

STUDIES OF ENCEPHALITOOZONOSIS IN VERVET MONKEYS (*CERCOPITHECUS PYGERYTHRUS*) ORALLY INOCULATED WITH SPORES OF *ENCEPHALITOOZON CUNICULI* ISOLATED FROM DOGS (*CANIS FAMILIARIS*)

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ABSTRACT

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Encephalitozoonosis was induced in 35 of 38 vervet monkeys (*Cercopithecus pygerythrus*). They were either directly (orally) inoculated with *Encephalitozoon cuniculi* or indirectly exposed to this protozoan parasite. Cell-culture-grown spores of *E. cuniculi*, isolated from the kidneys of dogs with natural, fatal disease, were administered orally to 29 of these monkeys. Another 5 were exposed in utero by orally infecting pregnant females, and 3 were exposed to horizontal infection by nursing infected infants. Only one was given an intravenous inoculation of spores. The disease was induced in non-gravid and late-pregnant adults, immunocompetent infants, and in infants that were immunologically compromised by parenteral steroid administration, as well as in one infant that was immunologically immature because of its premature birth. The effects of age, dosage, post-inoculation (PI) interval, passage level of the parasite in cell culture and immunological status of the host were correlated with macroscopical and microscopical lesions. The experimentally induced infection was confirmed either by re-isolation of the parasite in cell culture or by observation of spores in tissue sections. Both confirmatory methods were supported by serological examination. Re-isolation of the organism in primary cell culture prepared from kidneys usually resulted in more frequent isolates and larger yields of spores from infants than from adult vervets.

Infection with *E. cuniculi* invariably induced subclinical disease. Based on histology, lesions were minimal to moderately severe, depending on age, PI interval, and immunological status of the host. Alimentary tract infections were seen histologically as early as three days PI. Subsequently, infections resulted in detectable lesions most consistently in the liver, kidneys and brain. Lesions in these organs were generally granulomatous and were similar to those found in canine encephalitozoonosis. In addition, multifocal interstitial pneumonitis and myocarditis as well as vasculitis and perivasculitis were seen in other tissues and organs.

Infants had more severe and more widespread lesions than adults. Although lesions and spores were still present in the brain of one immunocompetent infant 36 weeks after initial infection, the disease in immunocompetent infants and adults is thought to be self-limiting. However, infection may persist.

Immunological depression favoured increased growth and multiplication of the organism, and resulted in detection of more spores within inflammatory lesions as well as more intracellular colonies of the organism that were free of inflammatory reaction.

Long-term growth of *E. cuniculi* in cell culture did not appear to alter its pathogenicity, nor did varying the numbers of spores (2×10^6 - 3×10^{10}) in the inoculum appear to influence the severity of the disease. Horizontal infection was readily established, and vertical transmission was demonstrated.

Results of this study suggest that spores of *E. cuniculi* shed by dogs may become agents of latent infection and disease in vervets, and probably other nonhuman primates as well. It further seems probable that these spores may also be potential agents of latent zoonosis of man.

INTRODUCTION

Encephalitozoonosis in dogs was apparently first observed by Kantorowicz & Lewy (1923) in Germany about one year after the aetiological agent of the disease in rabbits was initially described in the United States of America (Wright & Craighead, 1922) and at about the same time the organism was named *Encephalitozoon cuniculi* by Frenchmen (Levaditi, Nicolau & Schoen, 1923). Levaditi *et al.* (1923) considered their newly discovered protozoan to be a microsporidian, but this was not proven until nearly 40 years later when Nelson (1962) demonstrated the characteristic polar filament (tube) in the spore phase of the organism.

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The disease in canines and other carnivores has been reported in numerous countries including Germany (Kantorowicz & Lewy, 1923), France (Manouelian & Viala, 1924; 1927), England (Plowright, 1952), Tanzania (Plowright & Yeoman, 1952), Republic of South Africa (Basson, McCully & Warnes, 1966; Van Rensburg & Du Plessis, 1971; Van Dellen, Botha, Boomker & Warnes, 1978; Botha, Van Dellen & Stewart, 1979; Stewart, Van Dellen & Botha, 1979; Stewart, Botha & Van Dellen, 1979), Zimbabwe (McCully, Van Dellen, Basson & Lawrence, 1978), Czechoslovakia (Vavra, Blazek, Lavicka, Koczkova, Kalafa & Stehlik, 1971), Norway (Nordstoga, 1972; Arnesen & Nordstoga, 1977), and the United States of America (USA) (Buyukmihci, Bellhorn, Hunziker & Clinton, 1977; Shaddock, Bendele & Robinson, 1978; Cole, Sangster, Sulzer, Purcell & Ellinghausen, 1982).

Clinically, the disease in dogs has been confused with and is suggestive of canine distemper (Basson, McCully & Warnes, 1966; McCully, Van Dellen, Basson & Lawrence, 1978; Van Dellen, Botha, Boomker & Warnes, 1978; Botha, Van Dellen & Stewart, 1979). *Encephalitozoon* was also once suggested to be the cause of rabies (Manouelian & Viala, 1924; 1927). Encephalitozoonosis may be misdiagnosed clinically because it mimics other diseases, especially toxoplasmosis, which also affect the central nervous system (Shaddock & Pakes, 1971).

Cell culture isolation of *E. cuniculi* from dogs was

made in South Africa by Colin G. Stewart in 1976 (personal communication), and made it possible to conduct the studies reported here, *vide infra* (Stewart *et al.*, 1979a). A year later, by demonstrating spores within lesions in kidney and brain tissue sections, Lowell T. Sangster (personal communication, 1977) was the first to diagnose the disease in dogs in the USA, but the finding was not reported until five years later (Cole *et al.*, 1982), nor was the aetiological agent isolated. It was not until a second outbreak was discovered in dogs that the agent causing canine encephalitozoonosis was again isolated in cell culture and reported to occur in the USA (Shaddock *et al.*, 1978).

Non-human primates are apparently susceptible to infection by perhaps at least two genera of microsporidians. A natural, disseminated infection with severe encephalitis caused by *Nosema* (*Encephalitozoon*) *cuniculi* was reported in the squirrel monkey (*Saimiri sciureus*) (Brown, Hinkle, Trevethan, Kupper & McKee, 1973), as well as congenital encephalitis caused by *E. cuniculi* which was suggested to have been acquired *in utero* (Anver, King & Hunt, 1972). More recently, it has become apparent that squirrel monkeys raised in captivity may be at significant risk to acquire encephalitozoonosis (Zeman & Baskin, 1985). The specific microsporidian aetiology remained elusive, however, in another case in which *Nosema* sp. was only implicated as the cause of jejunal infection assumed to have been acquired by the host (*Callicebus moloch*) from eating insects (Seibold & Fussell, 1973).

Prior to the recognition of Acquired Immunodeficiency Syndrome (AIDS), microsporidial infections causing clinical disease in man were infrequently diagnosed. Only five, reasonably certain, cases were documented (Matsubayashi, Koike, Mikata, Takei & Hagiwara, 1959; Margileth, Strano, Chandra, Neafie, Blum & McCully, 1973; Ashton & Wirasinha, 1973; Pinnolis, Esbert, Font & Winter, 1981; Bergquist, Stintzing, Smedman, Waller & Anderson, 1984). In one of these five cases (Margileth *et al.*, 1973), the aetiological agent was retrospectively considered a "new" species and was later described as *Nosema connori* (n. sp.) (Sprague, 1974). By deduction, it was concluded that two other cases were probably caused by *Encephalitozoon* sp. (Matsubayashi *et al.*, 1959; Ashton & Wirasinha, 1973). In the fourth case, *Nosema* sp. was confirmed by transmission electron microscopy (Pinnolis *et al.*, 1981), and in the fifth case (Bergquist, *et al.*, 1984) encephalitozoonosis was diagnosed by serological tests.

Since the advent of AIDS and the increased awareness that microsporidiosis may occur in man, three additional cases with clinical manifestation of disease have been reported (Ledford, Overman, Gonzalvo, Cali, Mester & Lockey, 1985; Desportes, Le Carpentier, Galian, Bernard, Coehand-Priollet, Lavergne, Ravisse & Modigliani, 1985; Terada, Reddy, Jeffers, Cali & Schiff, 1987). All three of these were in AIDS patients, and one of them involved recognition of a new genus and species (Desportes, 1985). Thus, it seems probable, skeptical opinion notwithstanding (Bywater, 1979), that several genera of microsporidia are pathogenic for man.

A number of recent serological surveys appear to support this probability (Stewart, Van Dellen & Botha, 1981); Singh, Kane, Mackinlay, Quaki, Yap, Ho, Ho & Lim, 1982; W. H. O. Wkly. Epidem. Rec., 1983; Bergquist *et al.*, 1984; Bergquist, Morfeldt-Mansson, Pehrson, Petri & Wasserman, 1984; Hollister & Canning, 1985, 1987). It seems reasonably clear that man may be infected with microsporidia. Because of his close affiliation with the dog, he may be at greatest risk of infection with *Encephalitozoon*. This study was there-

fore conducted with *E. cuniculi* spores harvested from dogs in a species somewhat phylogenetically closer to man—the vervet—to enhance our understanding of encephalitozoonosis in primates, including man.

MATERIALS AND METHODS

Source of experimental animals and their husbandry

The vervet monkeys (*Cercopithecus pygerythrus*) studied in these experiments were obtained from free-living troops in the Kruger National Park¹. Individuals in the troops were considered free of encephalitozoonosis on the basis that 127 randomly collected sera tested by indirect fluorescent antibody (IFA) (*vide infra*) were interpreted as negative for antibodies to spores of *Encephalitozoon* sp. (Stewart, Van Dellen & Botha, 1981). Also, necropsies of more than 150 vervets from these troops did not reveal any lesions attributable to this disease (Van Dellen & DeVos, unpublished data, 1979). Vervets selected for experimental work were acclimated to laboratory captivity for periods ranging from one to several months. A commercial ration formulated and pelleted for primates was provided twice daily, and water was consumed *ad libitum*. This basic diet for adults was supplemented daily with pumpkins and sweet potatoes. One caesarean derived infant who never nursed on his dam (Table 3) and a 5-week-old orphan (Table 4) were hand-reared on human infant formula for 6 weeks and 9 weeks, respectively, prior to necropsy².

Each adult vervet was housed in a metal cage (1,0 × 1,5 × 2,0 m) with grated floor 0,6 m above the cement floor of an isolation stable, and in a presumably *Encephalitozoon*-free environment. Handling and examination of vervets were done with the aid of a sliding rear panel built into each cage to facilitate the trapping of vervets in a forward position so that a dissociative anaesthetic³ could be administered intramuscularly. The caesarean derived infant and the orphan were housed independently in a 0,5 × 1,0 × 1,0 m wire cage. Each was held by various human hands while being nursed with formula and provided with social comfort.

Inducing the infection

Of the 38 vervets which form the basis of this report, 29 were inoculated orally (Tables 1, 2 & 4), 5 were exposed *in utero* (via oral inoculation of pregnant vervets; Table 3), and 3 were exposed to horizontal infection (Table 6). One was given an intravenous inoculation of spores (not listed in tables).

Eight of the vervets were infants infected *per os* shortly after normal birth (one premature) and were reared by their dams (Tables 1 & 4). A ninth infant infected *per os* was a 5-week-old orphan that had been born in the wild and was hand-fed in captivity from approximately 1 week of age (Table 4). Three of the eight dams were selected to remain in their individual cages after their infected infants had been removed for necropsy; these dams were later examined for evidence of horizontal spread of the organism (Table 6). [The remaining five dams were removed to a vervet breeding colony which was being monitored serologically for horizontal spread of encephalitozoon infection.]

¹ The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, 2101 Constitution Avenue, N.W., Washington, DC 20418

² Lactogen®; Food & Nutritional Products (Pty) Ltd, 192 Hendrik Verwoerd Drive, Randburg, 2194

³ Ketamine hydrochloride (Ketalar®), Parke-Davis, Division of Warner-Lambert Inc., 201 Tabor Road, Morris Plains, New Jersey 07950

TABLE 1 Oral administration of spores of *Encephalitozoon* to immunocompetent infant vervets^(a)

Infant number	Age (days)	No. of spores given	Spores in tissue culture	Spores in tissue section	Macroscopical change			Microscopical change ^(g)			Postinoculation (weeks)
					Liver	Kidney	Brain	Liver	Kidney	Brain	
RM-23	1,5	2 × 10 ⁷	(b)	++ ^(f)	0	3	0	2-C	3-A	3-B	7
RM-32	62	1 × 10 ⁷	x ^(c)	++	0	2	0	1-B	3-A	3-B	9
RM-34	7	8 × 10 ⁷	x	++	0	3	0	2-A	3-B	2-B	7
RM-58	3	1 × 10 ⁷	x	++	0	2	0	2-A	2-A	3-B	8
RM-35	4	11 × 10 ⁷	- ^(d)	-	0	1	0	1-C	1-C	1-A	24
RM-37	1	8 × 10 ⁷	[x] ^(e)	[+] ^(e)	0	2	0	2-B	[3B] ^(e)	2-B	36
			x	++					2-A		
			[x] ^(e)	[+] ^(e)					[3B] ^(e)		

^(a) Results of horizontally infected dam, RM-58, are in Table 6; other dams were returned to breeding colony

^(b) Primary kidney cell culture did not grow

^(c) x = positive tissue culture

^(d) - = negative tissue culture

^(e) Specimen from unilateral nephrectomy at 12 weeks PI

^(f) Spores in Tissue Section.

- = not found

+ = scarce

++ = few

+++ = numerous

++++ = exuberant

^(g)Classification of lesions

Magnitude and frequency	Stage of Disease	Type	Dominant histological character
0 = none	Immunolymphoid	"A"	plasmacytes and lymphocytes
1 = minimal	Granulomatous	"B"	macrophages, epithelioid cells, giant cells, astrocytes (brain), some eosinophils and rarely neutrophils
2 = mild			
3 = moderate	Resolution	"C"	lymphocytes, fibrocytes and usually some eosinophils
4 = massive			

TABLE 2 Oral administration of spores of *Encephalitozoon* to adult vervets

Adult number	Number of spores given	Spores in tissue culture	Spores in tissue section	Macroscopical change			Microscopical change ^(f)			Postinoculation (weeks)
				Liver	Kidney	Brain	Liver	Kidney	Brain	
IRM-104 ^(a)	3,0 × 10 ¹⁰	ND ^(d)	+ ^(b)	0	0	0	0	0	0	3 days]
RM-56	1,0 × 10 ¹⁰	ND	+ ^(b)	0	0	0	1-B	1-A	0	1
RM-62 ^(b)	4,0 × 10 ⁷	—	- ^(f)	0	1	0	2-B ⁽ⁱ⁾	1-A	0	2
RM-61 ^(b)	4,0 × 10 ⁷	—	+	1	1	0	2-B	1-A	0	3
RM-47	4,0 × 10 ⁷	—	+	2	1	0	3-B	1-A	0	3
RM-60 ^(b, c)	3,0 × 10 ⁷	—	+	1	3	0	3-B	2-A	0	4
RM-63 ^(b)	7,0 × 10 ⁷	—	- ^(f)	1	1	0	2-B ⁽ⁱ⁾	2-A	0	4
RM-10	5,0 × 10 ⁷	ND	+	NS ^(d)	NS	NS	3-B	2-A	1-B	6
RM-50	6,0 × 10 ⁷	(e)	+	3	3	0	2-B	3-A	1-B	7
RM-51	6,0 × 10 ⁷	—	+ ^(f)	3	3	0	4-B ⁽ⁱ⁾	3-A	1-B	7
RM-52	5,0 × 10 ⁷	x ^(f)	+	1	2	0	3-B	3-A	0	8
RM-53	7,0 × 10 ⁷	—	+	1	3	0	2-B	3-A	0	8
RM-54	5,0 × 10 ⁷	—	+	1	3	0	2-B	3-A	0	8
RM-9	3,0 × 10 ⁷	ND	+	NS	NS	NS	1-B	3-B	1-B	9
RM-59 ^(b)	3,0 × 10 ⁷	—	—	1	1	0	1-C	2-C	1-B	11
RM-46	4,0 × 10 ⁷	(e)	—	1	2	0	1-A	2-A	0	12
RM-69	2,0 × 10 ⁶	—	+	1	2	0	1-C	2-A	0	13
RM-48	4,0 × 10 ⁷	—	—	1	2	0	1-C	2-C	0	14
RM-68	2,0 × 10 ⁶	(g)	—	0	1	0	2-B	1-A	0	16
RM-18	4,0 × 10 ⁷	—	—	1	1	0	2-C	2-C	1-A	18

^(a) This vervet is in fact an "old" juvenile and tabled here for comparison with RM-56, a "young" adult; both given high numbers of spores

^(b) Results of infants nursed by these females (or hand-reared) appear in Table 3

^(c) Female in late gestation whose infant was delivered by caesarean section and hand-reared on human infant formula (see Table 3)

^(d) ND=Not done; NS=Not seen

^(e) Primary kidney cell culture did not grow

^(f) Reisolated organisms were used to inoculate a dog

^(g) Fungal contamination of cell culture

^(h) Spores observed in small intestines, all other observations of spores were in liver or kidney or brain

⁽ⁱ⁾ "Lifted" granulomas contained degenerating spores, Fig. 8

^(j) See "Classification of lesions", (g) of Table 1

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TABLE 3 Oral administration of spores of *Encephalitozoon* to females in late gestation, ^(a) with resultant lesions in the infants

Infant or foetus number	Number of spores given to dam	PI time <i>in utero</i> (wks/days)	Clinical signs	Spores in tissue culture	Spores in tissue section	Macroscopical change			Microscopical change ^(e)			Time of necropsy (after birth) (or abortion)
						Liver	Kidney	Brain	Liver	Kidney	Brain	
RM-59 ^(b)	3 × 10 ⁷	3/21	Normal birth	x	++	1	1	0	1-B	2-B	1-B	8 wks
RM-60 ^(c)	3 × 10 ⁷	4/32	Caesarean delivery	ND	++	1	2	0	2-B	1-A	2-A	6 wks
RM-63	6 × 10 ⁷	4/32	Caesarean delivery	ND	—	0	0	0	0	0	0	10 h
RM-62	4 × 10 ⁷	2/13	Abortion	—	—	0	0	0	0	0	0	0,5 h
RM-61	4 × 10 ⁷	3/24	Abortion	(d)	ND	Dead, autolytic foetus			ND	ND	ND	—ND

^(a) Results shown are those of the infants; results of examination of their dams are found in Table 2

^(b) Infant was nursed by its dam

^(c) Separated from dam at delivery and hand-reared on human infant formula (Lactogen[®])

^(d) Primary kidney cell culture did not grow

^(e) See "Classification of lesions", (g) of Table 1

TABLE 4 Oral administration of spores of *Encephalitozoon* to immunocompromised, nursing infant vervets^(a)

Infant number	Age and/or mass	Number of spores given	Immuno-incompetence or suppression	Spores in tissue culture	Spores in tissue section	Macroscopical change			Microscopical change ^(g)			Postinoculation Weeks
						Liver	Kidney	Brain	Liver	Kidney	Brain	
RM-66 ^(b)	168 g	1 × 10 ⁷	Premature birth ^(b)	x	++++	0	2	0	2-B	2-B	2-B	6
RM-67	1 day (350 g)	2 × 10 ⁶	Steroid ^(d) 8 mg/wk, IM ^(e)	x	+++	1	1	0	3-B	1-B	1-B	4
RM-70 ^(c)	5 wks (500 g)	3 × 10 ⁷	Steroid ^(d) 1.3 mg/wk, IM ^(e)	(f)	+++	1	1	0	3-B	3-B	1-B	4

^(a) Results shown are those for the infants; results of horizontally infected dams are in Table 6

^(b) Infant 50–65% of normal mass

^(c) Orphan, hand-reared on human infant formula (Lactogen[®])

^(d) Methylprednisolone acetate

^(e) IM = intramuscular

^(f) Primary kidney cell culture did not grow

^(g) See "Classification of lesions", (g) of Table 1

Five adults in late gestation were infected *per os* (Table 3). Three of the five progeny lived; one infant was necropsied 8 weeks after normal delivery, and two infants were necropsied 10 h and 6 weeks after caesarean delivery. The dam of the first infant was inoculated 3 weeks prior to giving normal birth, while the dams of the latter two infants were inoculated 4 weeks prior to the caesarean sections. Two of the five inoculated females aborted (Table 3), but only one foetus was histologically examined, since the other was too autolyzed. These dams were inoculated 2 and 3 weeks prior to abortion, respectively.

Nineteen normal adult vervets (males and non-pregnant females) were non-concurrently infected *per os*, and were necropsied at varying intervals so that a PI interval ranging from 1–18 weeks was obtained (Table 2). [The twentieth vervet listed first in Table 2 is an "old" juvenile, RM-104, and placed here for comparison of data from RM-56, a "young" adult.]

To determine possible infectivity changes due to repeated passage of the organism in cell culture, two adults were infected *per os* (Table 5) with passage 17 of isolate DK₂. Their data were compared to those of two other adults inoculated with passage 75 of isolate DK₂ (see also Table 2).

An exceptionally large dose of spores (1–3 × 10¹⁰) was given orally to two vervets (RM-104 & RM-56, Table 2) in an effort to demonstrate multiplication of *E. cuniculi* in the gastro-intestinal tract. Numerous histological sections were made from several specimens taken from the stomach as well as the small and large intestine of these animals, in addition to the routine sections made from specimens of liver, kidney and brain.

Inoculum

The cell-culture-grown spores of *E. cuniculi* which comprised the inocula were originally isolated from renal tissues of two pups with encephalitozoonosis (Stewart *et al.*, 1979a). These terminally ill pups originated from two separate kennels where outbreaks of the disease had occurred.

The spores were typical for the genus in that they were approximately 1.5 μm in diameter, 2.5 μm long, slightly curved and bacillary in shape. They stained poorly with haematoxylin and eosin in tissue section, stained very well with tissue Gram's stain, and they were polarizable, acid-fast and PAS-positive. Each spore contained a single nucleus as well as a polar filament (tube) coiled in six loops.

Initial growth of spores in primary kidney cell culture was subsequently maintained in established kidney cell lines. Spores were then harvested from these kidney cell lines for oral inoculation into vervets. The first isolate, designated DK₁, was maintained in MDCK cells (dog kidney cell line), and the second isolate, designated DK₂, was maintained in LLCMK₂ cells (monkey kidney cell line). Isolate DK₂ was passaged every 4–7 days for a total of more than 75 passages. Spores were harvested at various passage levels, concentrated by centrifugation and resuspended in phosphate-buffered saline. An aliquot of the suspension was counted in a haemocytometer just prior to administration of inocula. The number of spores in each inoculum varied somewhat for different vervets, as indicated in Tables 1–4. Each inoculum was administered orally as a single dose to each vervet.

Nephrectomy

Unilateral nephrectomies were performed under general anaesthesia using Sernylan[®] on two selected infants

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TABLE 5 Effect of cell culture passage level on the pathogenicity of *Encephalitozoon* for adult vervets

Vervet number	Number of spores orally given	Passage level	Isolate used	Spores in tissue culture	Spores in tissue section	Microscopical change ^{(c)(d)}			Postinoculation (weeks)
						Liver	Kidney	Brain	
RM-9	3 × 10 ⁷	17	DK ₂	ND	+	1-B	3-B	1-B	9
RM-10	5 × 10 ⁷	17	DK ₂	ND	+	3-B	2-A	1-B	6
RM-50	6 × 10 ⁷	75	DK ₂	^(a)	+	2-B	3-A	1-B	7
RM-52	5 × 10 ⁷	75	DK ₂	^(b)	+	3-B	3-A	0	8

^(a) Primary kidney cell culture did not grow

^(b) Reisolated organisms were used to inoculate a dog

^(c) For macroscopical changes in liver and kidney of RM-50 and RM-52 see Table 2

^(d) See "Classification of lesions", (g) of Table 1

(RM-35 & RM-37) at 12 weeks PI (Table 1). The left kidney was removed and divided; one-half was fixed in 10 % neutral buffered formalin for histopathological examination, and the other half was used in the preparation of primary cell cultures for concurrent reisolation of *E. cuniculi*. One infant (RM-35) was subsequently necropsied at 24 weeks PI and the other (RM-37) at 36 weeks PI.

Immunosuppression and immuno-incompetence of infants

Two nursing infants (RM-67, on its dam; RM-70, a hand-reared orphan) received their first intramuscular dose of methylprednisolone acetate⁵ simultaneously with their oral inoculum (Table 4). The dosage of 26 mg/kg was repeated three times at 7-day intervals for a total of four doses. These infants were necropsied at 4 weeks PI.

A third infant (RM-66), the product of an uncomplicated birth, was born prematurely at 168 g (50-65 % normal mass). Although he appeared healthy and alert and nursed well, because he was premature he was considered immuno-incompetent. The inoculum was given orally 18 h after birth, and he was necropsied at 6 weeks PI (Table 4).

Necropsy and histopathology

A complete post-mortem examination was performed on each animal after euthanasia was accomplished by exsanguination under deep general anaesthesia. Tissues from all major organs were fixed by immersion in 10 % neutral buffered formalin. The brain was fixed in approximately 25 % unbuffered formalin. Sections for histopathological examination were cut from paraffin-embedded blocks at 5-6 μm thickness and were stained with haematoxylin and eosin (H&E). The Brown-Hopps (B&H) modification of the tissue Gram's stain (Luna, 1968) was used for routine scanning of sections to confirm the presence of spores in each vervet. Some blocks were cut serially when organisms were difficult to find, and at least 12 B&H-stained sections of liver, kidney or brain were thoroughly examined before concluding that gram-positive spores could not be found.

Numerous B&H-stained, serial sections of the gastrointestinal tract of RM-56 and RM-104 only were examined until spores were demonstrated. In addition, the Ziehl-Neelsen (ZN) stain and periodic acid-Schiff (PAS) reaction were used to demonstrate acid-fast and PAS-positive features of these spores.

Other tissues that were routinely examined for lesions and spores usually included heart, lung, spleen, pancreas, stomach, small and large intestine, urinary bladder and lymph nodes.

Lifting from histological sections for transmission electron microscopy

Sparse, focal lesions were lifted for examination by transmission electron microscopy from paraffin-embedded and plastic-embedded (Epon) histological sections of formalin-fixed tissue that were approximately 6 μm thick. Tissue was processed for "lifting" and transmission electron microscopical examination as previously described (Van Dellen, 1978). Thin sections, approximately 60 nm thick, were cut and subsequently stained at room temperature with Reynold's lead citrate and 4 % uranyl acetate in 50 % methanol. This special technique was used selectively to demonstrate the presence of the aetiological agent within selected microgranulomas if spores could not be found in them with the B&H stain.

Indirect fluorescent antibody test (IFA)

The IFA test on sera collected before and after infection was done as previously described (Stewart *et al.*, 1979b). Spores of *E. cuniculi* were used as the antigen for this test. They were obtained from supernatant cell culture fluid adjusted to contain 8 × 10⁶ spores per ml and were then spotted onto glass slides. After fixation in cold acetone, these antigen spots were flooded with doubling dilutions of test sera and were then incubated for 30 min in moist chambers at room temperature. After washing, slides were flooded for 30 min with fluorescein-conjugated rabbit anti-monkey serum⁶. They were then washed and counter-stained in a 0.04 % solution of Evan's blue dye for 5 min and examined under a fluorescent microscope. The titre of each sample was defined as the reciprocal value of the highest serum dilution showing strong peripheral fluorescence of 50 % of the spores.

Reisolation of *E. cuniculi* spores

A whole kidney from infants and approximately one-half of a kidney from adults were aseptically removed at necropsy for reisolation of *E. cuniculi* by preparing primary cell cultures. Renal tissue was trypsinized for dissociation of cells. Growth media consisted of Minimum Essential Media (MEM) enriched with 10 % normal bovine serum. When cells of the primary culture revealed signs of degeneration or failed to grow, cells of a dog kidney cell line (MDCK) or a monkey kidney cell line (LLMCK₂) were added to the culture flask. Cell cultures were passaged when the cells had formed a monolayer in the culture flask. They were examined twice weekly with an inverted microscope for the presence of spores.

RESULTS

Classification of lesions

Inflammatory lesions were classified and scored according to dominant histological characteristics (Tables 1-6). Lesions designated type "A" occurred in a stage of the disease which was generally dominated by plasmacytes and large numbers of lymphocytes. In such

⁴ Phencyclidine hydrochloride (no longer available)

⁵ Depomedrol®. The Upjohn Company, 7000 Portage Road, Kalamazoo, Michigan 49001

⁶ Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland, USA 20795

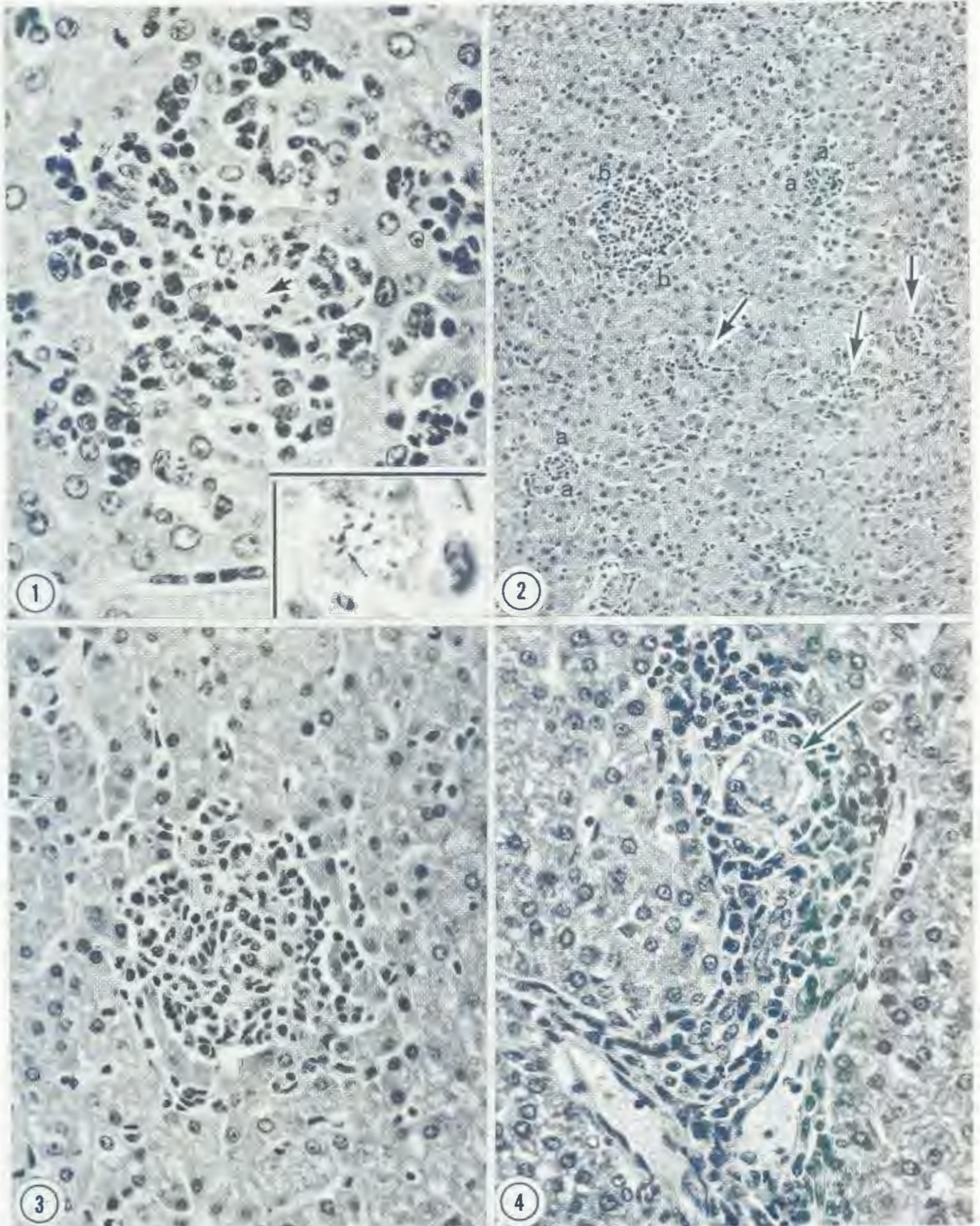


FIG. 1. Liver. Early, developing microgranuloma. The center of this lesion contains necrotic Kupffer's cells, hepatocytes, various inflammatory cells, and particulate structures suggestive of spores (arrow): HE \times 630. Inset, a serial section and higher magnification of this lesion revealing gram-positive spores. Note the anterior vacuole of the spore (arrow): B&H \times 1000

FIG. 2. Liver. Multiple microgranulomas organizing within sinusoids (a) and expanding into surrounding parenchyma (b) of liver. There is focal Kupffer's cell hypertrophy and hyperplasia (arrows): HE \times 160.

FIG. 3. Liver. Expanding microgranuloma and contiguous, degenerating hepatocytes: HE \times 400

FIG. 4. Liver. Portal vein and surrounding tissue containing giant cell (arrow) and infiltrate of mononuclear cells: HE \times 400

TABLE 6 Horizontal spread of infection to dams nursing infected infants^(a)

Dam number	Age (mass)	Contact with infant	Free of infant	Lesion development time	Serology ^(c)		Spores in tissue culture	Spores in tissue section	Macroscopical change			Microscopical change ^(d)		
					Before nursing	At necropsy			Liver	Kidney	Brain	Liver	Kidney	Brain
RM-58	Adult (3.5 k)	8 wks	5 wks	13 wks	0	160	—	—	0	0	0	1-B	2-B	0
RM-66 ^(b)	Adult (3.5 k)	6 wks	8 wks	14 wks	0	1 280	ND	—	0	0	0	2-B	3-B	0
RM-67 ^(b)	Adult (3.5 k)	4 wks	8 wks	12 wks	0	320	—	—	1	1	0	1-C	1-C	0

^(a) Results shown are those for the dams; results of infected, nursing infants are in Tables 1 and 4
^(b) RM-66 and RM-67 were caged together for 4 weeks after infants were removed
^(c) Titre is reciprocal value of highest serum dilution
^(d) See "Classification of lesions", (g) of Table 1

lesions, these cells together formed a dense infiltrate which severely displaced normal cellular architecture. Focal lesions frequently coalesced to form large, irregular inflammatory masses. The infiltrate did not have a remarkable granulomatous component; there were no epithelioid cells, and only rarely did they contain a few scattered macrophages. The kidney cortex was most conspicuously affected by this characteristic lesion.

In contrast, lesions designated type "B" were found in a distinctly granulomatous aspect of the disease and were most common in the liver and brain, and to a lesser extent the kidneys as well. This type of lesion also contained lymphocytes and plasmacytes, but a circumscribed aggregate of macrophages was always present which, when present in the kidneys, was usually centrally disposed to the lymphoid cells. Epithelioid cells, giant cells and occasionally eosinophils and neutrophils were included in varying numbers. Large, irregular lesions contained coalesced components of multiple, focal exudates which apparently reflected multiple sites of infection; granulomatous lesions with this pattern were prominent in some kidneys. In the liver, microgranulomas consisted almost exclusively of macrophages, and, in the brain, this principal inflammatory cell predominantly formed microgranulomas that had a close association with blood vessels or capillaries. Many reactions were distinctly nodular and circumscribed blood vessels. The lesions in the brain, however, included astrocytes.

Lesions designated type "C" consisted chiefly of lymphocytes, fibrocytes and usually a few eosinophils, but there were no significant numbers of macrophages or plasmacytes. Lesions in this stage of the disease were focal to diffuse. Isolated foci could be found in most organs and tissues.

In addition to varying in cellular composition, lesions also varied in size and number, and for this reason lesions in the liver, kidneys and brain were given a magnitude and frequency designation (Tables 1-6). Despite the presence of lesions of considerable magnitude and frequency (especially in immunocompromised infants), none of the vervets were noted to exhibit unequivocal signs of clinical disease.

Macroscopical and microscopical pathology

Small intestine. Multiplication of *E. cuniculi* was detected with the B&H stain in the small intestine of immunocompetent vervets given a large inoculum (Table 2). Colonies of spores were observed within epithelial cells, as well as within endothelial cells of capillaries in the lamina propria 3 days PI in RM-104 ("old" juvenile) and 7 days PI in RM-56 ("young" adult) (Table 2). A significant inflammatory response to the multifocal, sparse infection was not apparent, although there was a small increase in the number of plasmacytes and histiocytes normally present in the lamina propria. Neither spores nor lesions attributable to encephalitozoon infection were found in the gastrointestinal tract of the other vervets.

Liver. Macroscopical lesions were not seen in the livers of immunocompetent infants who were inoculated orally (Table 1). But, they were present in the livers of immunosuppressed infants (Table 4) and in infants born of females in late gestation (Table 3). Macroscopical liver lesions were most consistently present in adults, however (Table 2). In adults, they were best observed at about 7 weeks PI as a moderate number of greyish-white, poorly demarcated foci measuring up to 1 mm in diameter. Lesions were readily observed and widely dispersed as seen through the capsule, but they were not easily seen on the cut surface of the parenchyma.

What was interpreted as early histological change at

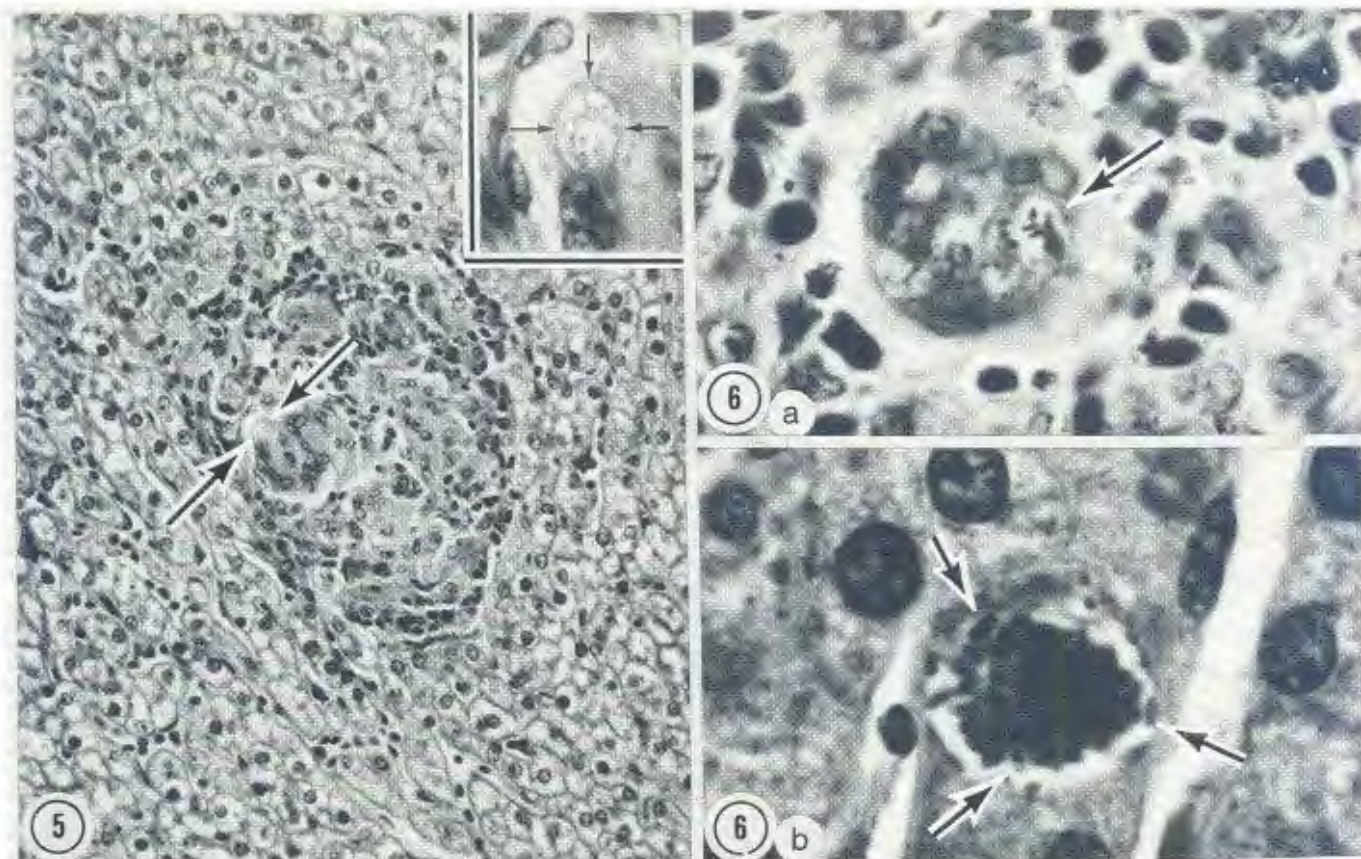


FIG. 5. Liver. Granuloma 2 weeks after intravenous inoculation of spores. The lesion is composed of macrophages, epithelioid cells, lymphoid cells and multiple giant cells, one of which contains a colony of encephalitozoon spores (arrows): HE \times 250. Inset, the colony of spores is within the parasitophorous vacuole of a giant cell: \times 1000

FIG. 6(a). Liver. Spores (arrow) within giant cell surrounded by mononuclear cells: B&H \times 1000. 6(b). Liver. Colony of encephalitozoon spores within hepatocyte and no attendant inflammatory reaction. Arrows indicate individual spores: B&H \times 1575

initial sites of infection within sinusoids was seen in both adults and infants. This change was comprised of mild, focal Kupffer's-cell hyperplasia and hypertrophy with mixed inflammatory cell infiltration, including lymphocytes, plasmacytes and a few neutrophils. Minimal microfocal necrosis was centrally disposed in this lesion. Such multifocal reactions to initial infection were typical and were thought to be developing microgranulomas. These were most conspicuous and photogenic in immunosuppressed infants at 4 and 6 weeks PI (Fig. 1). However, the majority of intrasinusoidal lesions had a predominantly distinct microgranuloma appearance, and were comprised of numerous macrophages, a few epithelioid cells and an occasional giant cell. Lymphocytes and plasmacytes infiltrated these apparently expanding lesions, but there were no neutrophils nor was there any evidence of necrosis. The magnitude and frequency of these lesions were most severe from about 2–8 weeks PI in adults (Table 2). They were scored "mild" to "moderate" in severity at 4–6 weeks PI in immunocompromised infants (Table 4), and were randomly distributed (Fig. 2).

Hepatocellular injury was minimal. The centres of early, developing microgranulomas (Fig. 1) sometimes contained necrotic hepatocytes, while the periphery of expanding microgranulomas included degeneration of contiguous hepatocytes (Fig. 3). Although spores were rarely seen in microfoci of necrosis (Fig. 1, inset), hepatocytes were seen to support the development of spores with no attendant inflammatory reaction (Fig. 6b), apparently until necrosis occurred. Portal triads of both adult and infant vervets contained cellular exudate which usually consisted of lymphoid cells and macrophages.

Giant cells and epithelioid cells were rarely seen in the portal areas (Fig. 4).

Histological changes in the livers of adults at 11–18 weeks PI (Table 2) were characterized by a decreasing number of inflammatory foci with changing cellular constituents. The proportion of macrophages to lymphocytes and plasmacytes decreased, giant cells disappeared altogether, and a few eosinophils were eventually included in the infiltrate. Only focal collections of lymphocytes and a few fibrocytes ultimately marked the remains of microgranulomas.

Demonstration of encephalitozoon spores within lesions was usually difficult, and the use of serial sections cut through focal lesions was often necessary to reveal them. Degenerating spores, not histologically detectable within widely dispersed microgranulomas, were demonstrated ultrastructurally with the "lifting" technique (Fig. 7 & 8). Spores degenerating within the cytoplasm of Kupffer's cells or macrophages retained an intact wall, which was depicted as a narrow, electron-dense, corrugated shell (exospore) circumscribing a discrete electron-lucent halo (endospore). The distinctly preserved spore wall usually surrounded only a central, homogenous mass of destroyed organelles (Fig. 7). (These degenerating spores were also demonstrated within microgranulomas in the brain.)

To facilitate optimum demonstration of the association of spores with lesions, a large dose of spores was administered intravenously to one, young adult vervet (RM-65, not in tables). Examination at 2 weeks PI revealed large numbers of microgranulomas, most of which included giant cells containing large colonies of gram-positive

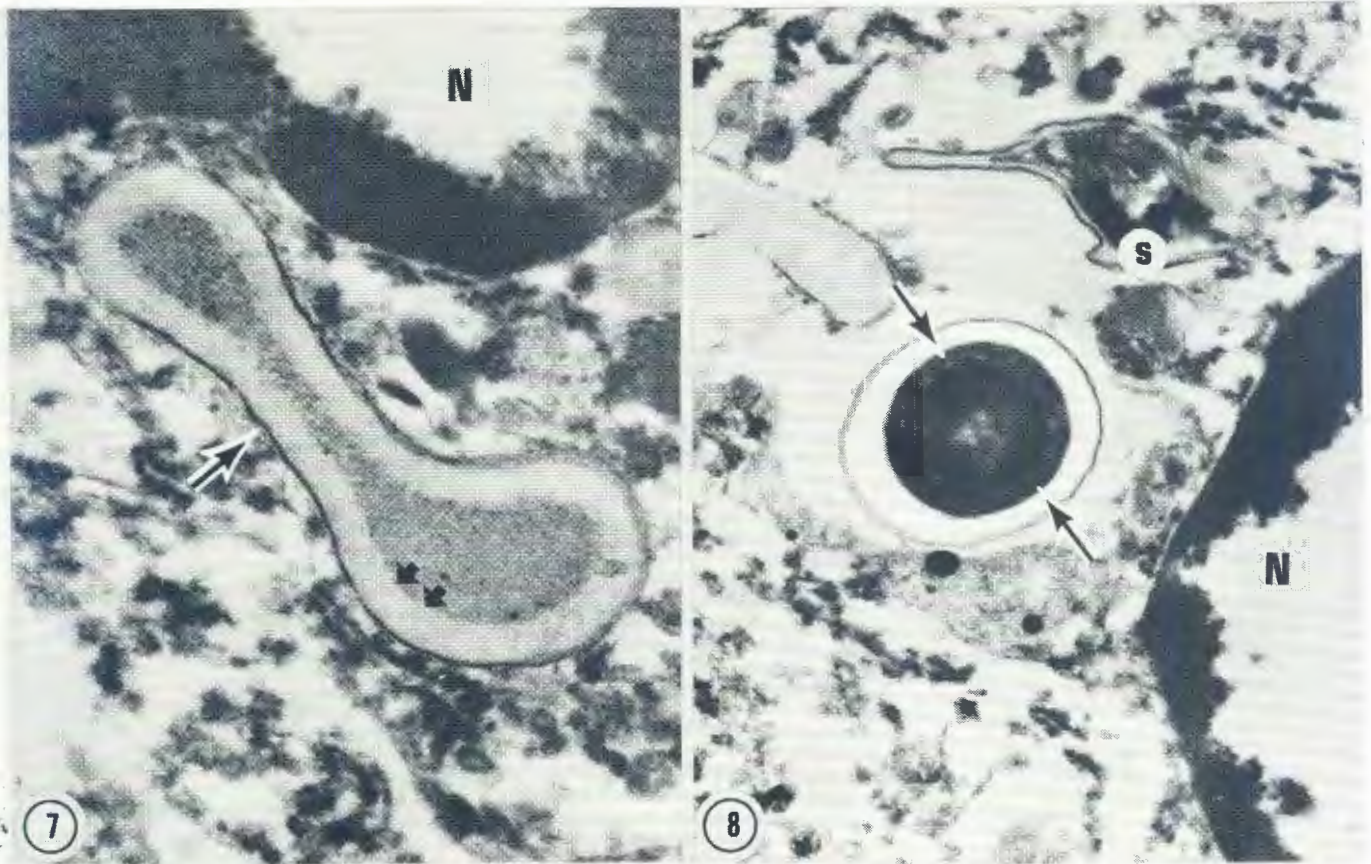


FIG. 7. Liver. Electron photomicrograph of degenerating spore. Note the electron-dense, outer, somewhat corrugated spore wall (exospore, large arrow) with its inner, wide, electron-lucent part (endospore, double arrow). N = nucleus of macrophage; HE, lead citrate, uranyl acetate $\times 40\ 000$

FIG. 8. Liver. Electron photomicrograph of intracellular, degenerating spore within a macrophage of a microgranuloma. The spore's size, well-preserved wall (endospore and exospore) and the cross sections of polar filament (arrows) identify it as *Encephalitozoon*. N = nucleus of macrophage; S = spore; HE, lead citrate, uranyl acetate $\times 30\ 000$

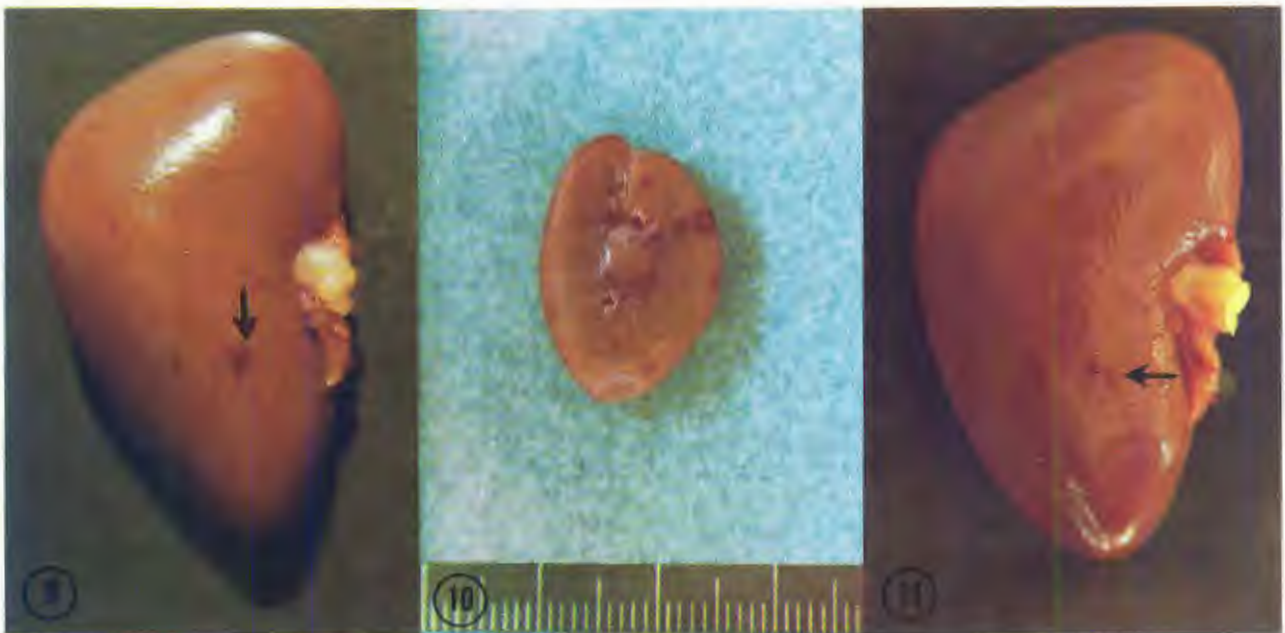


FIG. 9. Kidney. Focal, cream-coloured and reddish subcapsular lesions. Note the coronal hyperaemic appearance of the lesions depicted by the top, right arrow. The other arrow depicts the smaller and more typical dense, reddish lesion; Kodachrome $\times 1,5$

FIG. 10. Kidney. Subcapsular lesions in cross section. The centers of several cream-coloured foci are surrounded by a hyperaemic corona (arrows); Kodachrome $\times 1,5$

FIG. 11. Kidney. Discrete, circumscribed reddish subcapsular lesions. Note the central, dense focal lesion without the coronal appearance. This lesion is typical of the majority of the focal inflammatory lesions seen in kidneys; Kodachrome $\times 1,5$

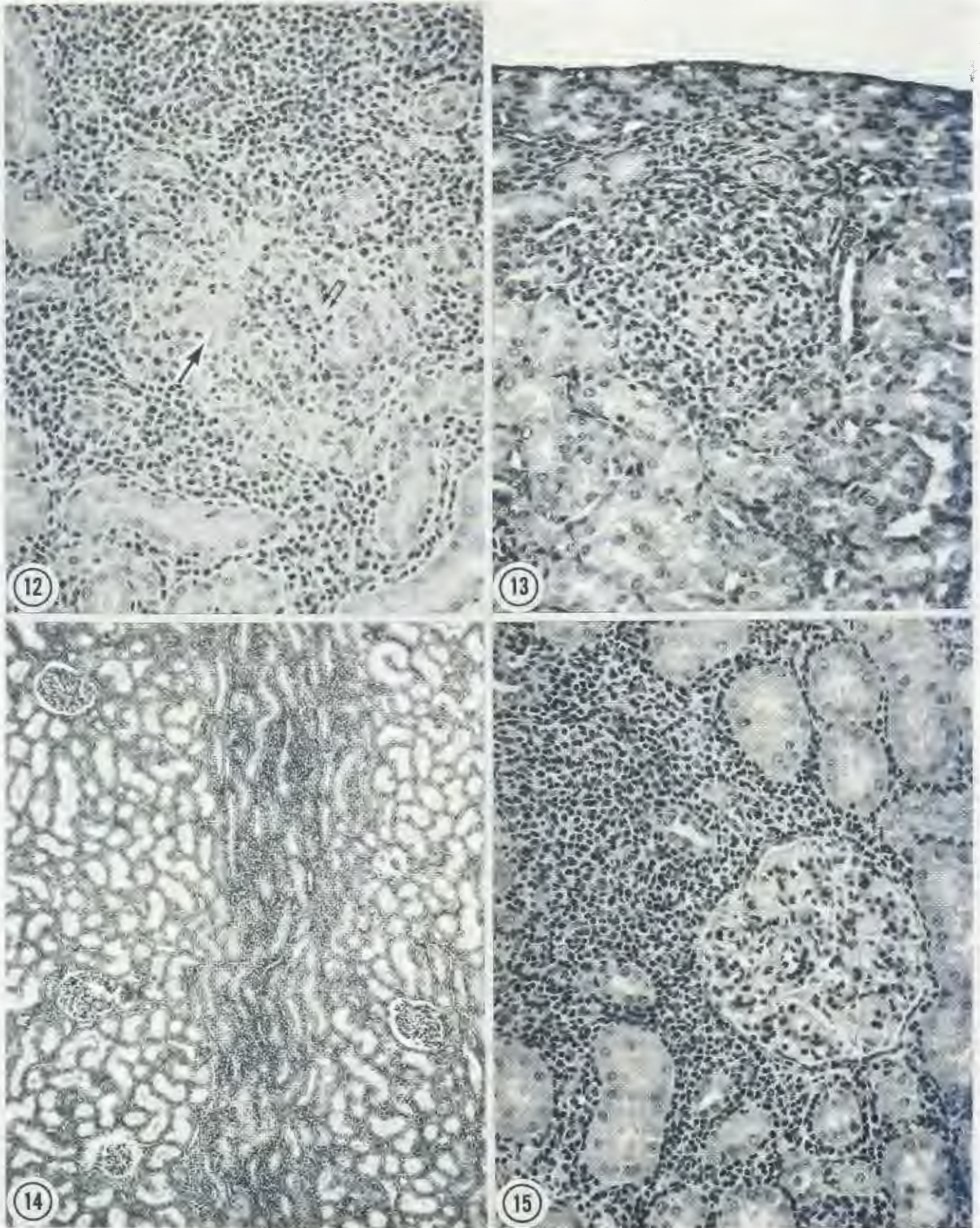


FIG. 12. Kidney. Dense, interstitial infiltrate of lymphocytes and plasmacytes. There are occasional epithelioid cells, a giant cell (arrow) and an epicentre of karyorrhectic cells (double arrows) indicative of necrosis: HE \times 250

FIG. 13. Kidney. Subcapsular microgranuloma: HE \times 250

FIG. 14. Kidney. Interstitial, mononuclear cell infiltrates along medullary ray. These linear infiltrates extended from the corticomedullary junction into the cortex and the medulla: HE \times 160

FIG. 15. Kidney. The intense reaction of lymphoid cells in the interstitium surrounds a relatively unaffected glomerulus: HE \times 250

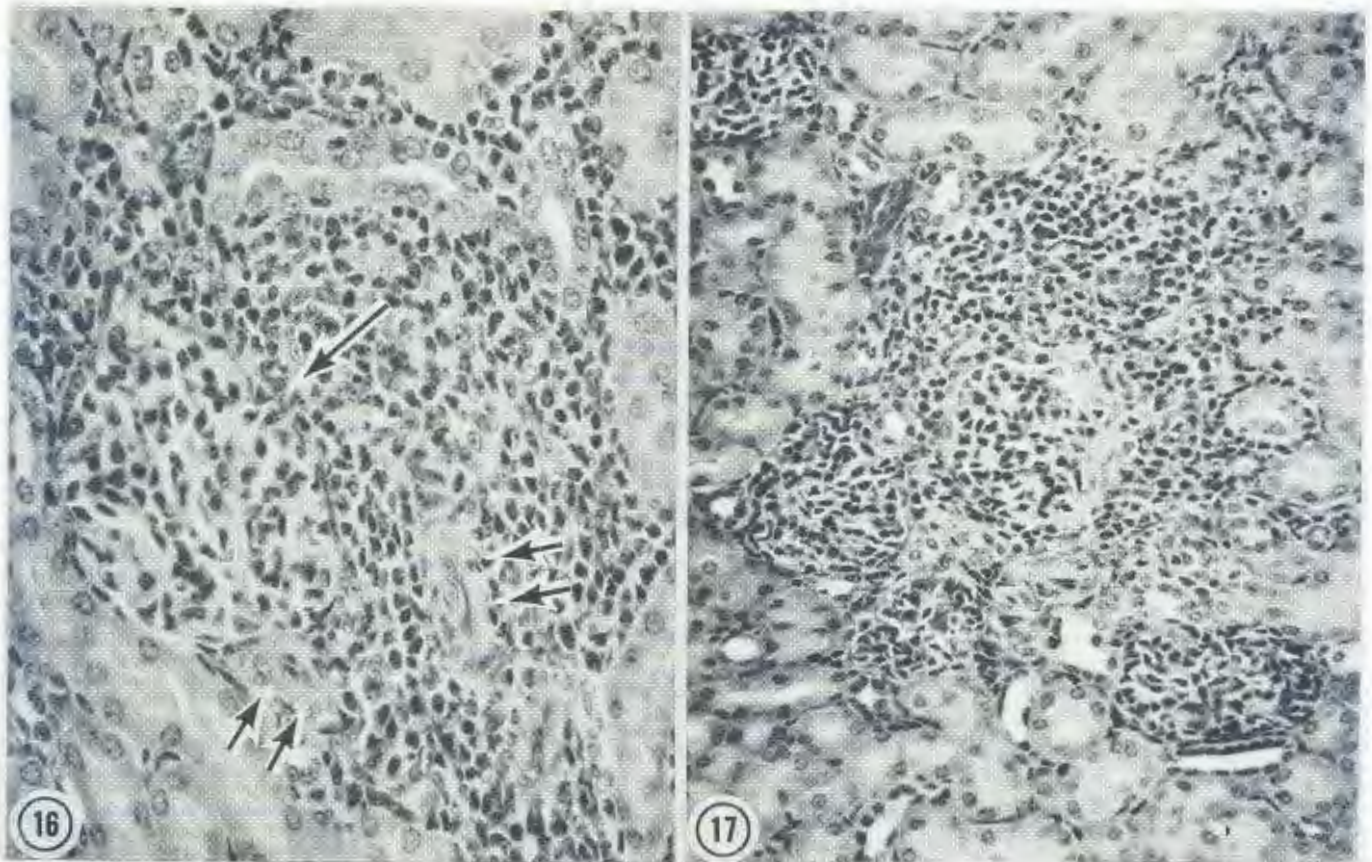


FIG. 16. Kidney. Glomerulitis and interstitial nephritis. The inflammatory reaction is incorporating the afferent arteriole (double arrow), Bowman's capsule (single arrow) and adjacent glomerular tufts: HE \times 400

FIG. 17. Kidney. Infiltration of mixed inflammatory cells into Bowman's capsule and surrounding interstitium, with destruction of adherent glomerular tufts containing proliferating mesangial cells: HE \times 250

spores (Fig. 5 & 6a). Colonies of spores were also seen within hepatocytes to which there was no corresponding inflammatory reaction (Fig. 6b) in both intravenously and orally inoculated vervets.

Kidneys. Macroscopical lesions in the kidneys of both adults and infants (Tables 1–4) were characterized by small, cream-coloured or reddish, well-demarcated foci that measured up to 2 mm in diameter. These multifocal lesions were widely scattered under the capsule (Fig. 9, 10 & 11). Most lesions were smaller than a millimeter and difficult to see without magnification. They were largest and most conspicuous when circumscribed by a narrow hyperaemic corona (Fig. 9 & 10). The majority of these lesions, however, appeared without this corona and were typical of the representative middle lesion seen in Fig. 9 & 11 which is best depicted in Fig. 11 (shift of specimen, focus and lights).

Microscopically, minimal multifocal lesions were seen in the cortex of the adult kidneys as early as 1 week PI (Table 2). At about 4–6 weeks PI, lesions were most numerous in the cortex and at the cortico-medullary (C-M) junction; at about 8–10 weeks PI, they were most numerous at the C-M junction and in the medulla.

The principal histological change in adults was contained interstitially, and the most prevalent inflammatory components of this change were lymphoid cells of which plasmacytes were usually dominant (Tables 2 & 5). Lesions were most numerous and intense at approximately 4–9 weeks PI (Table 2). Although granulomatous lesions were not common in the kidneys of adults, they were moderately severe in one vervet at 9 weeks PI (RM-9), Tables 2 & 5), and in one female (RM-66, Table 6) 14 weeks after her initial exposure to an infected infant (RM-66) she had nursed for 6 weeks (Table 4). Within

infants, the reaction was also interstitial, but lesions in them were more frequently granulomatous (Tables 1, 3 & 4) than they were in adults. In addition to peripheral lymphoid cells, many of these focal lesions contained a relatively small, central area of confluent macrophages, an occasional epithelioid cell and rarely a giant cell as well as minimal evidence of necrosis (karyorrhexis) (Fig. 12). Macrophages and lymphoid cells were often intermingled in various proportions, but a well organized arrangement of cellular components recognizable as constituents of a microgranuloma was unusual in the kidneys of both infants and adults (Fig. 13).

Mononuclear cell exudate was frequently observed as a linear, interstitial infiltrate extending along the medullary rays and coursing from the C-M junction to the outer cortex as well as into the medulla (Fig. 14). Relatively unaffected glomeruli were occasionally surrounded by dense interstitial exudate (Fig. 15), but they were often included when the afferent arteriole and Bowman's capsule were in the centre of cellular reaction (Fig. 16). Within lesions of glomeruli, tufts adhered to Bowman's capsule which was infiltrated by lymphoid cells and macrophages (Fig. 16 & 17). Glomeruli without surrounding inflammation occasionally contained focal, degenerative lesions in several tufts with adjacent, focally proliferative mesangial cells (Fig. 18). Coalescing lesions of the cortical interstitium incorporated degenerating tubules and glomeruli containing diffuse mesangial proliferation. Necrosis of tubular epithelium, although infrequently seen, evoked a mixed inflammatory cell reaction, including neutrophils and mononuclear cells as well as interstitial oedema (Fig. 19).

A change of renal lesions in the adult was first noticeable commencing at approximately 11–12 weeks PI

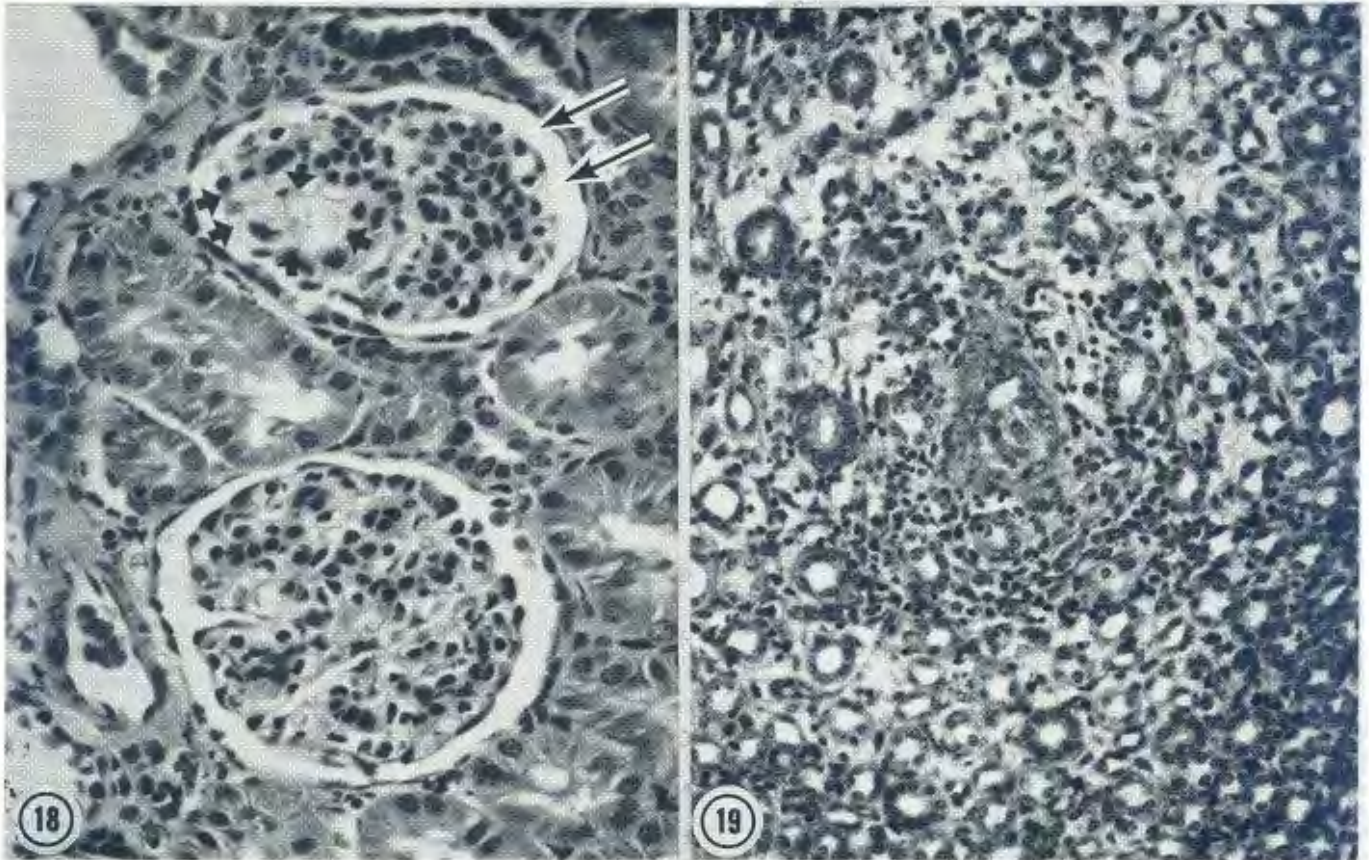


FIG. 18. Kidney. Focus of degenerating glomerular tufts (short arrows) and adjacent proliferating mesangial cells (long arrows); HE \times 400

FIG. 19. Kidney. Focal acute inflammatory reaction (mononuclear cells, few neutrophils) destroying a collecting tubule in the medulla. Interstitial oedema separates contiguous tubules; HE \times 250

(Table 2) with the disappearance of macrophages, a shift from predominantly plasmacytic mononuclear cells to lymphocytic cells, infiltration of a few eosinophils and mild fibroplasia. Fibrosis in both adults and infants appeared to develop along vascular pathways within the cortical interstitium and around shrunken glomeruli surrounded by thickened Bowman's capsules (Fig. 20). Fibrosis in the interstitium of the medulla was more prominent than in the cortical interstitium (Fig. 21).

Spores of *E. cuniculi* were very difficult to find within exudate and even diligent examination of serial sections often proved unrewarding. However, with little or no corresponding inflammatory reaction, epithelial cells of convoluted and collecting tubules of many vervets frequently contained conspicuous colonies of spores (Fig. 22). They were most numerous and were easier to find in the medulla, where spores were frequently seen to egress into the lumen of the affected tubule (Fig. 23). Reisolation of spores was readily accomplished from primary cell cultures of infant kidneys but they were less frequently reisolated from those of adult kidneys (Tables 1-6).

Brain. Macroscopical lesions in the brain and spinal cord were not evident in adults or infants (Tables 1-4 & 6). Microscopically, however, meningo-encephalomyelitis was characterized by discrete microgranulomas that were widely scattered throughout the brain and spinal cord (Fig. 24). Microgranulomas were almost always detected in the infants (Tables 1, 3 & 4) and much less frequently in the adults (Tables 2, 5 & 6).

Differences in the disease of the two age groups were evident when compared at the same PI interval. Lesions either failed to develop or were minimal in the brains of adults at 7-9 weeks PI, whereas lesions at this time were

consistently present in the brains of infants and were of remarkable size and usually were moderately numerous. Furthermore, microgranulomas in the meninges of infants tended to be somewhat more diffuse and especially prominent in the depths of sulci (Fig. 25).

Constituents of microgranulomas included hypertrophied and hyperplastic perithelial cells. These proliferative components were accompanied by infiltrates including various numbers of lymphocytes, plasmacytes, astrocytes, and usually a few eosinophils (Fig. 26). Eosinophils were occasionally disproportionately numerous in some lesions (Fig. 27). Altogether they formed a segmental nodular reaction, either eccentrically or concentrically, around vessels. Lesions associated with small vessels or capillaries were not always readily discernible, and they frequently required serial sectioning for confirmation (Fig. 27). Astrocytosis was often most prominent in the peripheral vicinity of the lesions and it appeared to increase toward the epicentre of the lesions as well. Necrosis, giant cells and perivascular cuffing with only lymphoid cells were rarely seen in the brain. Microgranulomas were still detectable as long as 36 weeks after inoculation with spores (Fig. 28).

Finding encephalitozoon spores within lesions of the brain, as in the kidneys, usually proved very difficult and tedious, especially within lesions of immunocompetent vervets, although spores were confirmed in most of them (Tables 1-3, 5 & 6). Within lesions of immunocompromised vervets (Table 4), however, spores were relatively abundant and therefore more easily found (Fig. 29 & 30). Within both immunocompetent and immunocompromised vervets, spores were occasionally seen in small (10-20 μ m) and sometimes larger (up to 30-40 μ m) compact colonies without attendant inflammatory reac-

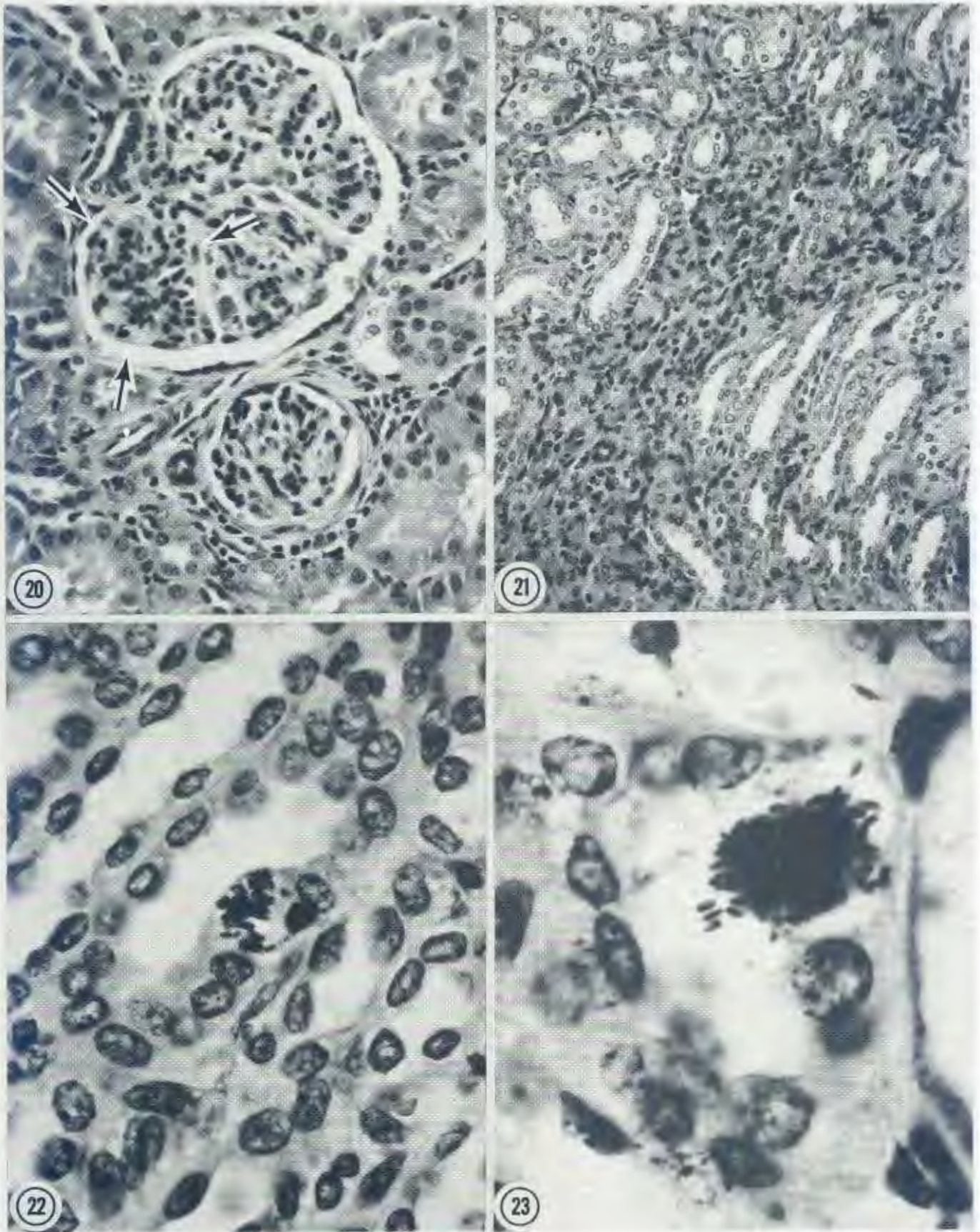


FIG. 20. Kidney. Periglomerular fibrosis. Upper glomerulus is of normal size, though some tufts contain proliferating mesangial cells (arrows). Lower glomerulus is shrunken and surrounded by a thickened, fibrous Bowman's capsule and a few mononuclear inflammatory cells: HE $\times 400$

FIG. 21. Kidney. Irregularly linear fibrosis in the interstitium was most prominent along vascular pathways and most conspicuous in the medulla: HE $\times 250$

FIG. 22. Kidney. Tubular epithelial cell containing a colony of *Encephalitozoon* and no corresponding inflammatory reaction: B&H $\times 1\ 000$

FIG. 23. Kidney. Mature colony of encephalitozoon spores. Spores are erupting from an epithelial cell into the lumen of a collecting tubule: B&H $\times 1\ 575$

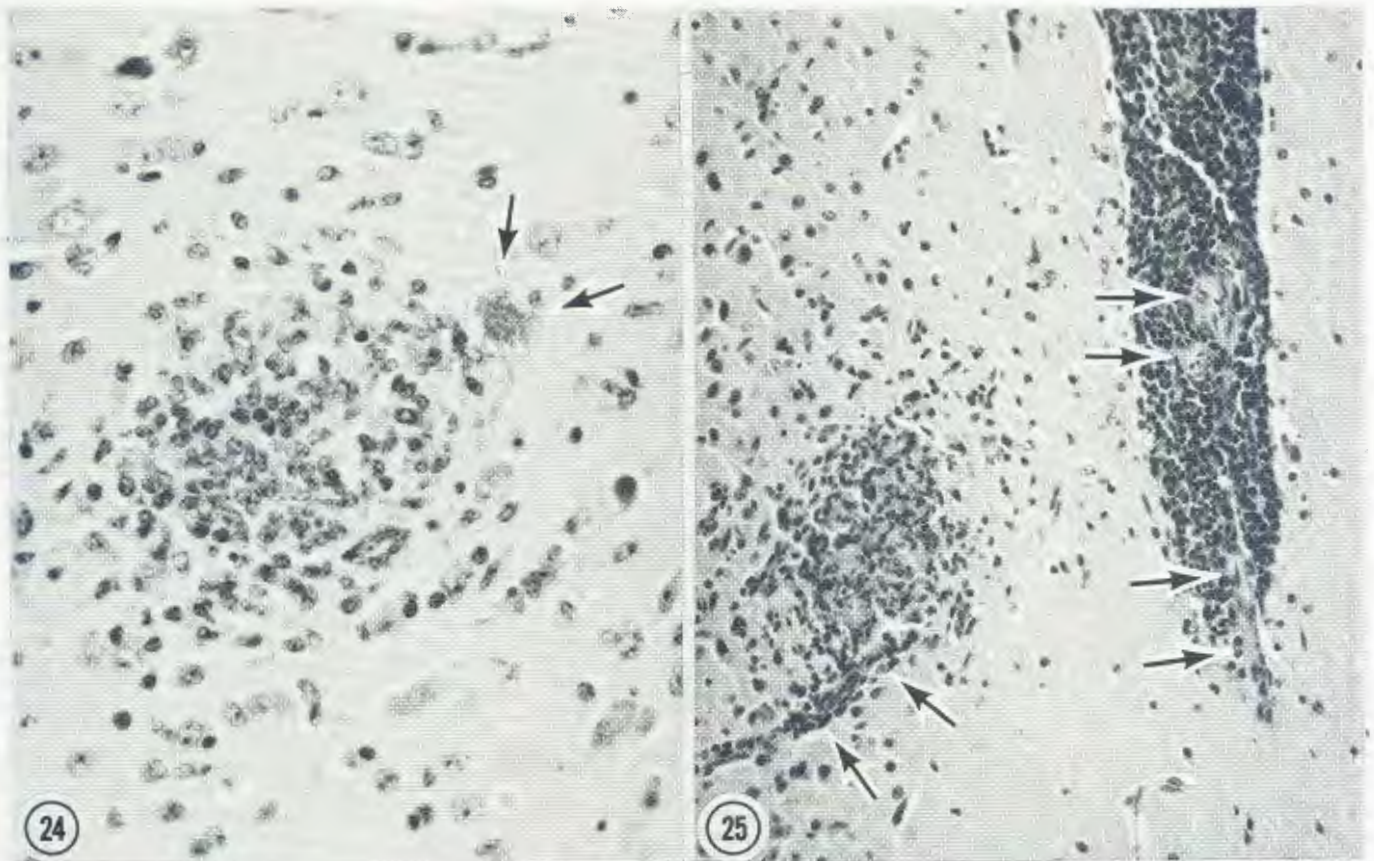


FIG. 24. Brain. Microgranuloma (glial nodule) with a colony of spores (arrows) on its periphery. Inflammatory cell components are mixed and include more than glial cells: HE \times 400

FIG. 25. Brain. A microgranuloma in grey matter (lower left) and diffuse mononuclear cell exudate in meninges (deep sulcus, upper right). The intense cellular reactions involve vessels (arrows) in both lesions: HE \times 250

tion. These colonies were believed to be within distended endothelial cells of occluded capillaries (Fig. 31). Spores were rarely seen within neurons or other cells.

Lungs. Evaluation of lung lesions caused by encephalitozoon infection was inconclusive in the adults because pre-existing granulomatous lesions that were caused by crystalline (mineral) deposits complicated interpretation. However, no such complication was found in the lungs of several infants. The lungs of immunocompromised infants contained a few macroscopical, subpleural, reddish-brown and irregularly circumscribed foci less than 1 mm in diameter (attempts to photograph these were unsuccessful). Microscopically, these lesions were present in various parts of lobules of different lobes and consisted of irregular, focal areas of alveolar septae that were thickened by sparse interstitial infiltrates of lymphoid cells and macrophages (Fig. 33a & 33b). Lymphoid cells frequently formed small cuffs around bronchial vessels. Although spores were extremely difficult to demonstrate in pulmonary tissue, they were rarely seen interstitially and they once were seen in the wall of a vessel with granulomatous vasculitis (Fig 32a & 32b).

Other organs and tissue. Histological lesions ascribed to infection with *E. cuniculi*, although sparse, were found in connective tissue, the pancreas and the heart. Reactions usually consisted of focal accumulation of lymphocytes, a few plasmacytes and macrophages often in association with blood vessels. These exudates were frequently found in the epicardium and were in the interstitium. The lesions included fibrinoid, necrotizing vasculitis (Fig. 34). Also, nodular granulomatous vasculitis without contiguous inflammatory reaction was seen in the pancreas (Fig. 35). Spores were not found in either of these vascular lesions.

Follicular lymphoid hyperplasia was present in the mesenteric lymph nodes and the spleens of most of the infected vervets, but it was particularly prominent in RM-37 36 weeks PI (Table 1). In this infant, numerous macroscopical nodules measuring 1–2 mm in diameter were raised above the capsule and were found throughout the parenchyma of the spleen. Histologically, these were reactive and greatly enlarged follicles with expanding germinal centres surrounded by a narrow, dense corona of lymphocytes.

Reisolation of spores of E. cuniculi in primary kidney cell culture

Successful primary cell cultures of kidneys were prepared from 26 kidney specimens, 10 of which were from infants, 15 from adults, and 1 from a foetus (Tables 1–6). *E. cuniculi* was reisolated in cultures from nine infant specimens, including those from infants that were immunocompromised, and from only one adult specimen. Reisolation was successful from a kidney removed by nephrectomy from one of the infants (RM-35) at 12 weeks PI, but was unsuccessful from the contralateral kidney when the same animal was necropsied at 24 weeks PI, despite the fact that spores were seen in sections of tissue obtained at necropsy. From another infant (RM-37) cultures were positive at 12 weeks PI (unilateral nephrectomy) and also at 36 weeks PI (necropsy). Satisfactory initial growth of primary cultures from 6 of the 15 adults was not self-perpetuating and the attempt to reisolate spores from them was maintained by adding MDCK cells. However, five of these six rejuvenated cultures remained negative for *E. cuniculi* even though spores were seen in tissue sections; in only one culture from adults were spores seen following the addition of MDCK cells. Two cultures from adults and three cul-

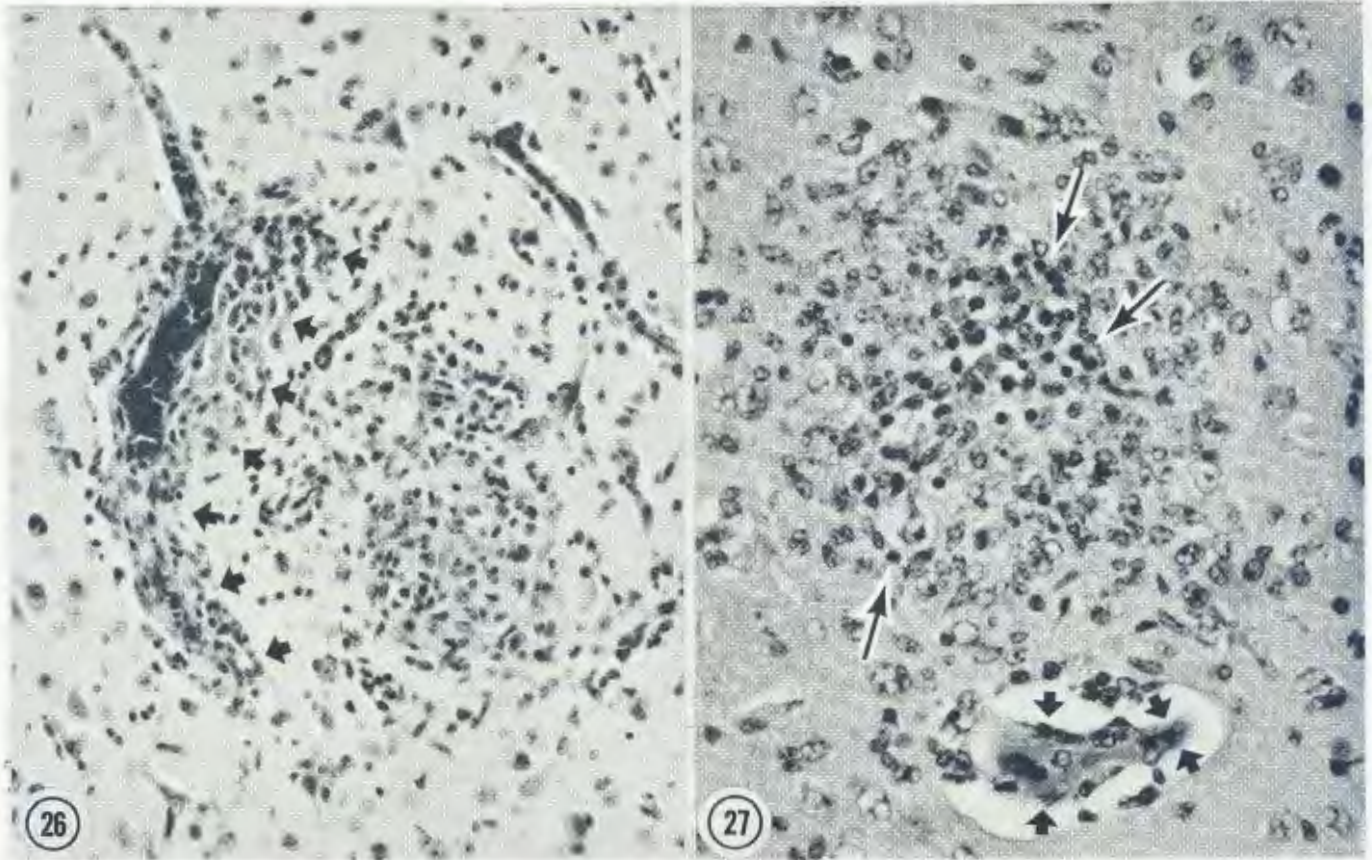


FIG. 26. Brain. Microgranuloma, vasculitis and perivasculitis. Arrows delineate proliferating perithelial cells along a vessel with severe vasculitis: HE \times 250

FIG. 27. Brain. Microgranuloma. Eosinophils (long arrows; small, dense cells) were disproportionately numerous in some lesions. Note the associated, but only the peripheral segment, of a vessel (short arrows) which courses through part of the lesion in another plane of the tissue not shown: HE \times 400

tures from infants were discarded because they failed to form satisfactory primary growth, and in these cultures the attempt to reisolate spores was not successful by the addition of MDCK cells. One of these was from an immunologically compromised infant. Also, one culture from an adult was discarded because of fungal contamination.

It was essential for reisolation of *E. cuniculi* in cell culture to maintain cultures for sufficient time to allow development, growth and spread of the parasite. Cultures were maintained for 47–95 days before discarding them as negative. The number of passages for reisolation varied from 1–11, with a mean of 4. In only one culture were spores seen before the first subculture at seven days, and one culture was passaged 11 times before spores were observed.

Comparing resistance of adults and infants to the growth and multiplication of E. cuniculi

Considering immunocompetent animals, all that were inoculated and those that were infected horizontally or exposed to infection *in utero* (Tables 1, 2, 3, 5 & 6) developed systemic infection and lesions; but, organisms were easier to find and appeared more numerous in tissue sections prepared from infants (Tables 1 & 3) than in those prepared from adults (Tables 2, 5 & 6). This difference of spore population between age groups was even more pronounced when infants were immunocompromised (Table 4). The apparent decreased resistance of infants to the growth and multiplication of *E. cuniculi* is also suggested by culture results.

Specifically, reisolation of the organism was more successful in primary kidney cell cultures prepared from

infants than in those prepared from adults. Again, considering only immunocompetent infants and excluding aborted foetuses, 7 isolates in 8 cultures were obtained from infants (Tables 1 & 3), whereas only 1 isolate in 15 cultures was obtained from inoculated and horizontally infected adults (Tables 2 & 6); an 87,5 % success rate for immunocompetent infants and 6,7 % for all successfully cultured adults. The reisolation success rate was slightly higher for infants when the cultures from immunocompromised infants (Table 4) were included, i.e., 90,0 % (9 isolates in 10 cultures). Also, the reisolation rate is higher for adults when only inoculated animals are included in the calculations, i.e., 7,7 % (1 isolate in 13 cultures; excludes horizontally infected dams). Thus, from the results of tissue section examination and isolation from kidney cell culture, *E. cuniculi* was more numerous in the tissues of infants than adults.

Lesions and organisms within immunologically compromised infants

Two infants immunologically compromised by steroid injection and one infant compromised by its premature birth had extensive systemic disease (Table 4). Lesions were granulomatous in the brain, liver and kidneys of each infant. Organisms were numerous to exuberant, particularly within lesions in the brain of the premature infant in which several colonies of spores were often present within a single lesion (Fig. 30). The organism was reisolated in primary kidney cell culture prepared from two (RM-66 & RM-67) of the three infants. Reisolation of the organism was not accomplished from the third infant (RM-70) because renal cell cultures did not grow, although spores were easily demonstrated in renal tissue sections. Spores within tissues and inflammatory

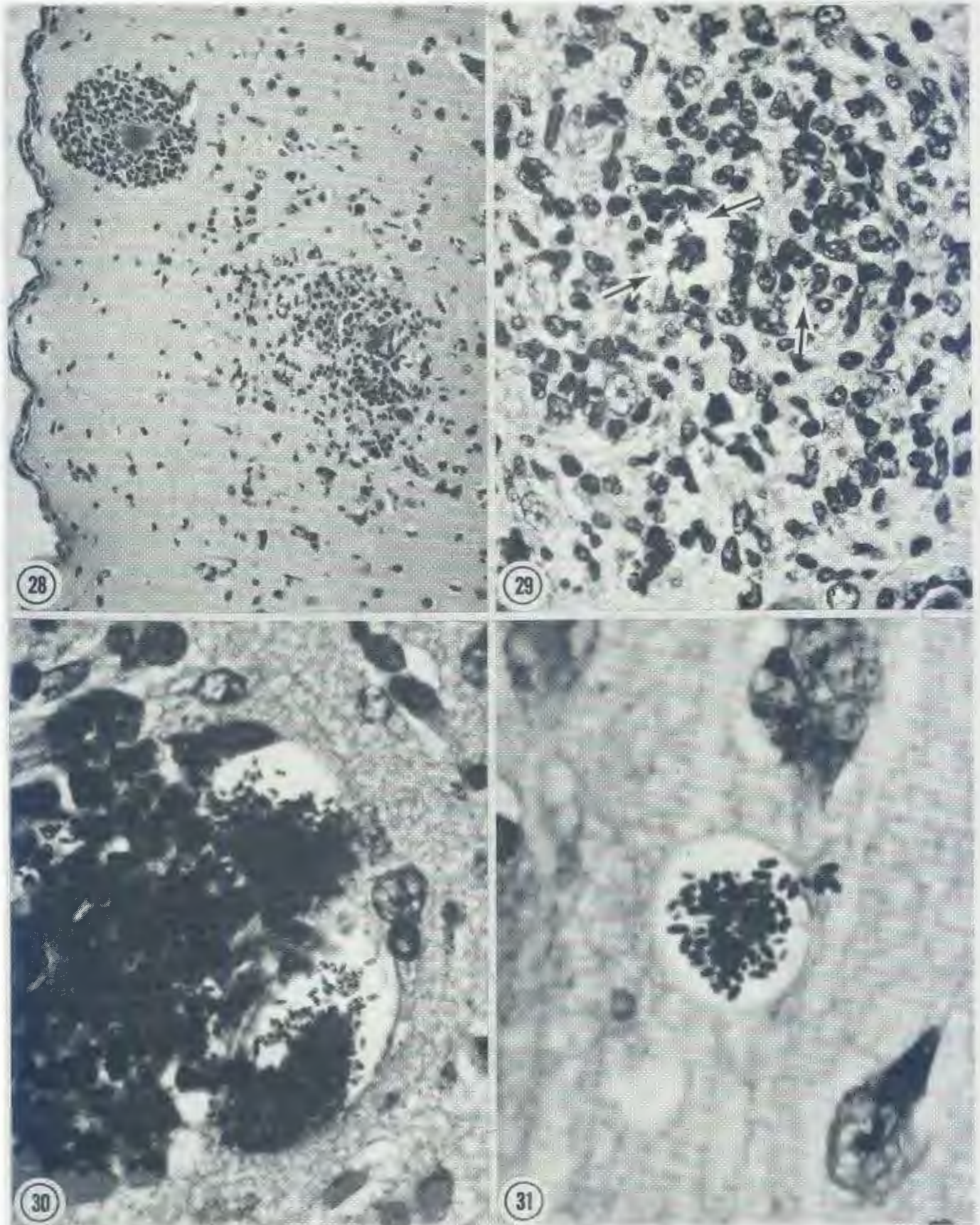


FIG. 28. Brain. Dense perivascular cuff of lymphoid cells and a loosely organized microgranuloma: HE \times 250

FIG. 29. Brain. Spores of *Encephalitozoon* (arrows) near the center of a microgranuloma: B&H \times 630

FIG. 30. Brain. Two colonies of encephalitozoon spores within parasitophorous vacuoles on the edge of a microgranuloma: B&H \times 1 000

FIG. 31. Brain. A colony of encephalitozoon spores free of inflammatory reaction. This colony is thought to be within the parasitophorous vacuole of an endothelial cell of an occluded capillary: B&H \times 1 575

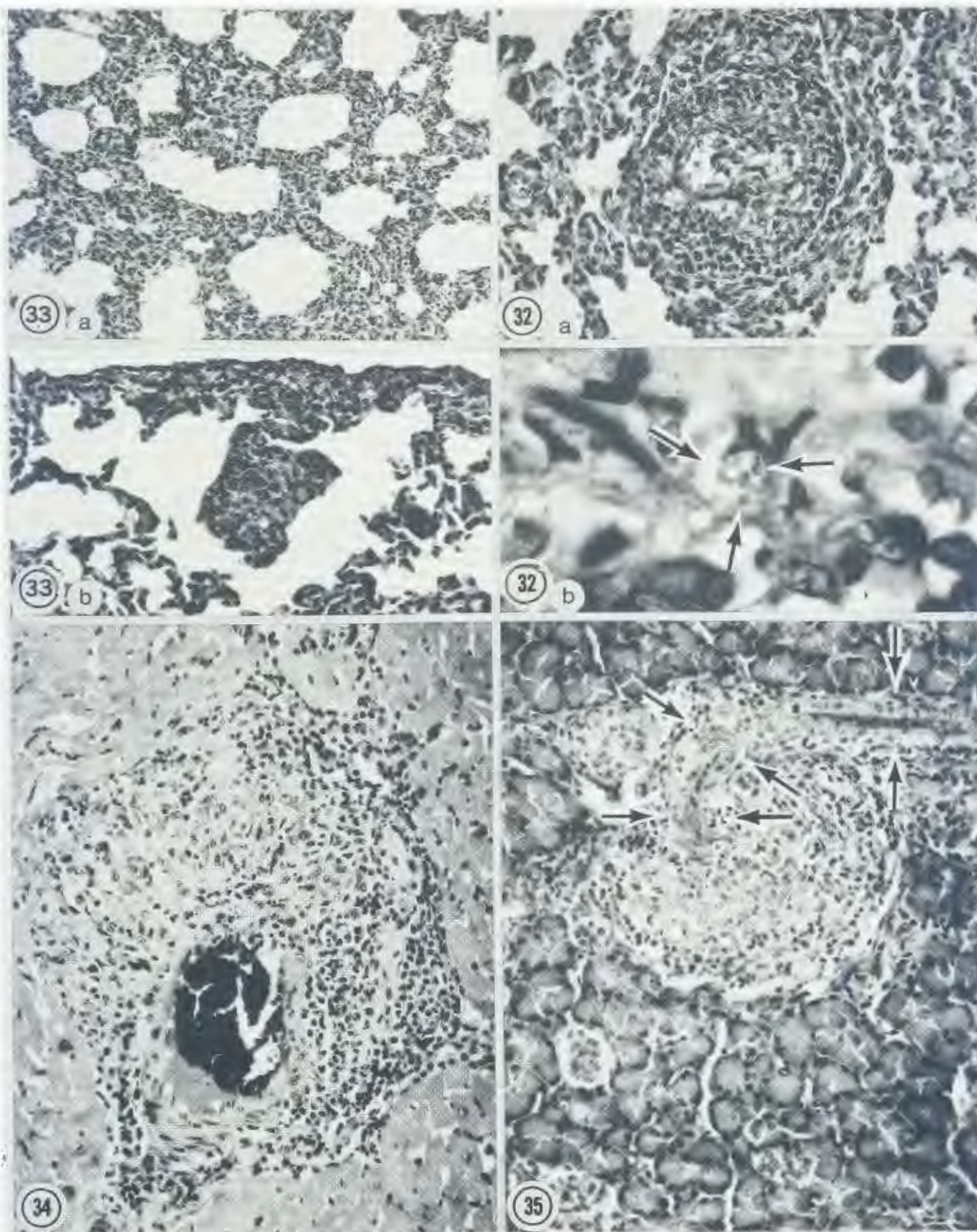


FIG. 32(a). Lung. Granulomatous vasculitis of a bronchial vessel: HE \times 250. 32(b). Lung. Gram-positive spores (arrows) in the wall of the vessel depicted in 32(a): B&H \times 1 575

FIG. 33(a). Lung. Interstitial pneumonitis. Alveolar septae are thickened by infiltrates of mononuclear cells: HE \times 160. 33(b). Lung. Subpleural, interstitial nodule. Intense, focal interstitial mononuclear cell reaction contributed to formation of nodules: HE \times 250

FIG. 34. Heart. Fibrinoid, necrotizing, granulomatous vasculitis: HE \times 250

FIG. 35. Pancreas. Nodular, granulomatous vasculitis. Arrows delineate the involved vessel: HE \times 250

lesions of immunocompromised infants grew and multiplied obviously more than in those of immunocompetent infants or adults.

Effect of cell culture passage level on pathogenicity of E. cuniculi

Cell culture passages 17 and 75 (a difference of 58 passages) were compared in four adult vervets (Table 5). Two monkeys were inoculated for each of the two passage groups. Lesions in the livers, kidneys and brains of all four vervets were essentially similar to those previously described for adults (Table 2).

Lesion development in the new-born after foetal infection (vertical transmission of spores)

Of five pregnant vervets simultaneously inoculated late in gestation, two aborted 2 and 3 weeks PI, two underwent caesarean section 4 weeks PI, and one delivered a normal infant 3 weeks PI (Table 3). Histological examination of one of the two aborted foetuses (the other was too autolytic) and the placenta of both revealed no lesions or spores. The cause of the abortions was not determined.

Macroscopical and histological examinations of the infant necropsied 10 h after it was delivered by caesarean section also revealed no lesions or spores. Examination of the other infant that was delivered by caesarean section, but who was hand-reared on human infant formula and necropsied 6 weeks after delivery, revealed subclinical encephalitozoonosis with lesions in the liver, kidneys and brain similar to those described above for infants. Spores were not difficult to find in tissue sections of this infant (Fig. 22 & 23).

The infant delivered via normal birth, raised by its dam and necropsied 8 weeks after delivery, also had lesions of subclinical encephalitozoonosis in its liver, kidneys and brain similar to those described above for infants. Spores were readily found in tissue sections and they were reisolated in primary kidney cell culture. The five dams of all these infants had mild lesions and subclinical disease as previously described (Table 2).

Lesion development in dams nursing infected infants (horizontal transmission of spores)

Three dams each nursing an infected infant were exposed to the spores of *E. cuniculi* for a period of time ranging from 4–8 weeks (assuming that spores were shed by the infants from the first day of their infection). Lesions were allowed to develop in the dams for 12–14 weeks; dams were free of their infants from 5–8 weeks (Table 6) of this time. As a result, minimal to moderate lesions such as those previously described for adults (Table 2) were found in the livers and kidneys of these dams. As in adult animals experimentally infected *per os*, the disease in the dams remained subclinical and generally mild. In one of the dams (RM-67), the lesions were generally of the "C" type, whereas the other two dams (RM-66 and RM-58) had predominantly "B" type lesions. None of the dams had lesions in the brain. Although organisms were not found in cell cultures or tissue sections, the serum of all three dams converted from a negative to a positive titre.

Induction of lesions in the dog with spores of E. cuniculi reisolated from the vervet

Canine encephalitozoonosis was induced with spores that were reisolated in cell culture from one vervet (RM-52, Table 2) inoculated with the 75th passage of cell-culture-grown organisms (Table 5). The subclinical disease and experimental lesions in the dog at 8 weeks PI were essentially the same as previously reported (Botha *et al.*, 1986).

DISCUSSION

Encephalitozoon isolated in cell culture from the dog is remarkably infectious for the vervet monkey. Of 37 vervets examined for this report, 34 are shown to have contracted encephalitozoonosis directly or indirectly from orally induced infection. Intravenous inoculation (38th monkey), as expected, produced widespread infection and moderately severe lesions in multiple organ systems. However, oral administration of spores also readily caused infection. Within 1 week (3 days) after inoculation *per os* it was observed that colonies of spores are present within epithelial and endothelial cells of the small intestine. Although we did not find spores in the gastric mucosa, we think that *E. cuniculi* may also have multiplied within epithelial cells of the stomach since colonies of spores have been seen within gastric epithelium of the dog (Van Dellen, unpublished observation, 1976). Multiplication of the parasite in gastric epithelium with the release of second-generation spores into the gastric lumen would have numerically enhanced the orally administered infective dose which reached the small intestine. Thus, by oral administration of the inocula, we initiated numerical enhancement of spores which augmented systemic spread of the organism to other major organs beyond the gastrointestinal tract.

We are inclined to believe the infection is progressive by way of major organ systems. The parasite appears to disseminate from the gut to induce remarkable lesions in the liver and kidneys within the first 3–4 weeks after inoculation. However, moderately severe granulomatous lesions were recorded only in the liver during this time, while rather minimal to mild lymphoid cell reaction was seen in the kidneys, and no lesion in other organs. Thus, during the early weeks of gastrointestinal infection, it appears that the liver is the first major target organ. Dissemination to the liver presumably occurs predominately via the portal vascular system because lesions were most prominent in portal triads and sinusoids.

Multiplication of the parasite in the liver, which should additionally enhance systemic dissemination, occurs within endothelial cells and hepatocytes and possibly within Kupffer's cells. However, we think that in the immunocompetent host, reproduction of the parasite within Kupffer's cells is generally a "dead-end" process, for with transmission electron microscopy of canine liver (Van Dellen, unpublished observation, 1977) we have only seen degenerated spores within them.

As seen in the liver of adults, response of the cellular immune system to infection was morphologically detectable approximately as early as 1 week after oral inoculation. The cell-mediated immunity in the liver, principally executed by Kupffer's cells and subsequent generation of macrophages, appears to be the paramount mechanism to limit the spread of infection. In adults, in contrast to infants, most of the lesions in the liver by 7–9 weeks PI were prominent microgranulomas which contained histologically undetectable, degenerated spores. It may be that the more marked granulomatous reaction in the liver of adults decreases the propensity of the infection to spread to other organs and the chance of finding or isolating spores in adults. This was supported in part by cultures in which the success rate of reisolation of spores from inoculated adults was only 7.7% in comparison to the 87.5% success rate from immunocompetent infants; and in part also in that spores within tissue sections of adults were judged to be "scarce", whereas we could more easily find a "few" spores in the tissue sections of infants.

By the 4th week PI, kidney lesions in the adult had reached remarkable development only in the cortex including the C-M junction. Renal lesions had a pattern

which suggested that once infection was established in the vasculature it spread in the nephron along the afferent arterial system to the glomerulus, and from there down the efferent arterial system toward the venous system as well as down the tubular system to the medulla. We think that when a progressive infection and flow of spores (detectable by the corresponding inflammation) reaches the glomerulus, endothelial and epithelial cell infection in the glomerulus is established and subsequently showers spores toward the medulla down the vascular and tubular systems of the affected nephron.

The usually predominant plasmacytic, interstitial inflammation in the kidneys was often conspicuously linear, and was primarily seen along the medullary rays in response to a vascular infection we perceived advancing within vessels. Although infection of tubular epithelia was common, especially in the medulla, it did not usually appear to elicit an interstitial inflammatory reaction. Interstitial exudate probably reflects infection of the vascular systems, whereas infection of tubular epithelia from spores flowing in the tubular lumen, we think, evades the cellular defense mechanism of the host. The exception to this may occur when a focus of tubular necrosis is established, but this was not commonly observed.

Dissemination of spores and development of lesions in the kidney, then, seems to progress from the C-M junction into the cortex and then to the medulla, primarily along the vasculature and secondarily along the tubules. This is supported by the following observations: (1) Mild, early inflammatory lesions first affected vessels at the C-M junction; this is the junctional site and origin of vessels supplying the nephron. (2) A linear spread of inflammatory reaction primarily occurred along the medullary rays in the cortex; through this region vessels course to and from the glomerulus. And (3), interstitial lesions with spores were seen earlier in the cortex than in the medulla. Later, during apparent resolution of the systemic disease when spores and lesions were rare in the cortex, they were more frequently detected and were relatively numerous in the collecting tubules and interstitium, respectively, of the medulla.

While renal lesions in the adult reached remarkable development by the 4th week PI, brain lesions were not detected until several weeks later at 6 weeks PI. Even then, granulomatous lesions in the brain were small and minimal but were otherwise similar to lesions as described in the dog (Van Dellen *et al.*, 1978). This delayed, and less severe, involvement of the brain suggests its inherent resistance to infection. Furthermore, when brain lesions in both adults and infants are compared at the same PI interval, it is suggested that inflammatory reaction in the brain decreases with increasing age. At 7-9 weeks PI, the infant usually had a moderate number of granulomatous lesions, while at this time in the adult they were minimal or absent. Thus, the vervet brain appears to be endowed with a natural resistance to systemic infection with *E. cuniculi*, and this resistance seems related to age.

That resistance to Encephalitozoon infection is dependent on age and perhaps indicative of the host's ability to retard growth and multiplication of the organism, is suggested by the observation that a greater number of spores were present in tissue sections from normal infants than in sections from adults. Relatively better success of reisolating the organism in cell culture from infants supported this histological impression; organisms grew in 7 out of 8 successful cultures from immunocompetent infants for a reisolation success rate of 87,5 % and in only 1 out of 13 successful cultures from inoculated adults for a success rate of 7,7 %. Thus, age dependent resistance to

infection with *E. cuniculi* is apparently a function of the host's ability to somehow retard the growth and multiplication of the organism. This resistance is more effective in adult than infant vervets.

Primary lesions of the vasculature in several major organs and in sinusoids of the liver were striking. Indeed, one cannot escape viewing the disease as principally an infection and an immunopathological entity of the vascular system. A large part of the injury is an indirect result of the intense immunogenic cellular response around infected vessels, and much of the injury affects vessels directly. Primary vasculitis is clearly a significant part of the pathogenesis of encephalitozoonosis as demonstrated in the brain, lungs, liver, kidneys, heart and pancreas of the vervet. While some investigators have emphasized an immunogenic mechanism as the cause of primary vasculitis in spontaneous encephalitozoonosis of the blue fox (Nordstoga, 1972; Nordstoga & Westbye, 1976), it has also been demonstrated by others that direct (mechanical) injury of the vasculature by infection with the aetiologic agent is likewise of major importance when the disease naturally occurs in dogs (Van Dellen *et al.*, 1978). Whatever the complete pathogenesis may be, this study of encephalitozoon infection in vervets corroborates the significance of vasculitis as a fundamental lesion of encephalitozoonosis as previously reported (Van Dellen *et al.*, 1978). More recently, Zeman & Baskin (1985) also reported on remarkable findings of vasculitis with encephalitozoonosis of squirrel monkeys raised in captivity.

Whether immunity in the infant was suppressed with parenteral steroid or was incomplete because of premature birth (compared to a full-term, new-born vervet), compromised immunity altered the character of lesions which was reflected by an increase in the number of spores found in tissue sections. Microgranulomas in the brain of the immunologically compromised infants seemed more compact, and a larger number of spores was found within and outside of these lesions. Compromised immunity, we think, decreases the ability of the infant to restrict parasite multiplication, apparently because components of the microgranuloma are less able to effectuate the resistance of the host. This was most evident in the premature infant.

Systemic infection is readily established, however, clinical manifestation of encephalitozoonosis may require a severely immunocompromised host. The host-parasite balance must undoubtedly be significantly disequibrated for the disease to become clinically evident, and for this to occur we think the host's immunity is paramount to the virulence of the aetiological agent. Although we did not record clinical signs of disease in any of the vervets, the premature infant did have severe lesions with the greatest number of spores seen in any of the animals. Clinical abnormalities (lethargy and slight head tremor in retrospect probably attributable to encephalitozoonosis) were difficult to interpret considering the premature status of the infant. In retrospect, had he not been killed, he could have been expected to have developed a more obvious manifestation of his disease. Impressions obtained from this study suggest that the magnitude and frequency of lesions, and thus the severity of encephalitozoonosis, may be inversely proportional to the immunological status and the age of the host. Predictably, the disease will be more severe and more likely apparent clinically when the host is infected early in life and/or is increasingly immunocompromised.

We describe and illustrate that canine isolates of *Encephalitozoon* are infectious and pathogenic for the vervet monkey in contradistinction to *E. cuniculi* from rodents which has been reported non-infectious for the

Rhesus monkey (Shadduck *et al.*, 1979). Furthermore, the *Encephalitozoon* reported as causing disease in canines may be more pathogenic for its host than *E. cuniculi* is for rabbits, rats, mice and hamsters (Plowright, 1952); McCully *et al.*, 1978; Shadduck *et al.*, 1978; Van Dellen *et al.*, 1978; Stewart *et al.*, 1979(a); Botha *et al.*, 1979; Shadduck *et al.*, 1979). Shadduck *et al.* (1979) suggested that differences in the morphology and distribution of lesions induced by *E. cuniculi* in rats, mice, rabbits and Rhesus monkeys (no lesions) may be the result of differences in the response of various hosts rather than some characteristic unique to the parasite. On the other hand, it may also be (at least in part) a discriminating characteristic of the parasite that modulates and complements the immunopathologic response of the host. The ability of this parasite to influence the immune response of the host (Cox, 1977); Niederkorn, Shadduck & Schmidt, 1981) suggests that it influences the morphogenesis of lesions. This characteristic surely is of prime value (along with other criteria) in assessing the true identity of the parasite.

The marked infectivity and pathogenicity of our canine isolates for a non-human primate suggest that they may be distinct from other *E. cuniculi*. Additional definitive characteristics based on more inter-species infectivity study, serology, biochemistry, DNA homology, electrophoresis of spore wall polypeptides (Fowler & Reeves, 1974; Street, 1979) and spore isozyme analysis (Joslyn, Kelly, Knell & Dillard, 1979) will be necessary to further characterize these canine isolates. It is interesting to note, though, that in some countries (i.e., Europe and USA) where encephalitozoonosis is common in rabbits it is relatively rare in dogs, whereas elsewhere (i.e., Republic of South Africa) the disease is relatively common in both rabbits and dogs. Also of note is that in Norway the parasite has remarkable pathogenicity for the blue fox, but apparently not for the dog. Epidemiologically, then too, it is suggestive that there are, perhaps, more pathogenic strains of *E. cuniculi*.

We found experimental pathogenicity of our isolates of *E. cuniculi* in vervet monkeys to be similar to that observed in the dog (Botha *et al.*, 1986), and also stable. Spores of the 75th cell culture passage produced similar lesions in both hosts, and the pathogenicity of the organism did not appear attenuated when lesions produced from the 75th culture passage were compared to those induced with spores of the 17th passage. Thus, the organism was shown (on a limited scale) to be a stable pathogen when cultured, and that it caused similar subclinical disease in a phylogenetically distant host.

Our finding that oral administration of spores readily induces systemic lesions in a non-human primate, strengthens a previous suggestion (McCully *et al.*, 1978) that natural infection acquired by ingestion of spores (harboured in the environment) may play a significant role in the epidemiology of encephalitozoonosis in man and animals. We now suggest that spores of *E. cuniculi* shed by the dog may be agents of zoonotic infection and disease, albeit latent. Additional serological examination of man should be done to reinforce this hypothesis. Furthermore, that development of lesions with manifestation of clinical disease in any host usually depends upon suppression of the host's immunological competence or the involvement of an immunologically poorly protected anatomic site, correlate well with case reports of disease in man. Four of the eight case reports of human patients with clinical microsporidiosis were immunologically depressed through combined immunodeficiency (Margileth *et al.*, 1973) or AIDS (Ledford, 1985; Desportes, 1985; Terada, 1987), and two had infection located in an im-

munologically privileged site—the cornea (Ashton & Wirasinha, 1973; Pinnolis *et al.*, 1981). The remaining two human patients with encephalitozoonosis had manifestation of neurologic disease, but were apparently immunologically normal (Matsubayashi *et al.*, 1959; Bergquist *et al.*, 1984).

We believe *E. cuniculi* is a potential zoonotic disease agent of significant proportion, especially when the host is immunocompromised. This study demonstrates again that *E. cuniculi* is indeed not host specific, as others have also reported (Shadduck *et al.*, 1979), and that still another host—the vervet, a primate—should be included in its infectivity range. One could reasonably speculate, therefore, that man may also be included in its host range. The disease in an immunologically normal host, including man, should be expected to be subclinical. Reports of positive serology of humans support this probability (Cox & Pye, 1975; Wilson, 1979; Stewart *et al.*, 1981; Singh *et al.*, 1982; Bergquist *et al.*, 1984; Hollister & Canning, 1987). Encephalitozoonosis probably is a zoonosis!

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