PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 150, 318–323 (1975)

The Pathogenesis of Simian Varicella Virus in Cynomolgus Monkeys[1] (39027)

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For the past four years we have attempted to discover why workers in the field of varicella have been unable to recover, except rarely, varicella virus from body parts other than the skin in uncomplicated illness. Our efforts match those of another (1) inasmuch as we have been unable, even during early and late incubation intervals to rescue virus from urine, pharyngeal cells or secretions of the pharynx.

At that point we turned to study a newly discovered varicella-zoster (VZ) virus of primates (2, 3) in the hope that some of the epizootic and pathogenetic features of simian varicella might provide approaches useful in human infections. In this report we present data on *some* features of infection following use of a primate strain of VZ virus in cynomolgus monkeys.

Materials and methods. Monkeys. Cynomolgus monkeys, weighing between 1.9 and 2.7 kg were nonreactive to old tuberculin. All were in excellent health. Serum samples were procured prior to virus inoculation.

Virus. The Medical Lake Macaque (MLM) strain of VZ virus was provided by Dr. James Nakano, Center for Disease Control, United States Department of Health, Education and Welfare. The virus had been passaged 12 times consecutively in cultures of rhesus thyroid cells and thereafter 19 times in Vero cell cultures; seven of the passages in Vero cells were made in our laboratory. The virus passaged for inoculation had a titer of $\geq 10^{4.7}$ tissue culture infective units per milliliter.

Inoculations. Intradermal, intracardiac and/or intravenous portals were used for delivery of virus. Approximately 1.0 ml was inoculated in parallel rows consisting of eight intradermal blebs on the anterior thorax. The volume delivered either intravenously or intracardially averaged 1.6 ml per monkey.

On the third and fourth days postinoculation oil of mustard was rubbed onto the skin of the chests of three monkeys. None of the animals developed dermal irritation following such application over a 5 cm^2 area.

Clinical studies. Monkeys were inspected twice daily after the third day of inoculation. Rectal temperatures were measured daily. Monkeys were bled for viremia, and leukocyte patterns on alternate days 2 through 12, and for antibody responses at appropriate intervals (see Tables I and III). Monkeys were sacrificed by exsanguination while anesthetized with sodium pentothal.

Autopsies were performed immediately after death. Specimens for virus studies were collected with individual sterile instruments. Tissues were fixed in formalin and Zenker's fluid; sections were stained with hematoxylin and eosin, and occasionally with a trichrome stain.

Virus studies. The leukocyte buffy coat was procured from heparinized venous blood. Urine was adjusted to neutrality. Tissues were minced with scissors in maintenance media to make a 10% suspension. These specimens were inoculated onto Vero cells for the detection of virus.

Antibody studies. Antigens used in complement fixation tests were prepared from the MLM simian virus and our Wu strain of human varicella virus. These antigens, as well as uninfected control antigens were derived from sonicated cell culture extracts. Both antigens were studied in box titrations against homologous antisera. In the test proper twofold dilutions of sera were incubated overnight at 4° in the presence of 2 units of antigen and 5 units of complement. Appropriate controls were included. Sensitized cells were added to appropriate tubes and after incubation for 30 min at 37° scored

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TABLE I. DATA PERTAINING TO MONKEYS AND THEIR RESPONSES TO THE MLM STRAIN OF VZ VIRUS.

Identity	Portal of inoculation: ID	IC	IV	Clinical expressions: Fever[b]	Vesicles	Day of sacrifice	Viremia (indicated days): 2	4	6	8	10	12	Antibody responses CF titers[c] at indicated intervals: Pre inoc	10	11–16	24	28	Number of specimens yielding virus[d]
M-34	+[a]	+[a]		+ (103°–5th)	+(14)	16	–	–	–	–	–	–	8	512	1024	–	–	0
M-35	+	+		0	±(13)	46	–	–	–	–	–	–	16	1024	ND	512	128	ND
M-36	+[a]	+[a]		0	±(13)	57	–	–	–	–	–	–	<8	1024	512	512	256	ND
M-37	+	+		0	+(12)	14	–	–	–	–	ND	ND	16	512	512	–	–	2
M-38	+[a]		+[a]	0	+(11)	59	–	+	–	–	–	–	8	1024	ND	256	128	2[e]
M-39	+		+	+ (104–8th)	+(9)	11	+	+	–	–	ND	ND	32	256	256	–	–	7
M-40	+[a]		+[a]	0	+(15)	16	–	–	–	–	–	C	32	1024	1024	–	–	2
M-41	+		+	0	+(10)	16	–	–	–	–	–	ND	16	512	256	–	–	1

Note: ID = intradermal; IC = intracardiac; IV = intravenous portals of inoculation; ND = not done.

[a] Virus concentrated by negative pressure dialysis using a collodion membrane retaining proteins of 25,000 mol wt (Schleicher and Schuell). The virus had $\geq 10^{4.7}$ infective tissue culture units.

[b] In excess of 103°F on days noted for two monkeys.

[c] Expressed as reciprocol of serum dilution.

[d] See also Table II.

[e] Visceral organs were not taken during the infective period; virus present in vesicle fluid, as well as blood.

for dilutions showing 3 to 4+ fixation of complement. The microtiter system was used.

Results. Clinical disease. Six of eight monkeys developed a vesicular eruption between the day 9 and day 15 postinoculation. Vesicle formation was observed principally on the chest and abdomen and proximal segments of the extremities. Vesicles ranged in size from 0.5 to 3.0 mm; occasionally clusters developed especially in areas where the skin had been rubbed with oil of mustard.

Vesicles developed in four monkeys initially at intradermal inoculation sites and then elsewhere on the trunk within 24–48 hr. As a rule truncal lesions emerged within 36 hr, and may have developed in narrow temporal stages in view of variations in size. Pustulation was not a significant feature of the dermal reaction. Red-hued, thin scabs formed within 36–48 hr and healed completely within 3 days. None of the animals had a demonstrable enanthem.

Two monkeys developed fever (Table I); one had recognized viremia (M-39); otherwise none developed signs of illness. A mild neutropenia and lymphopenia on the second day was followed by lymphocytosis between the fourth and tenth days postinoculation.

Recovery of virus. VZ virus was recovered from vesicle fluids and cutaneous biopsies of seven monkeys. Virus was recovered once from visibly uninvolved skin in one of four monkeys. VZ virus was detected in the leukocyte fraction of blood obtained from two monkeys on the second and fourth days. None was rescued from similar fractions in six monkeys, nor from these two positive monkeys between the 4th and 12th days postinoculation (Tables I and II).

Virus was recovered also from urine aspirated from the bladders of two monkeys on the days 9 and 15, respectively. Additionally, virus was detected in homogenates of bladder, kidney, liver and lymph node specimens procured at autopsy from two to five monkeys sacrificed for such studies (Table II).

Antibody responses. The specific antibody status of monkeys to MLM virus is tabulated in Table I. Seven of eight animals possessed low levels of antibody prior to challenge with MLM virus. The serologic responses were maximal by the tenth day postinoculation. A moderate reduction in titer values occurred during the ensuing weeks.

These same sera were studied also in relation to antibodies developing to human varicella virus (strain Wu). Preinoculation sera reacted to the Wu strain identically with MLM antigen (Table III). MLM infection raised antibodies to the human strain of VZ virus.

On the day 28 after inoculation with MLM

TABLE II. PRESENCE OR ABSENCE OF VIRUS AND PATHOLOGIC CHANGES IN SELECTED TISSUES AND BODY FLUIDS OBTAINED FROM FIVE ANIMALS SACRIFICED DURING THE ACUTE PHASE OF INFECTION.[a]

Identity	Presence or absence of	Tissue and body fluids											
		Skin	Trachea	Lung	Spleen	Lymph nodes	Liver	Bone marrow	Bladder	Kidney	Urine	Blood	Trigeminal G.
M-34	Virus										−		−
	Pathology	+					+	−			−		−
M-37	Virus	+							+		−		−
	Pathology	+					+	−			−		−
M-39	Virus	+				+	+		+	+	+	+	
	Pathology	+	±				±	−			−		−
M-40	Virus	+									+		−
	Pathology	+					+	−					
M-41	Virus	+											
	Pathology	+					+	−			−		−

[a] Legend: +, = isolation of MLM virus, and/or definitive histopathologic findings. −, = not tested. All open entries indicate negative tests for virus and normal histology, excepting the skin from M-34. The cultures from the negative tissues above were passaged once again on Vero cells and failed to yield virus. Many additional tissues, excluding the central nervous system were studied histologically; these include tongue, pharynx, alimentary tract, heart, pancreas, adrenals etc. All presented normal histologic features. Not included in this table is the viremia detected in M-38 (see Table I).

virus the three surviving monkeys received the Wu strain of varicella virus intradermally. One monkey (M-38) on the third day developed erythema, measuring 1.0 cm in diameter and a month later may have had a modest antibody recall (Table III).

Histopathology. The development of cutaneous lesions is illustrated in Fig. 1. An early lesion shown in Fig. 1A involved the epidermis, a hair follicle and sebaceous gland. The remaining photomicrographs (Figs. 1B, C, and D) illustrate progressive colliquation of epidermal cells, development of septa, fluid collection and vesiculation. The vesicle roof is covered by cells of the stratum cornium, while the floor is comprised of a thin mantle of basal epithelial cells, often in varying stages of cellular injury.

These lesions demonstrate the following changes: a major involvement of epithelial cells, principally of the stratum spinosum with progressive cell distortion and destruction leaving a fibrinoid reticulum filled with fluid. With further progression multinucleate cells develop and appear to be cast freely (Fig. 1D) into, or along amorphous strands (epithelial fibers, possibly) of the vesicle.

Cytologic features include distortion of epithelial cells, disunion of intercellular

TABLE III. COMPARATIVE SERORESPONSES TO MLM AND Wu* STRAINS OF VZ VIRUSES FOLLOWING INFECTION FROM MLM AND INTRADERMAL INOCULATION OF Wu IN CYNOMOLGUS MONKEYS.

Identity	Antigen	Reciprocal of titers on indicated days				
		Preinoculation	10–16	20–27	28	36–59
M-34	MLM	8	512	—	Inoculated intradermally with Wu strain of VZ virus	—
	Wu	<8	128	—		—
M-35	MLM	16	1024	512		128[b]
	Wu	16	256	128		256
M-36	MLM	<8	1024	512		512
	Wu	<8	512	128		256
M-37	MLM	16	512	—		—
	Wu	32	256	—		—
M-38	MLM	8	1024	256		1024
	Wu	16	256	512		1024
M-39	MLM	32	256	—		—
	Wu	32	256	—		—
M-40	MLM	32	1024	—		—
	Wu	32	256	—		—
M-41	MLM	16	512	—		—
	Wu	32	128	—		—

[a] The Wu strain was isolated from vesicle fluid of a child ill with varicella.

[b] M-35, data for serum 8 days postchallenge with Wu. Sacrificed on the 46th day when the blood sample was lost accidentally.

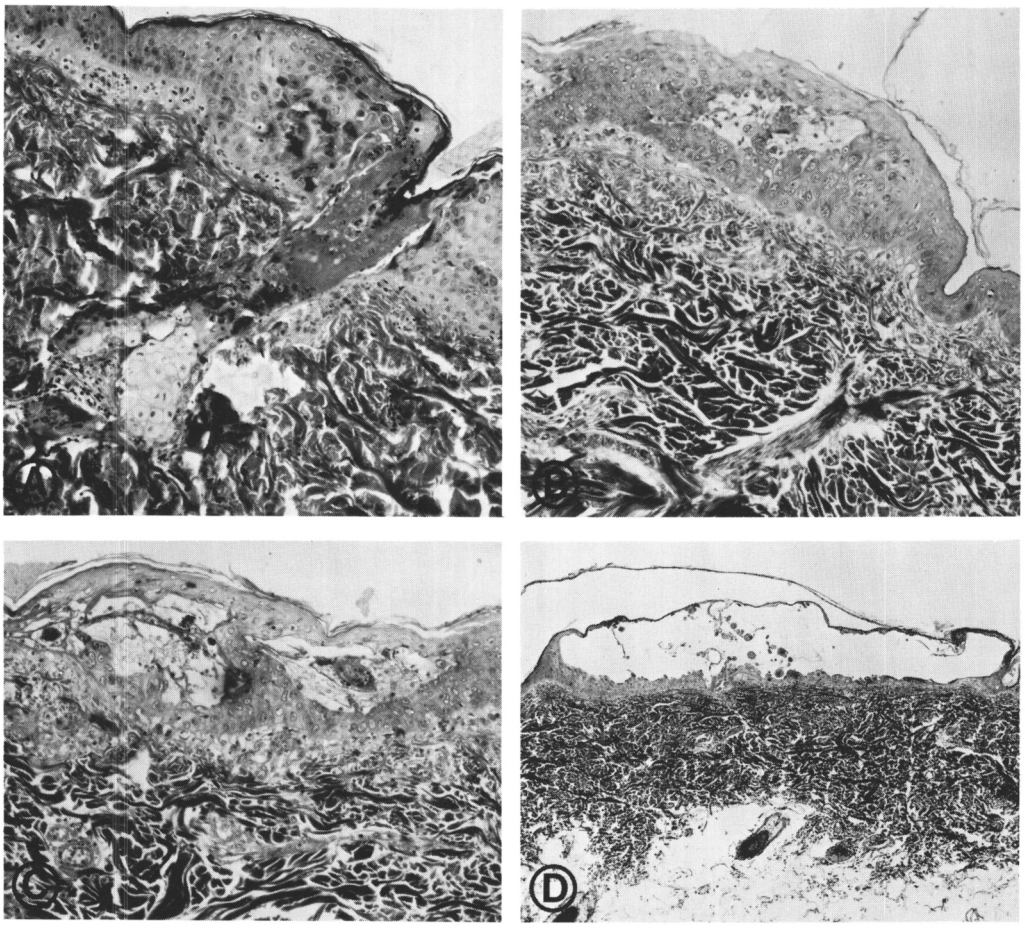

FIG. 1. The histopathology of cutaneous lesions observed in cynomolgous monkeys. Hematoxylin and eosin stain. Fig. 1-D = 40×; others = 60×. 1-A and 1-D are from M-39 sacrificed on the 11th day postinoculation; 1-B and 1-C are from M-40 sacrificed on the 16th day.

bridge and eventual separation from the epidermal syncytium. Nuclei appear swollen and the cytoplasm supplanted. The nuclei are often homogeneously pale tinctorially; sometimes they appear "empty"; otherwise they contain marginal bands of chromatin, or threading with a fine basophilic reticulum. Many of these cells contain eosinophilic intranuclear inclusions of varying sizes and shapes (Fig. 2A).

The dermis and adjacent tissues are involved also in the inflammatory process. The endothelium of adjacent capillaries appear to be swollen, hyperplastic, and sometimes surrounded by a thin mantle of mononuclear cells. Occasionally polymorphonuclear cells are found in adjacent areas. Inclusion bodies resembling those seen in the epidermis were not observed in endothelial, or other cells infiltrating the dermis and subcutaneous tissues.

Microabscesses scattered in the parenchyma of the liver were found in four of five monkeys. Two such lesions are illustrated in Figs. 2B and C. The first illustrates an acute lesion featuring necrosis of hepatocytes, polymorphonuclear and monocytic infiltration, and early repair. The second illustration depicts a more advanced stage of repair with mononuclear and phagocytic cells enmeshed among fibroblasts.

Similar microabscesses were found infrequently in regional lymph nodes. One such abscess is illustrated in Fig. 2D. Lymphoid

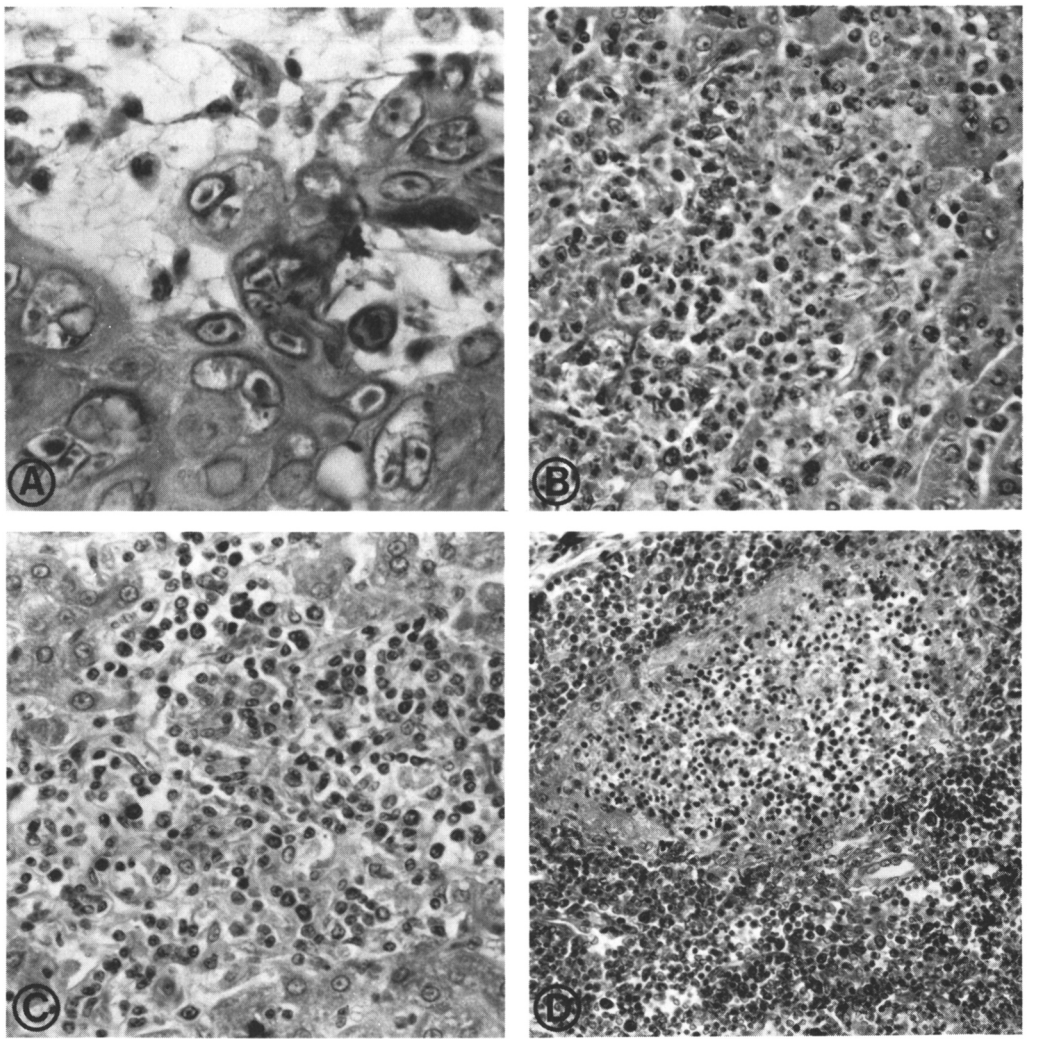

FIG. 2. Additional histopathological injuries. 2-A, cytological reactions within a cutaneous lesion from M-40 illustrating a reticulum interlacing epithelial cells in various stages of injury; some are multinucleate and contain "hollow" nuclei and inclusions, 500×. 2-B, C, lesions involving hepatocytes, in the acute (2-B) and reparative (2-C) phases, 192×. 2-D, acute phase reaction in lymph nodes of M-41, 120×. Hematoxylin and eosin stain.

cells in various stages of lysis are enmeshed in an amorphic matrix lying within this encapsulated follicle.

A few cells lying within hepatic microabscesses contain intranuclear inclusions. Such cells are few, and the inclusions somewhat smaller than those seen in epithelial cells. Definitive inclusions were not seen within cells of lymph nodes. Although MLM virus was not recovered from these two particular organs the same organs from companion monkeys yielded MLM virus. Attempts to label antigen within organs by fluorescence microscopy are in progress.

Discussion. The findings recorded herein on experimental infection of cynomolgus monkeys with the MLM strain of VZ virus are essentially similar to naturally occurring infections in macaques reported by Blakely *et al.* (2). The disease in cynomolgi was benign, and excepting for pathologic lesions involving the skin, liver and several lymph nodes none of our animals had the additional organic lesions noted in monkeys

dying of the disease (2, 3). Since MLM virus had been passaged continuously in cell cultures, a loss in virulence for monkeys may account for the prolonged incubation period noted in our study. In recent studies using a virus stock derived from M-39 the incubation period is shorter and the disease expression intensified.

The new data in our report relate to the recovery of virus from the blood during the first 4 or 5 days postinoculation, and its detection in visceral organs and urine during the eruptive period of infection. The early viremia probably represents primary multiplication *in vivo*, possibly in leukocytes or in sinusoidal endothelium preceding the involvement of hepatocytes and other susceptible cells. While we have not detected viremia late in the incubation period (our studies were sometimes deficient because of inadequate sampling) we infer its presence based on the detection of virus in kidney, bladder and urine. The lack of renal pathology may not exclude virus growth in renal cells. At this time in our studies we postulate that viruria represents renal clearance of virus from blood, possibly complexed with antibody.

The low levels of VZ antibodies found prior to challenge with MLM may have been another factor modulating the clinical expression of disease! Were these antibodies raised by prior experience from either *Herpesvirus varicellae* (simian or human) or *Herpesvirus simiae*? At this time there are no answers. The evidence is equivocal in view of the generalized infection in M-39, a monkey having antibodies prior to inoculation, and the restricted responses of M-36, a monkey free of such antibodies at the time of challenge. We are aware of the possibility that CF antibodies wane following preceding infections either in the wild or in captivity.

Following an epizootic Blakely *et al.* (2) found monkey sera reactive for VZ and *Herpesvirus hominis* antigens. About half of these sera (46%) were positive for VZ and a fourth (23%) for the human herpesvirus; the CF titers of each was significantly lower than for MLM virus. The MLM and VZ antibody responses in our cynomolgus monkeys were not as divergent, and may be an expression of a broader antigenic response to prior experiences with related antigens!

This varicella-like disease of monkeys may provide ways for unraveling pathogenetic and epidemiologic aspects of VZ infections of mankind. A number of interesting questions relate to the natural disease in human beings. A major obstacle in completely accepting droplet transmission has been the failure to rescue VZ virus from pharynx or pharyngeal secretions, either during the incubation period or during the eruptive phase of illness. The available evidence in generalized varicella supports hematogenous dissemination, and the probable reality of such viremia has been noted (1, 4). Hopefully studies in monkeys may provide information useful in further studies of human beings.

Summary. The MLM herpesvirus is infectious for cynomolgus monkeys. The disease in this species, possibly modulated by preinoculation antibody resembles human varicella. Virus has been recovered from blood during the early incubation period, and from liver, lymph nodes, kidney, bladder and urine during the eruptive period of infection. The major target organs were skin and liver; specific pathological changes developed in both. Appropriate antibody responses, including those to *Herpesvirus varicellae* followed infections mounted by parenteral inoculation of cynomolgus monkeys.

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Received May 12, 1975. P.S.E.B.M. 1975, Vol. 150.