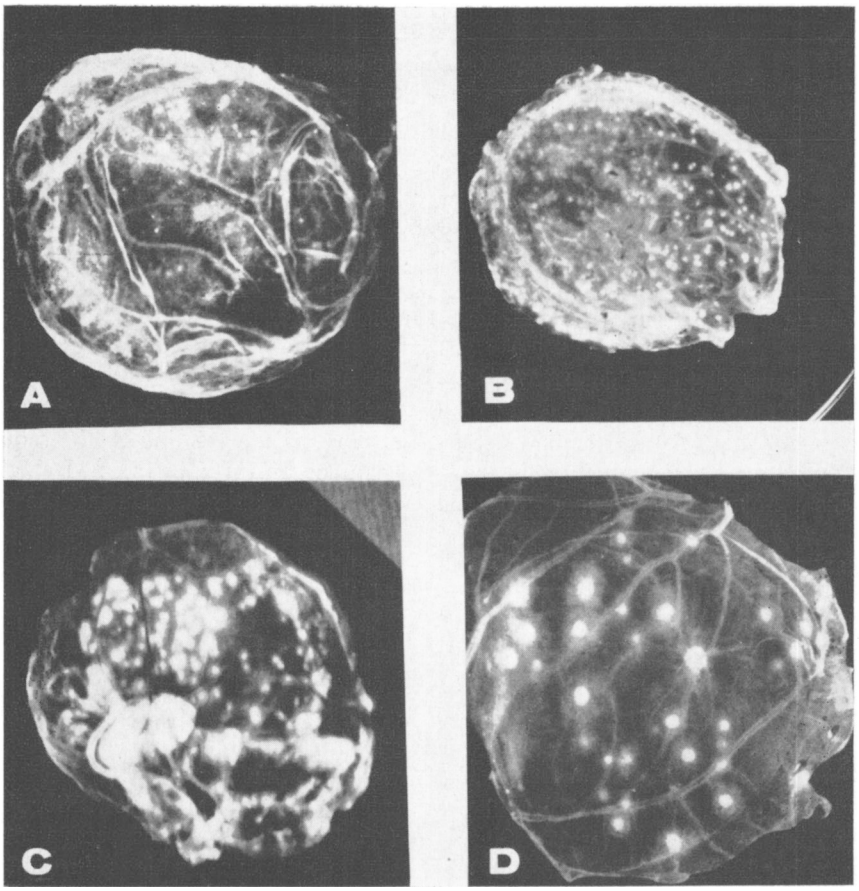

FIG. 1. Pocks produced on CAM of chick embryo by monkeypox and vaccinia viruses. MPV produces smaller pocks (*A, B, C*) than vaccinia virus (*D*). Primary isolation of the virus from blood of infected monkeys (*A*) showed edematous changes with minute pocks. Second (*B*) and fourth (*C*) passage of the viremic isolate yielded well-defined larger pocks.

discrete pocks were clearly discernible (Fig. 1A). With passage the edema was less pronounced and discrete pocks appeared; such are illustrated in Fig. 1C.

By the 4th passage on CAM monkeypox virus produced pocks of maximal size; thereafter both pock size and ID_{50} endpoints remained relatively constant. ID_{50} values of infected CAM ranged from $10^{4.5}$ to 10^5/ml when titrated on either CV-1 or Kappa cells. The dilution of CAM producing 1 to 10 pocks was about $10^{-4.8}$/ml.

The temperature dependency of MPV on pock development is summarized in Table I. Well defined pocks formed on CAMs incubated at temperatures of 33 and 37°, whereas none developed at 40°. The CAMs of embryos incubated at 33° were edematous, and yielded less distinctively outlined pocks than at 37°. Apparently the pock count was slightly reduced; however minute pocks may have been obscured by membrane edema.

Infectivity of MPV inoculated into the yolk sac. General aspects. In a preceding report relating to experimental chemotherapy (6) we reported on the lethal effects of MPV inoculated into the yolk sac of chick embryos. Six-day-old embryos inoculated with $1.7 \times 10^{4.0}$ pfu of virus had an average survival time of 6 days. In this study 11-day-old embryos were used in order to obtain adequate samples of various organs for evidence of virus replication.

The virus-dose responses in 11-day-old em-

TABLE I. Temperature Effect on Growth of MPV on CAM of Chick Embryos.[a]

Inoculum: 0.2 ml/CAM	Pfu/CAM (average/individual) at specified temperature		
	33°	37°	40°
$10^{3.5}$	26	31	0
	(10, 16, 30, 50)	(26, 28, 30, 40)	(0, 0, 0, 0)
$10^{3.0}$	86	114	0
	(70, 87, 100)	(111, 114, 118)	(0, 0, 0)

[a] Pfu = pock forming unit; CAM = chorioallantoic membrane.

bryos are tabulated in Table II. Approximately 500TC ID_{50} of MPV were required to kill 50% of the 11-day-old embryos. Surviving embryos hatched normally; the newborn chicks were normal in appearance and activity.

The results of virus–dose responses recorded in Table II were reaffirmed in some preliminary studies designed to delineate the distribution of MPV in chick tissues (Table III). In this study using a dose of $3.2 \times 10^{2.0}$ pfu to infect embryos, MPV was found in allantoic fluid, CAM, and blood. Viremia lasted only a day; MPV was not recovered from any tissue after the third day, and was never recovered from visceral organs. Surviving embryos hatched normally. However, embryos infected with a much larger dose of virus ($3.2 \times 10^{5.0}$ pfu) developed and retained virus in allantoic fluids and CAMs. MPV was found in visceral organs, of which the liver is but an example. None of the unsampled embryos remaining in the series survived. Therefore, $3.2 \times 10^{5.0}$ pfu of MPV was used in the subsequent studies.

TABLE II. Virulence of MPV for Chick Embryos.[a]

MPV (pfu/egg)	Number of chick embryos		
	Infected	Died	Hatched
$3.2 \times 10^{5.0}$	42	42	0 (0%)
$3.2 \times 10^{4.0}$	18	18	0 (0%)
$3.2 \times 10^{3.0}$	16	14	2 (12%)
$3.2 \times 10^{2.0}$	10	4	6 (60%)

[a] Results accumulated from four different experiments. All chick embryos were 11-day-old when they were infected with 0.2 ml of virus via the yolk sac route. Many other infected embryos harvested before hatching (21 days) were excluded from scoring.

Distribution of MPV in tissues of chick embryos. Data relating to the sequential appearance of MPV in infected chick embryos appear in Table IV. During the first 24 hr MPV was recovered in high concentrations from CAMs and allantoic fluids. Allowing for dilution and dispersion and amount of virus in these samples exceeded virus input. During this period and shortly thereafter MPV was recovered from bone marrow, liver, spleen, kidney, and skeletal muscle. The upsurge of virus during the second and third day was somewhat greater than elsewhere in liver, spleen, and bone marrow. Soon thereafter ($\leq$ 1 day) MPV was recovered in almost equivalent concentrations in all 12 tissues

TABLE III. Recovery of Virus from MPV Infected Chick Embryos.[a]

MPV (pfu/egg)	Tissues	Day after infection				
		1	2	3	4	5
$3.2 \times 10^{2.0}$	Allantoic fluid	+	+	+	0	0
	CAM	+	+	0	0	0
	Blood	+	0	0	0	0
	Liver	0	0	0	0	0
$3.2 \times 10^{5.0}$	Allantoic fluid	+	+	+	+	+
	CAM	+	+	+	+	+
	Blood	ND	ND	ND	ND	ND
	Liver	+	+	+	+	+

[a] Embryos were infected at 11 days of incubation with 0.2 ml of virus suspension via yolk sac route. Two embryos were harvested each day thereafter. In the 500 pfu ($3.2 \times 10^{2.0}$) study, other negative tissues included spleen, kidney, lung, heart, skeletal muscle, skin, intestine and brain. Outcome of remaining embryos in this series appears in Table II. + = positive for MPV, 0 = negative for MPV, ND = not done.

TABLE IV. Distribution of MPV in Tissues of Infected Chick Embryos.[a]

	Log_{10} $TCID_{50}$/g tissue of chick embryos (No. 1–10) on days after infection									
	Day 1		Day 2		Day 3		Day 4		Day 5	
Tissues No.:	1	2	3	4	5	6	7	8	9	10
CAM	4.8	4.3	6.5	4.3	7.5	5.5	6.8	5.5	7.3	7.5
Allantoic fluid	5.5	5.3	4.5	5.3	5.5	5.3	7.0	5.5	6.8	—
Bone marrow	0	1.7	0	2.5	4.3	5.3	5.8	5.0	6.5	6.5
Liver	0	1.7	0	3.7	6.3	6.0	7.0	6.0	7.5	8.3
Spleen	—	0	—	2.7	—	5.5	—	6.0	6.3	6.5
Kidney	0	1.7	2.7	2.7	3.7	4.8	7.3	5.5	6.5	7.5
Lung	0	1.7	0	0	3.7	—	5.3	4.3	6.3	7.5
Heart	0	0	0	3.3	4.3	4.8	5.8	5.5	6.5	6.5
Skeletal muscle	0	2.3	0	2.5	3.3	4.8	5.5	4.8	6.5	6.5
Skin	0	0	0	0	3.7	4.5	5.0	4.5	6.3	8.3
Intestine	0	0	0	0	2.3	4.3	6.0	5.5	6.5	7.5
Brain	0	0	0	0	1.7	5.0	4.8	3.8	5.0	5.5

[a] Eleven-day-old chick embryos were infected via yolk sac with 0.2 ml of virus suspension containing $3.2 \times 10^{5.0}$ Pfu. 0 = no virus isolated with 10% tissues suspensions; — = not done or insufficient tissue for study. All tissue suspensions were tested in CV-1 cells; infectivity titers are based on cytopathic effect in these cells.

that were studied. By this time (see below) histological evidence of viral injury had developed.

The available evidence indicates primary replication of MPV in the CAM and cells of the allantoic sac. MPV was not recovered from yolk sac or its membranes after the 24th hr. Although cells within bone marrow, spleen and liver were recruited as sites of early virus multiplication, we consider them as potential secondary targets in view of the later upsurge of virus in these organs.

No data are presented for the viremic phase, chiefly because we wished to avoid risks of contaminating the small organ samples. Viremia very probably occurred during the earliest period of infection but at low levels of detection since many organs with rich vascular systems not only failed to yield MPV, but also failed to show focal fluorescence with labeled antibody.

Pathology. Significant histological alterations of tissues were not observed in infected chick embryos during the first 72 hr. By the fourth or fifth day gross and microscopic lesions were seen in the various visceral tissues listed in Table V. The liver and spleen were enlarged slightly, and white–yellow lesions up to 0.2 mm in diameter were present on the surface, as well as inside these organs. Visible lesions were not seen in other organs.

Microscopically, the main features of infection were those of focal cellular degeneration, necrosis and infiltration by, or local proliferation of mononuclear cells. Foci of necrosis in liver and spleen were characterized by small centers of lysed cells, and peripheral zones of pale-stained cells, often

TABLE V. Histopathological Scores in MPV Infected Chick Embryos.[a]

	Lesions found on indicated day after infection				
Tissues	1	2	3	4	5
CAM	+	+	+	+	+
Liver	0	0	0	+	+
Spleen	0	0	—	—	+
Heart	0	0	0	±	+
Kidney	0	0	0	±	+
Lung	0	0	0	±	±
Skin	—	0	±	0	+
Skeletal muscle	0	±	0	±	+

[a] Other negative tissues include brain and intestine. The scores: 0, = no lesions; +, = positive lesions; ±, = questionable lesions; —, = not done, or insufficient tissue for interpretation.

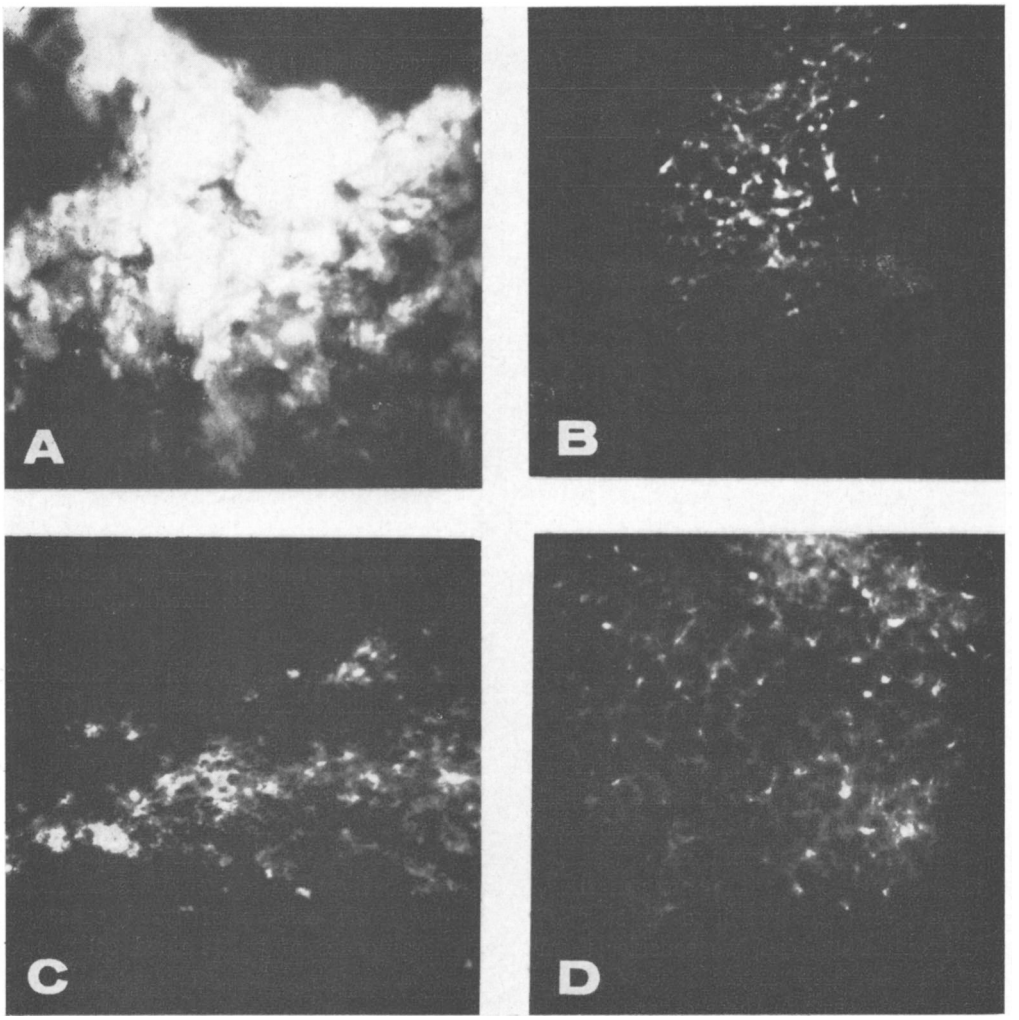

FIG. 2. Specific immunofluorescence of viral antigens in MPV infected chick embryo tissues. *A*. Pock on CAM, 100x. *B*. Spleen, 100x. *C*. Liver, 100x. *D*. Liver, 100x. See text relating to time postinfection.

with fragmented nuclei. Cellular infiltration of mononuclear cells was seldom seen in these areas. Focal infiltrates of mononuclear cells only were observed in heart, kidney, skeletal muscle, skin, and lung.

Histologically, the pocks produced in the CAM by MPV were indistinguishable from those produced by vaccinia virus. In both instances the inflammatory response consisted of edema, hemorrhage, focal necrosis and infiltration by mononuclear cells. Inclusion bodies were rarely found in tissues from infected chick embryos.

Labeling of tissues with FA conjugates. Selected tissues harvested on the fourth and fifth days yielded specific immunofluorescence by direct staining. Foci of labeled "cells," either discretely or diffusely stained were found in liver, spleen, kidney, lung and the CAM. The character of the staining pattern in several tissues is illustrated in Fig. 2. Histologically, areas of cellular necrosis were associated with specific labeling by anti-MPV antisera, as well as by labeled anti-vaccinia antisera.

Discussion. Embryonic chick tissues

provide a sensitive milieu for *in vivo* studies of MPV. Pock development is easily definable, and virus titrations are reproducible. However, the CAM, as used was no more sensitive than tissue culture systems for measuring MPV concentration in tissues, or in blood of infected monkeys (1). The responsiveness of the chick embryo to MPV presented further opportunity for studies on pathogenesis.

A rapid growth of MPV took place in extracorporeal ovular tissue (~ 12 to 18 hr) prior to the detection of virus (~ 24 to 36 hr) in liver, spleen and bone marrow. We assume that virus was transported hematogeneously to embryonic tissues wherein renewed replication of virus within reticuloendothelial elements resulted in cellular injury and further transport and recruitment (56–60 hr) of the remaining embryonic tissues entered in the study. Evidence of virus replication is based principally on intracellular labeling of MPV by specific fluorescent antibody.

A relatively large LD_{50} was necessary for induction of embryonic disease. The reasons are not explainable from data at hand. Strains of vaccinia and variola viruses differ in virulence and lethal responses for chick embryos (8, 9). Virulence *per se,* however, must relate to host response. Possibly a minimal effective concentration of virus needs to be reached in the chorioallantois for embryonic invasion. On the other hand, barriers to embryonic invasion may relate to metabolic changes developing in the embryo during the 21-day period *in ovo.* Virus replication in chick tissue may be retarded (10) between the 12th and 15th days *in ovo* during a time of rapid protein synthesis. Additional factors may relate to maturation of receptor sites on susceptible cells. Another barrier may relate to developing cellular immunity. Goodpasture (11) reported that chicks infected *in ovo* with vaccinia virus after hatching were immune to vaccinia virus.

Monkeypox virus is a member of the vaccinia–variola complex. Both variola and MPVs produce generalized disease with multiple organ involvement in cynomolgus monkeys. Vaccinia virus differs in that only local lesions develop after parenteral inoculation; discrete organ involvement in monkeys has not been described. On the other hand, pathogenetic features of MP and vaccinia (8) viruses *in ovo* are remarkably alike. Our strain of vaccinia virus however, after 10 passages *in ovo* failed to produce generalized disease in embryonated chick tissues. In contrast, MPV after one passage, produced generalized infection.

Our studies indicate that MPV is related more closely to variola than to vaccinia virus. The reasons relate to (a) a similar course of infection with MPV and variola in monkeys, (b) production of smaller pocks by variola and MPV than by vaccinia virus, and (c) similarities in ceiling temperatures of inactivation of MP and variola viruses. Failure of pock formation at 40.5° has been found a useful means of differentiating MPV and variola from vaccinia, cowpox, and rabbitpox viruses (12).

Summary. Chick embryos developed generalized infection from MPV. Sequential distribution of MPV *in situ* suggested that the virus was transported hematogenously, first to the extracorporeal membranes, and secondarily to embryonic tissues. Embryonic organs of the reticuloendothelial system were principal primary cell targets. The specificity of the cellular pathology was confirmed by retrieval of MPV from, and the immunological demonstration of fluorescent antigen in cells of affected organs.

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Received Sept. 20, 1971. P.S.E.B.M., 1972, Vol. 139.