Apoptosis and Phenotyping in Japanese Encephalitis Virus Infected Rhesus Monkey Challenge Models

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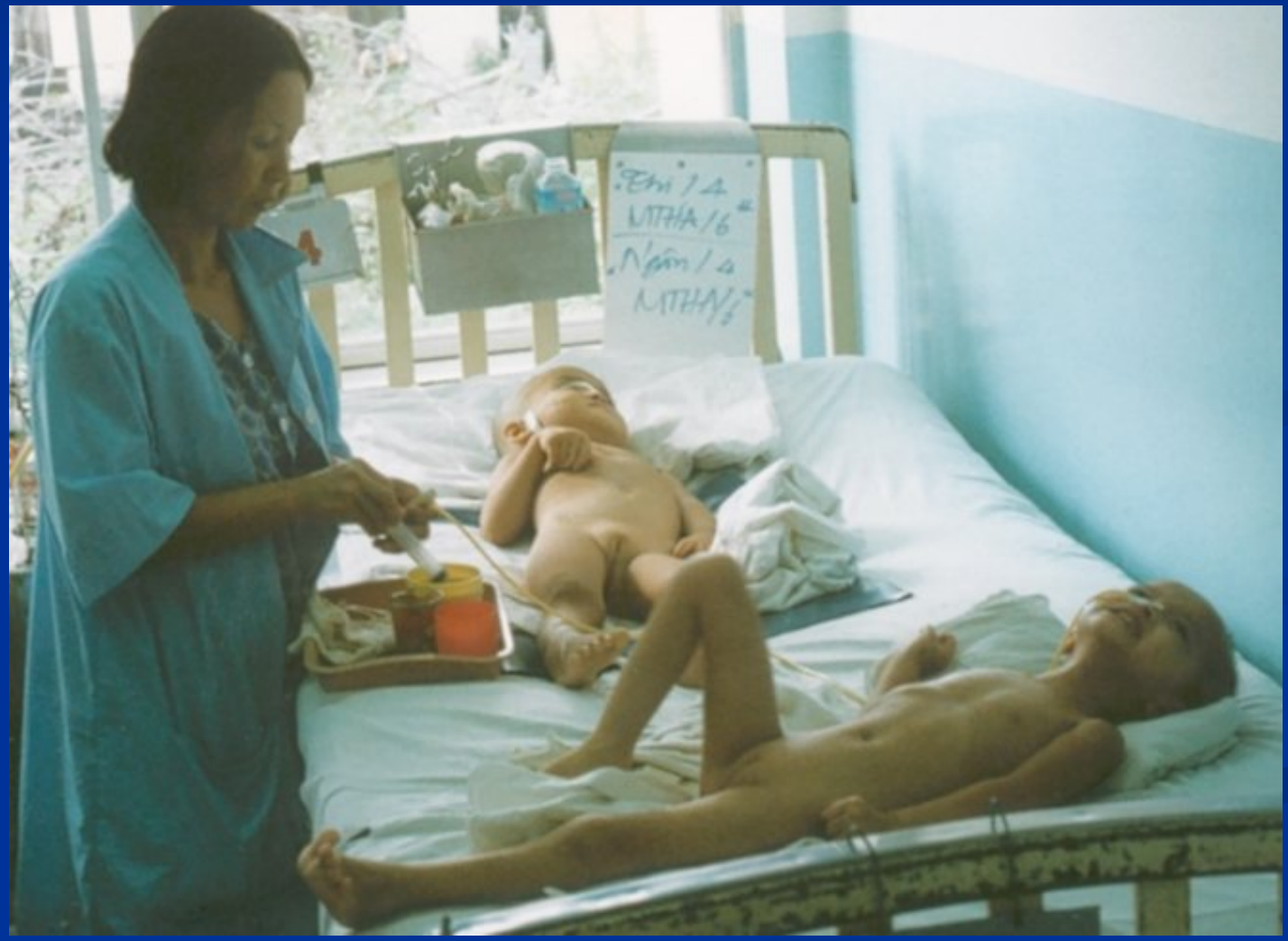
BACKGROUND

- Japanese encephalitis virus (JEV) continues to be the major cause of viral encephalitis endemic to Asia and the Western Pacific



BACKGROUND

- Annual estimated cases about 30,000-50,000 and 10,000-15,000 deaths
- Little is known about the pathogenesis of JE
- It is not known whether cell injury is due to viral cytopathic effects or the inflammatory response
- It is not clear whether only virus infected neurons die or whether JEV induces death of neighbouring uninfected cells
- Studies that address the mechanisms producing neuronal dysfunction and damage are required

OBJECTIVES

- To identify the mechanisms of neuronal damage in JE by exploring the role of apoptosis
- Phenotyping of inflammatory infiltrates

MATERIALS AND METHODS
Safety and Immunogenicity of recombinant JEV vaccines (1993-94)

- Twelve rhesus macaques (*Macaca mulatta*) challenged intranasally with a well characterized wild-type JEV

  Boonyos et al, 1999. An intranasal challenge model for testing Japanese encephalitis vaccines in rhesus monkeys. Am J Trop Med Hyg 60:329-337.

- Monkeys were euthanized when they developed stupor or coma (11-14 days post-inoculation)

TABLE 1. List of Rhesus monkeys that developed encephalitis after challenge with wild-type JEV

No	PHASE	Monkey No.	Challenge virus	Challenge dose (pfu)
1	Part I	DA-352	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^9
2	Part I	DA-378	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^9
3	Part I	DA-413	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^9
4	Part I	DA-443	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^9
5	Part II Stage I / Group 1	DA-470	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^{10}
6	Part II Stage I / Group 1	DA-525	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^{10}
7	Part II Stage I / Group 1	DA-526	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^{10}
8	Part II Stage I / Group 2	DA-379	KE93, AP61-1, C6/36-1	2.3×10^7
9	Part II Stage II / Group 1	DA-314	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	7.5×10^7
10	Part II Stage II / Group 1	DA-322	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	7.5×10^7
11	Part II Stage II / Group 1	DA-304	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	7.5×10^5
12	Part II Stage II / Group 2	DA-349	KE93, AP61-1, C6/36-1	6.6×10^6

SPECIMENS COLLECTED

- **Tissues**

- **All parts of brain and spinal cord**
  → cerebral cortex, brainstem, thalamus, cerebellum, olfactory bulb, meninges
  → cervical, thoracic, lumbar spinal cord
- **Liver, lung, spleen, lymph nodes**

- **Sera**

IMMUNOSTAINING

- Sections of thalamus and brainstem were stained for JEV antigen using an indirect immunofluorescence and a commercial DAB Elite Kit (Vector)

- Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end in-situ labeling (TUNEL) using Apoptag *In Situ* Apoptosis Detection kit (Chemicon)

IMMUNOSTAINING

- **Phenotyping** was done by cell specific markers
- **Dual staining** was done with TUNEL and cell specific markers to identify the apoptotic cells
- A laser scanning confocal microscope LSM 510 Meta (Carl Zeiss, Germany) with excitation wavelength 488 nm (Argon laser for FITC) and 543 nm (HeNe1 laser for Texas red) was used to detect apoptosis and JEV

IMMUNOSTAINING

- Appropriate controls included for each marker
  - uninfected monkey brain
  - positive control
  - control serum (mouse IgG/rabbit IgG)

- In each section the presence of positive cells was evaluated semi-quantitatively and scored as follows:

0	no positive cells
+	occasional isolated positive cells
+ +	a few nests of positive cells
+++	frequent positive cells

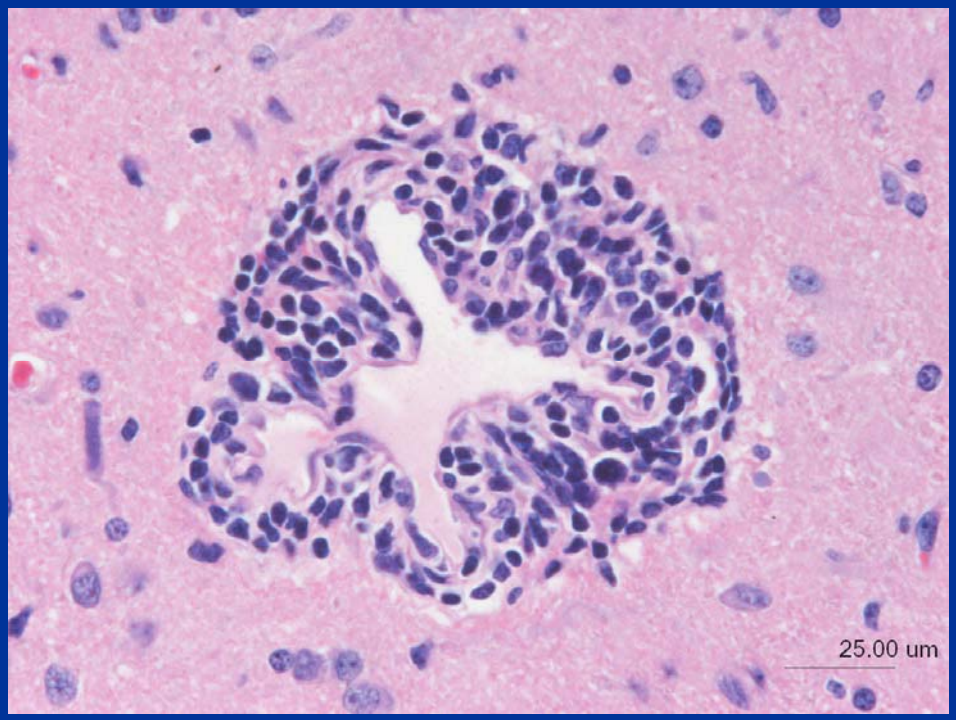
TABLE 2: Panel of antibodies utilized

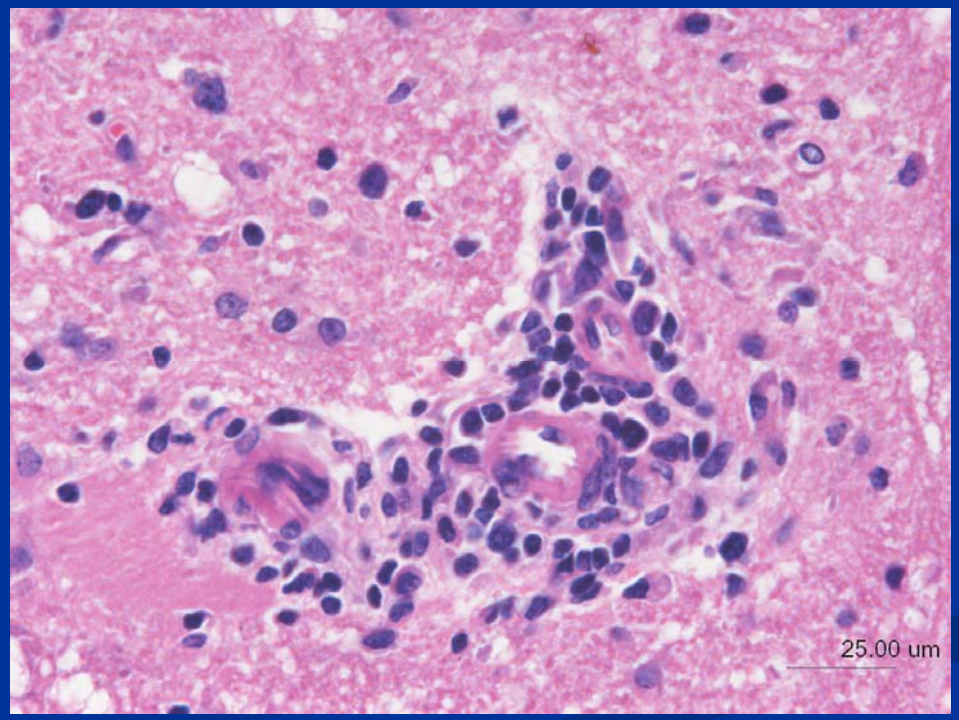
Antibody	Source	Specificity
Anti-Glial Fibrillary Acidic Protein (GFAP)	DAKO	astrocytes
Anti-neuronal nuclei (NeuN)	Chemicon	most neuronal cell types throughout the nervous system
CD3	DAKO	T cells
CD68 (KP1)	DAKO	macrophage, monocyte, microglial cells
CD20 cy (L26)	DAKO	B cells
Anti-Human HLA-DR Antigen (MHC II)	DAKO	B cells, activated T cells, macrophages, antigen presenting cells, some endothelial and epithelial tissues
Anti-Human Myeloid/Histiocyte Antigen (MAC 387)	DAKO	reactive tissue macrophages
Anti-Human macrophages (MCA 1478)	Serotec	macrophage, monocyte, histiocytes

RESULTS

PATHOLOGY

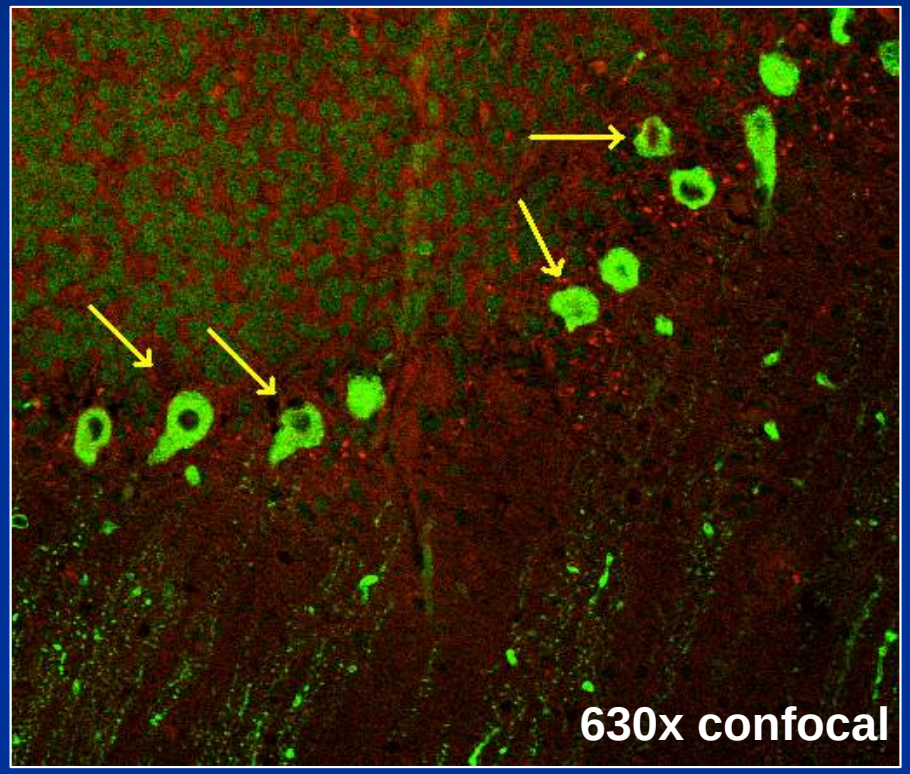
- non-suppurative meningitis
- mononuclear perivascular cuffing
- generalized microglial response



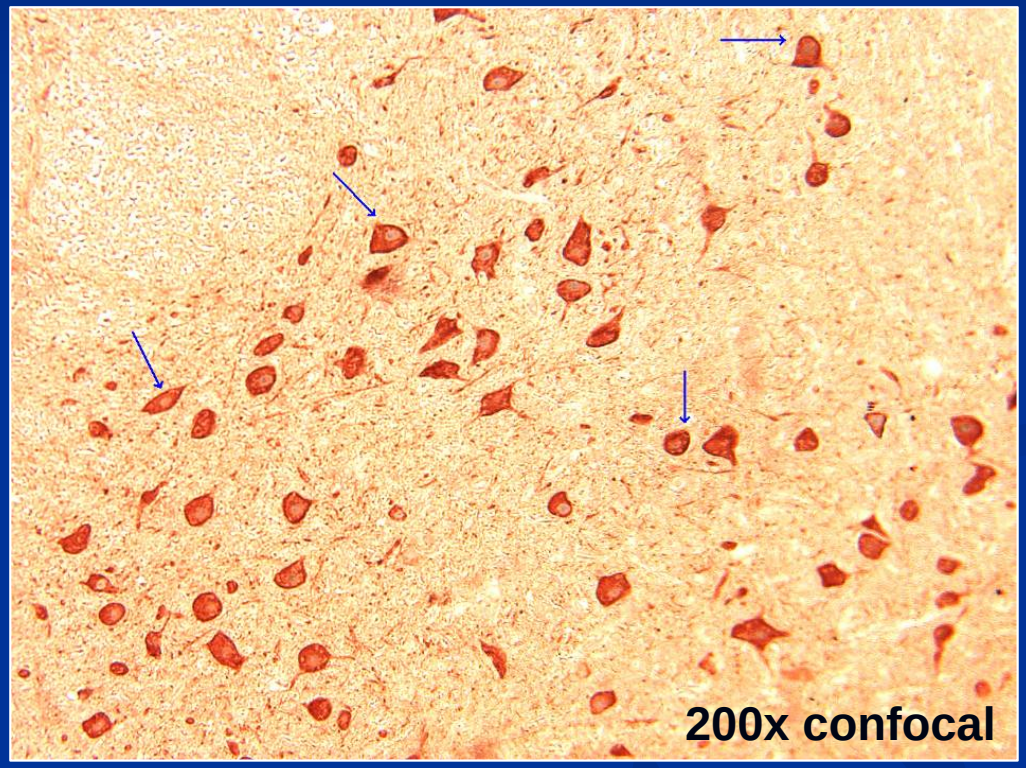


JEV Antigen Staining

Widespread antigen seen in thalamus, brainstem and cerebellum

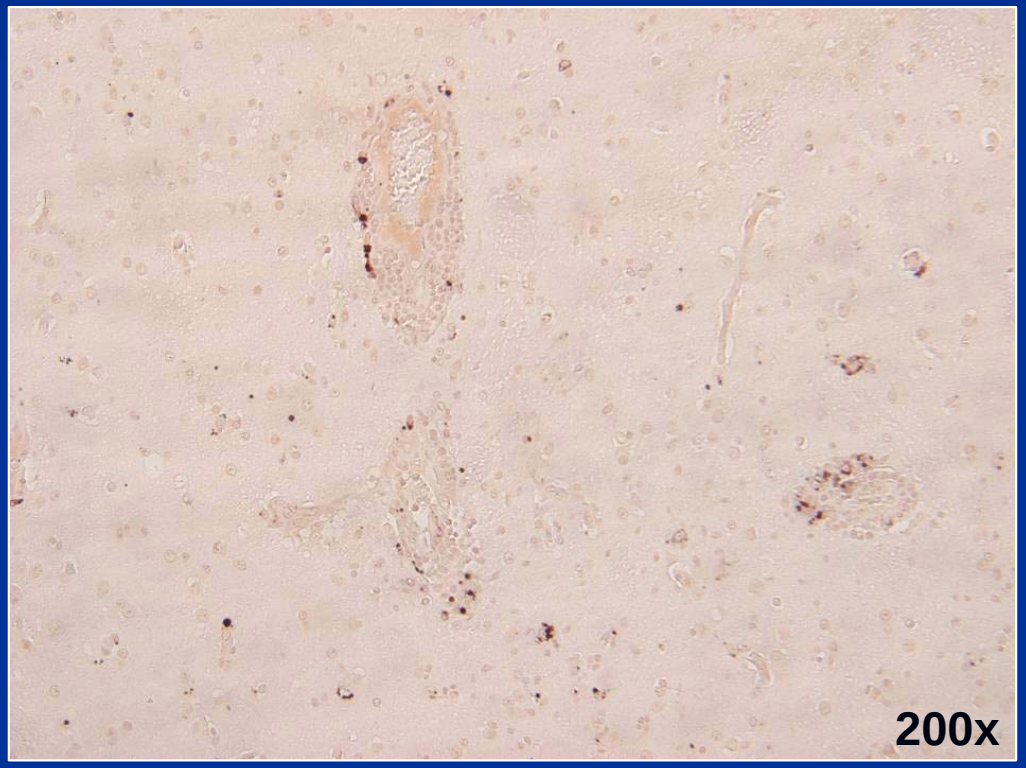


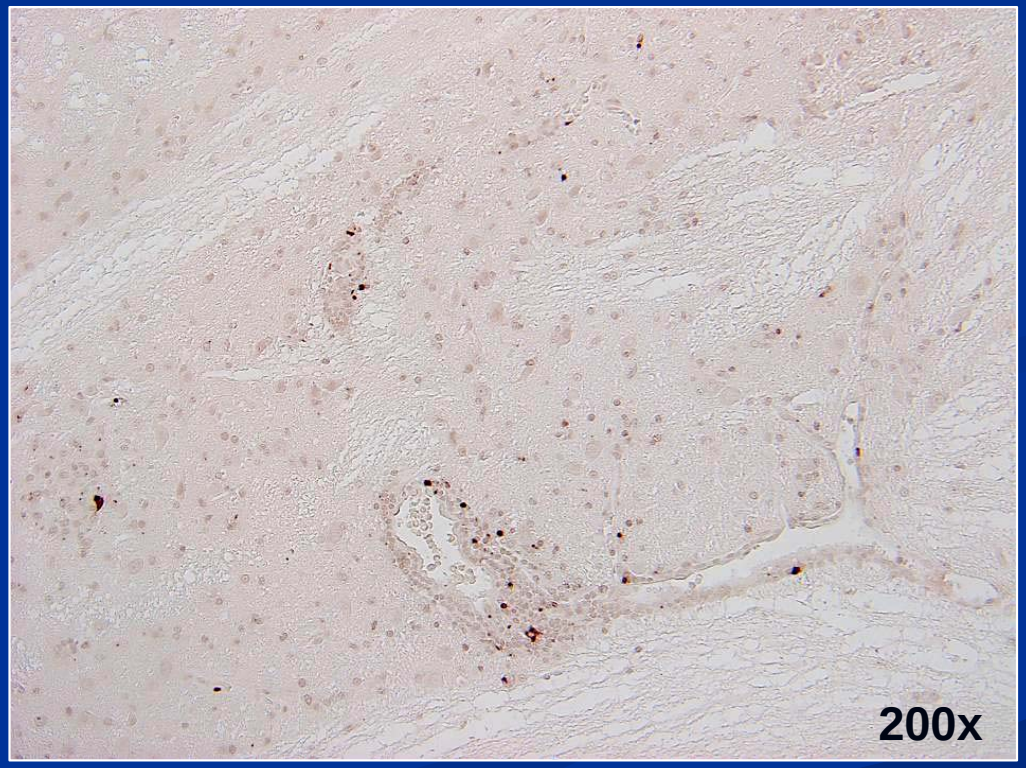
cerebellum



thalamus

Apoptosis Staining using TUNEL Assay (Chemicon)

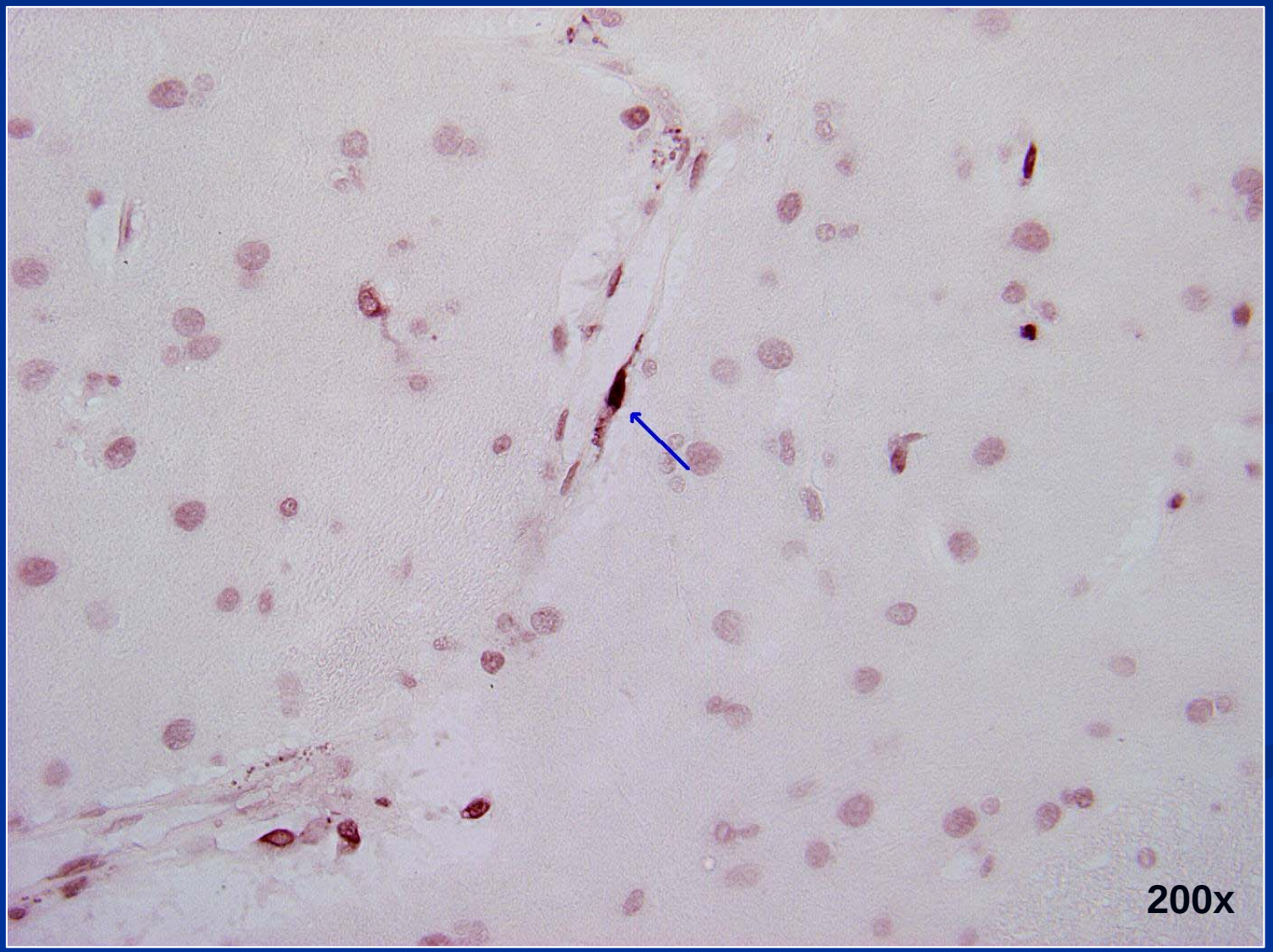




TUNEL-positive apoptotic cells were seen randomly distributed in the parenchyma and perivascular infiltrates

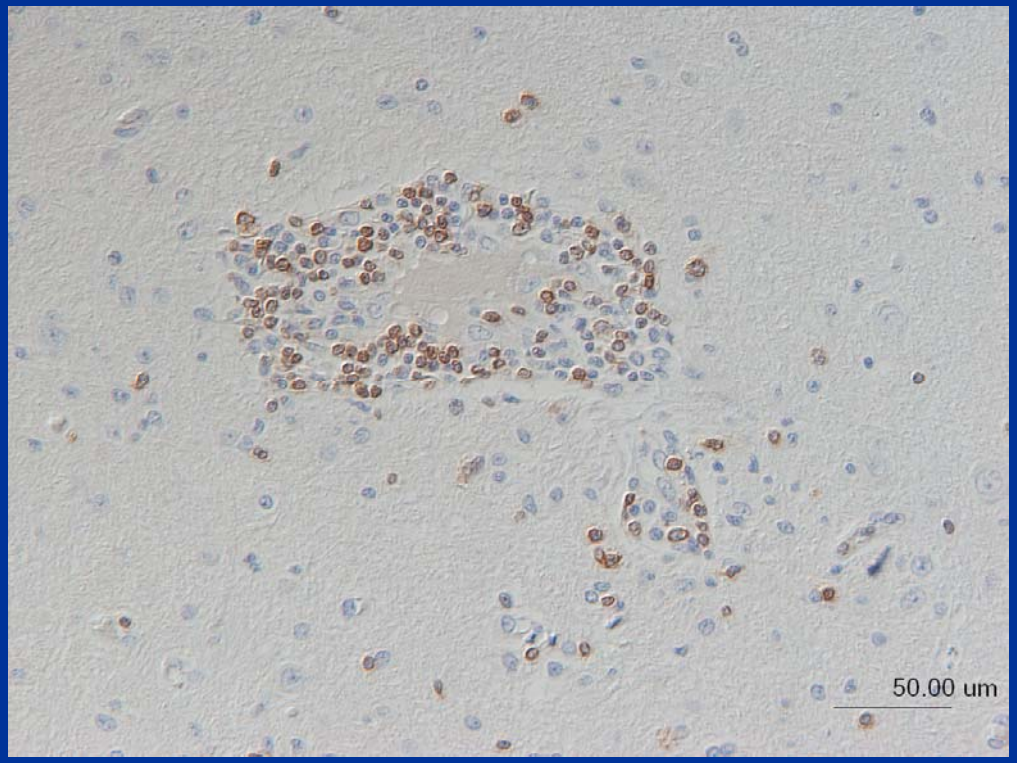
Apoptosis Staining using TUNEL Assay (Chemicon)

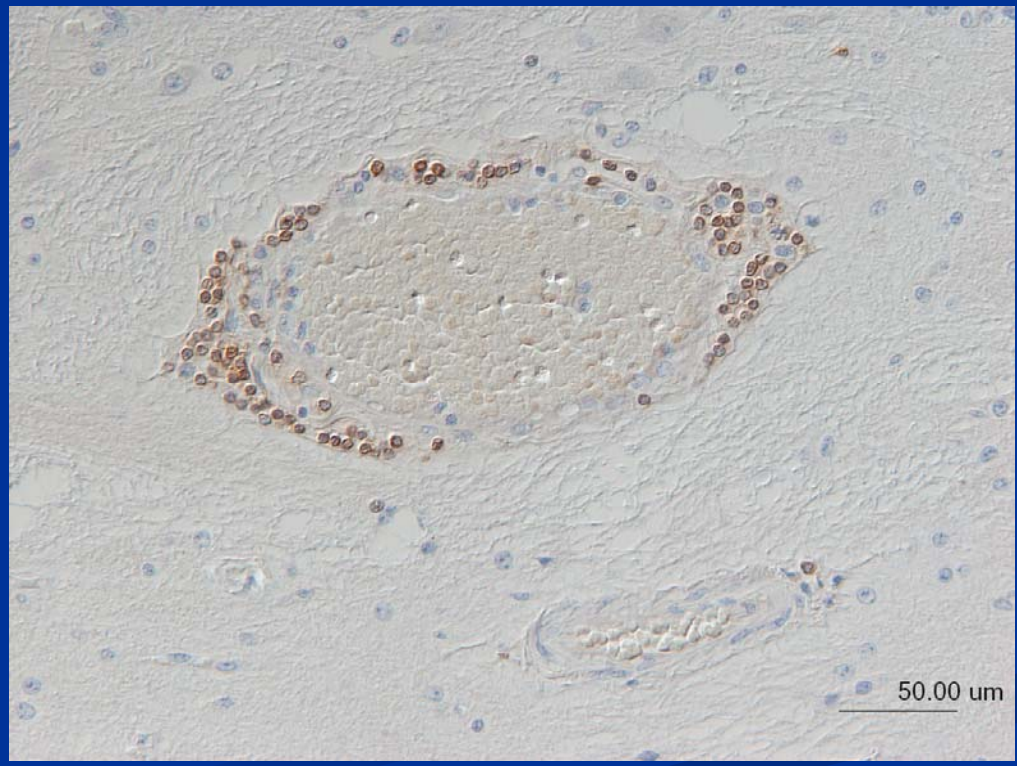
TUNEL-positive apoptotic cells were seen randomly in perivascular areas



RESULT

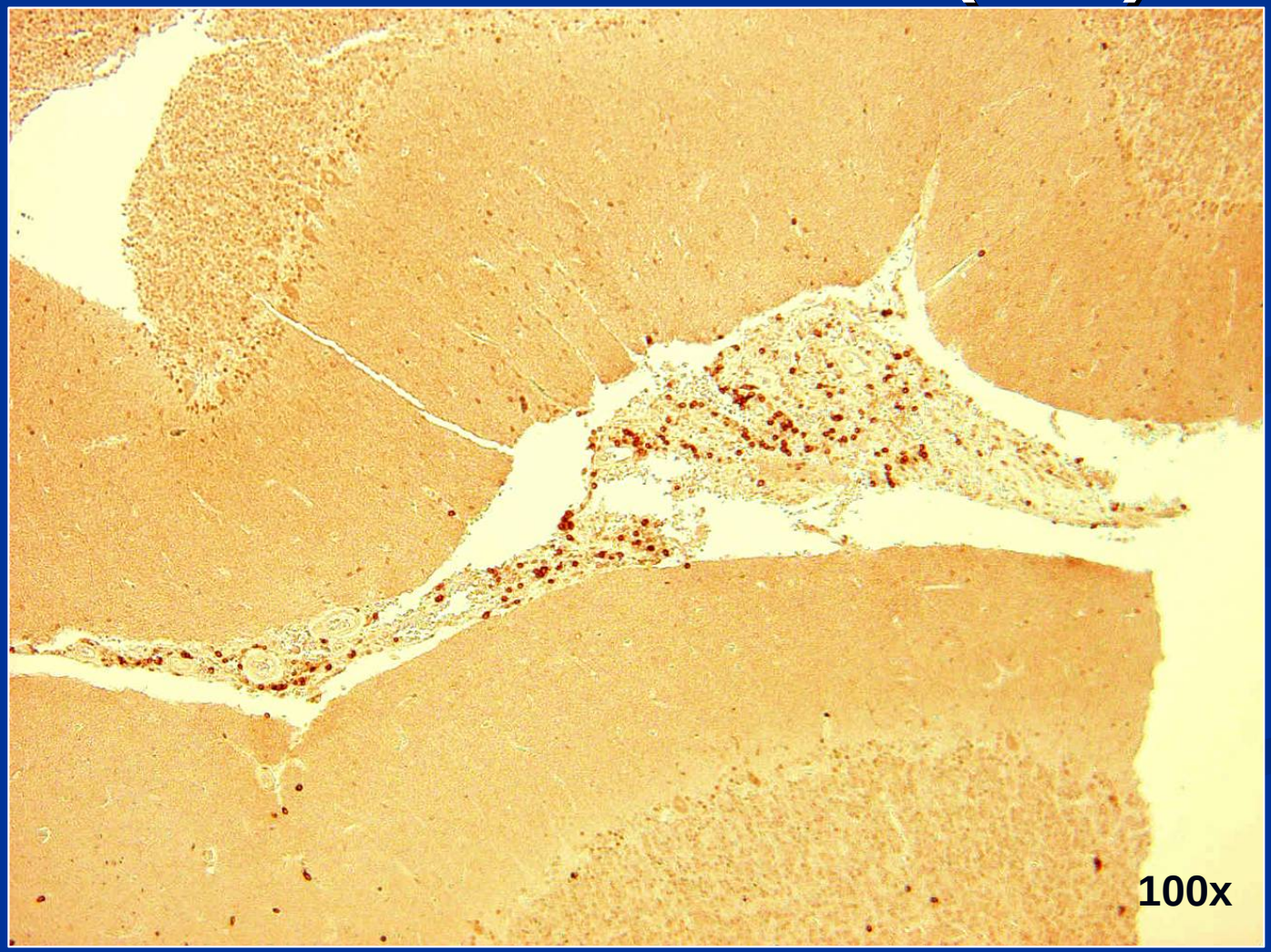
In mononuclear infiltrates CD3+ T cells are most numerous (IHC)





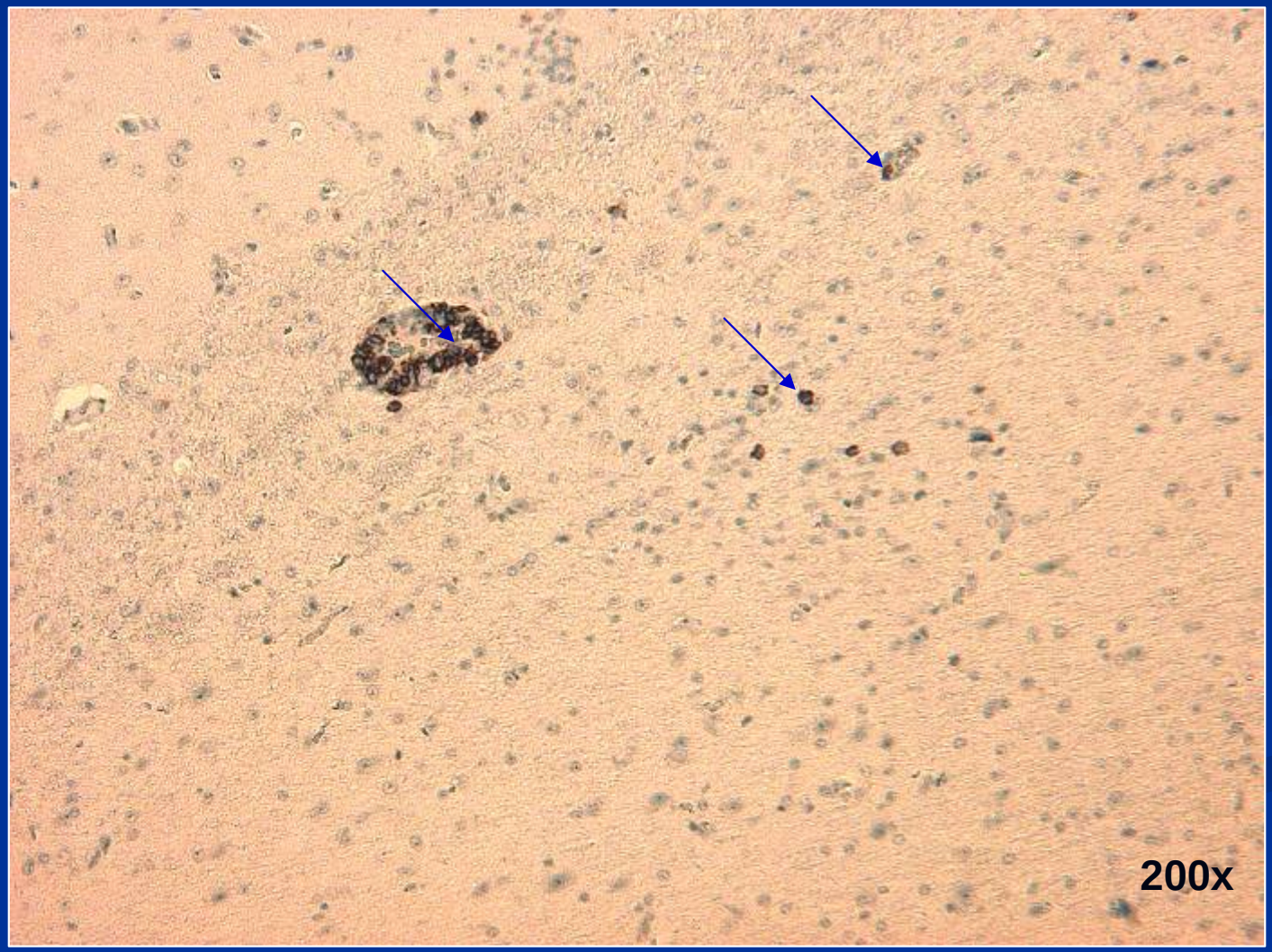
RESULT

In mononuclear infiltrates CD3+ T cells are most numerous (IHC)



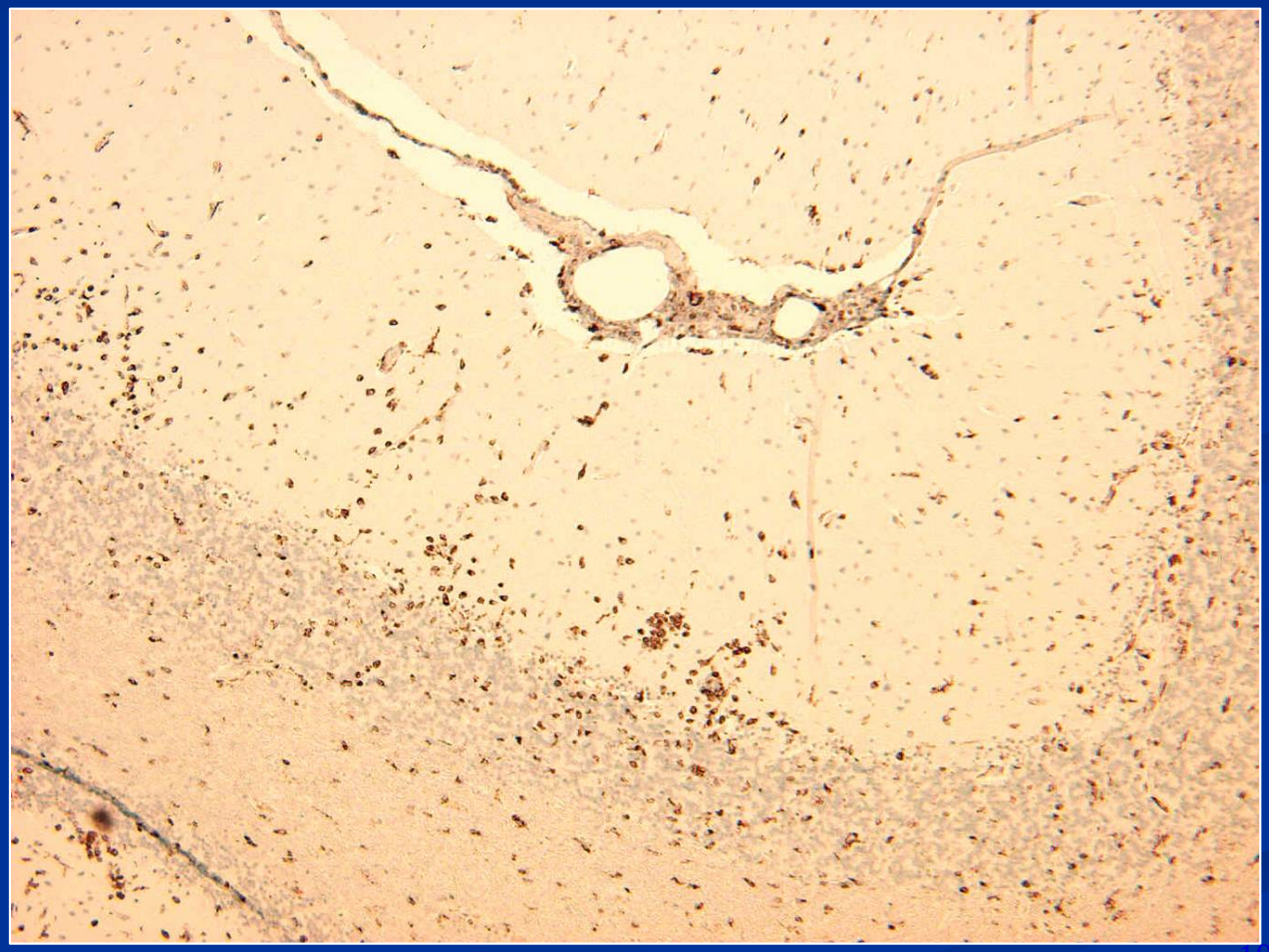
RESULT

Perivascular inflitrates also contained abundant CD20+ B cells



RESULTS

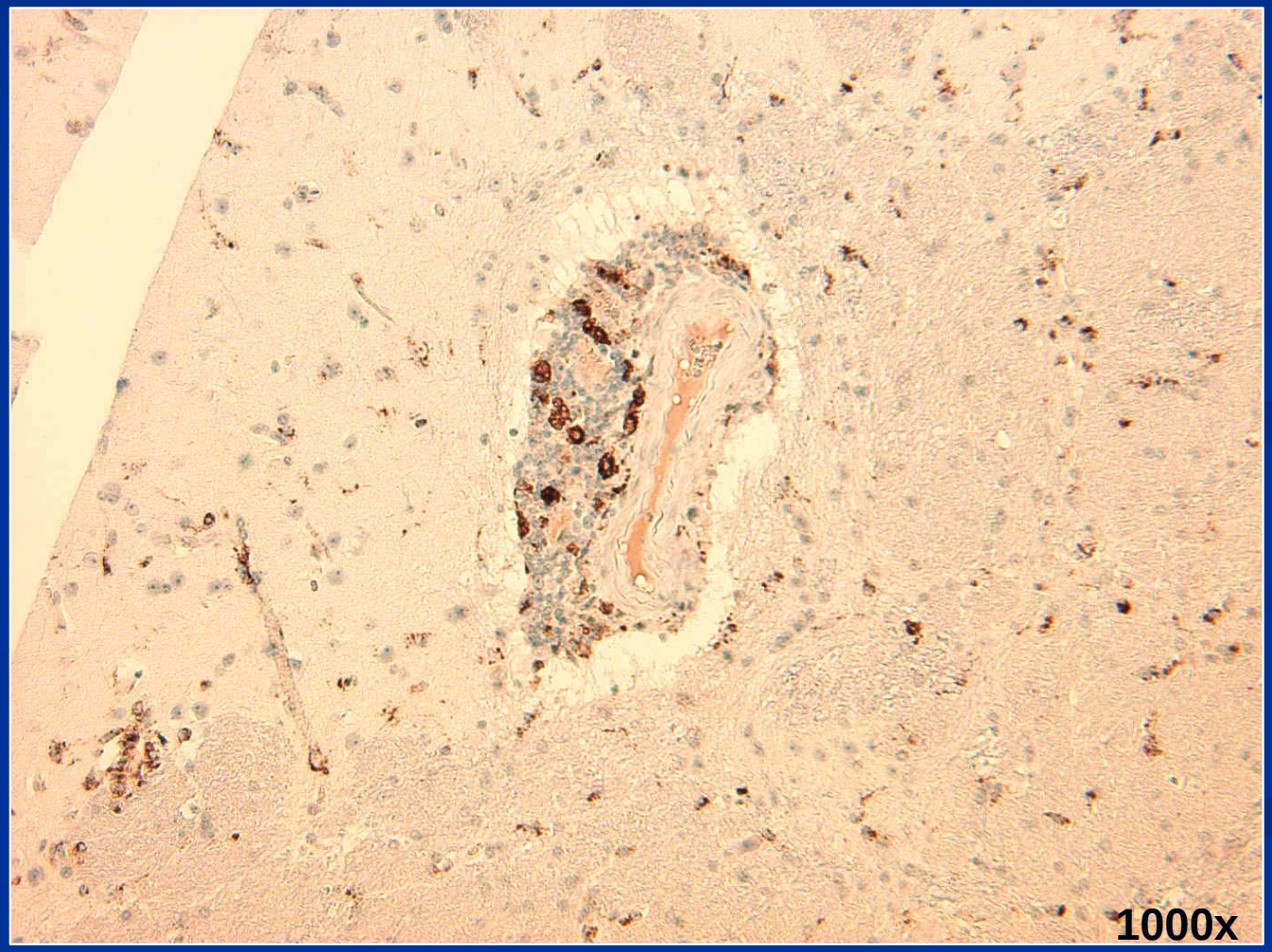
CD68^{+} cells (macrophages, microglia) were seen scattered in the parenchyma (IHC)



100x

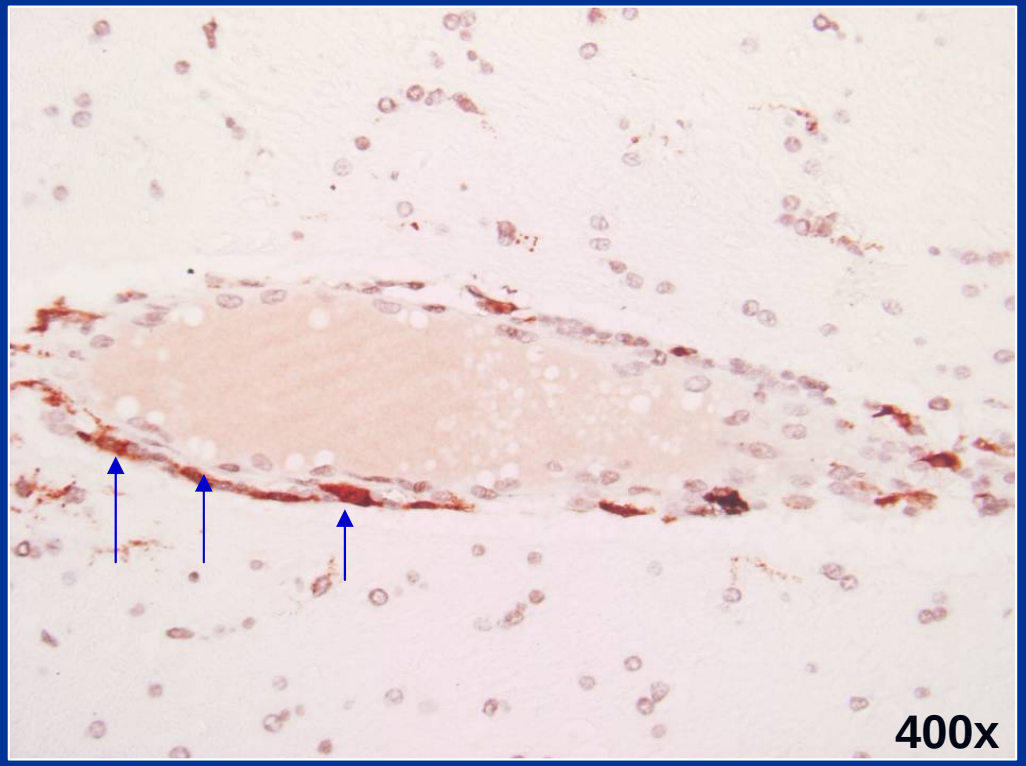
RESULTS

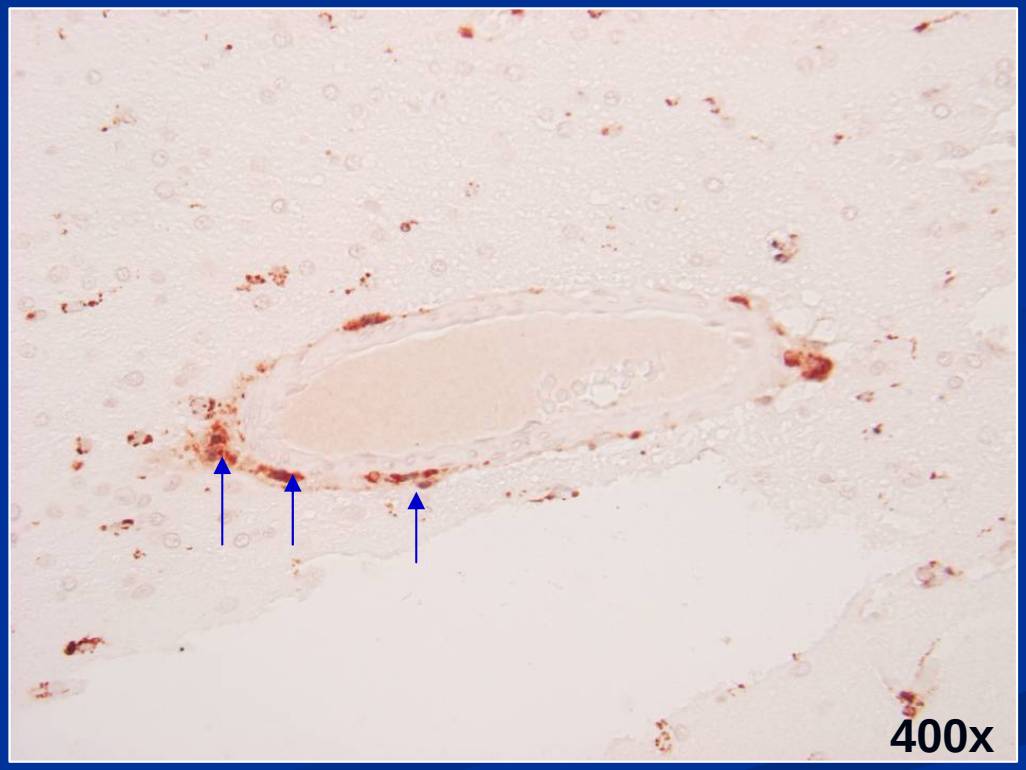
$CD68^+$ cells (macrophages, microglial cells) were seen scattered in the parenchyma (IHC)



RESULTS

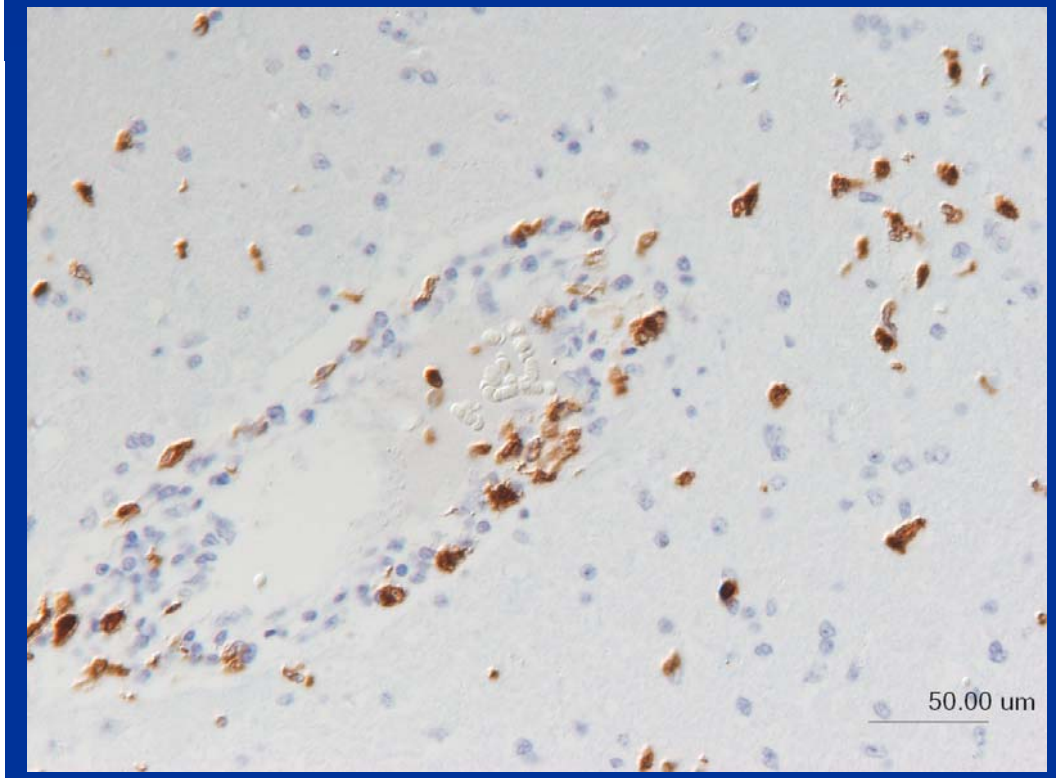
$CD68^+$ cells were seen in perivascular spaces (IHC)

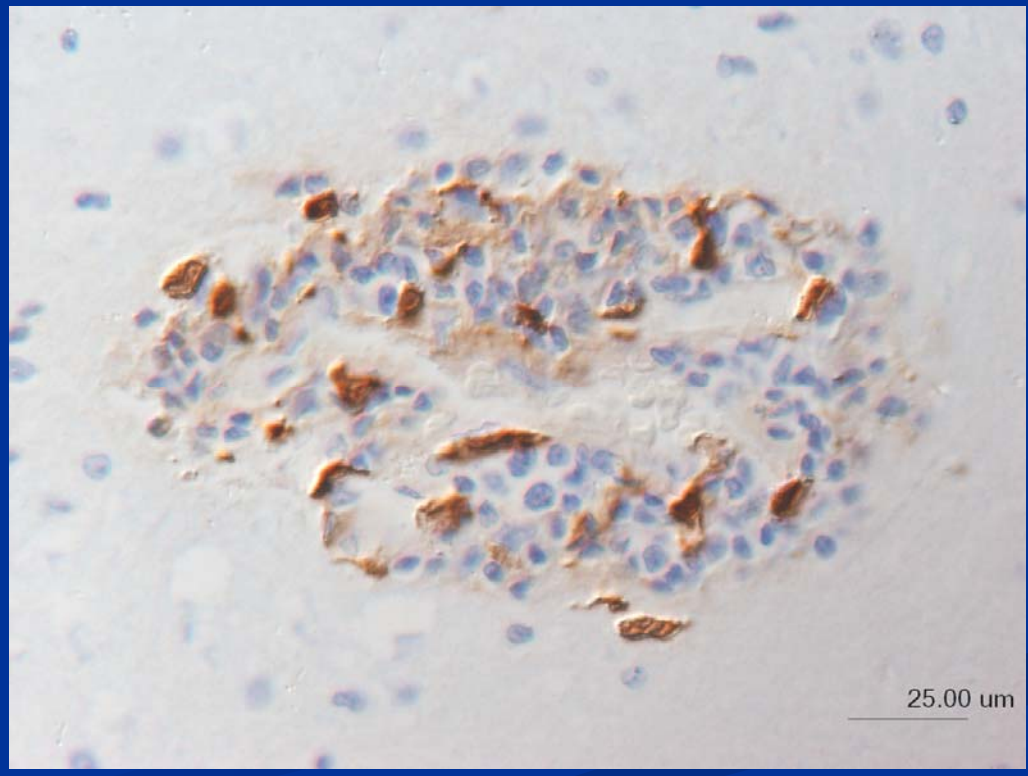




RESULTS

MAC387 cells were seen in perivascular areas (IHC)





Double staining for JE antigen (green) & GFAP (red) in JE infected monkey brain

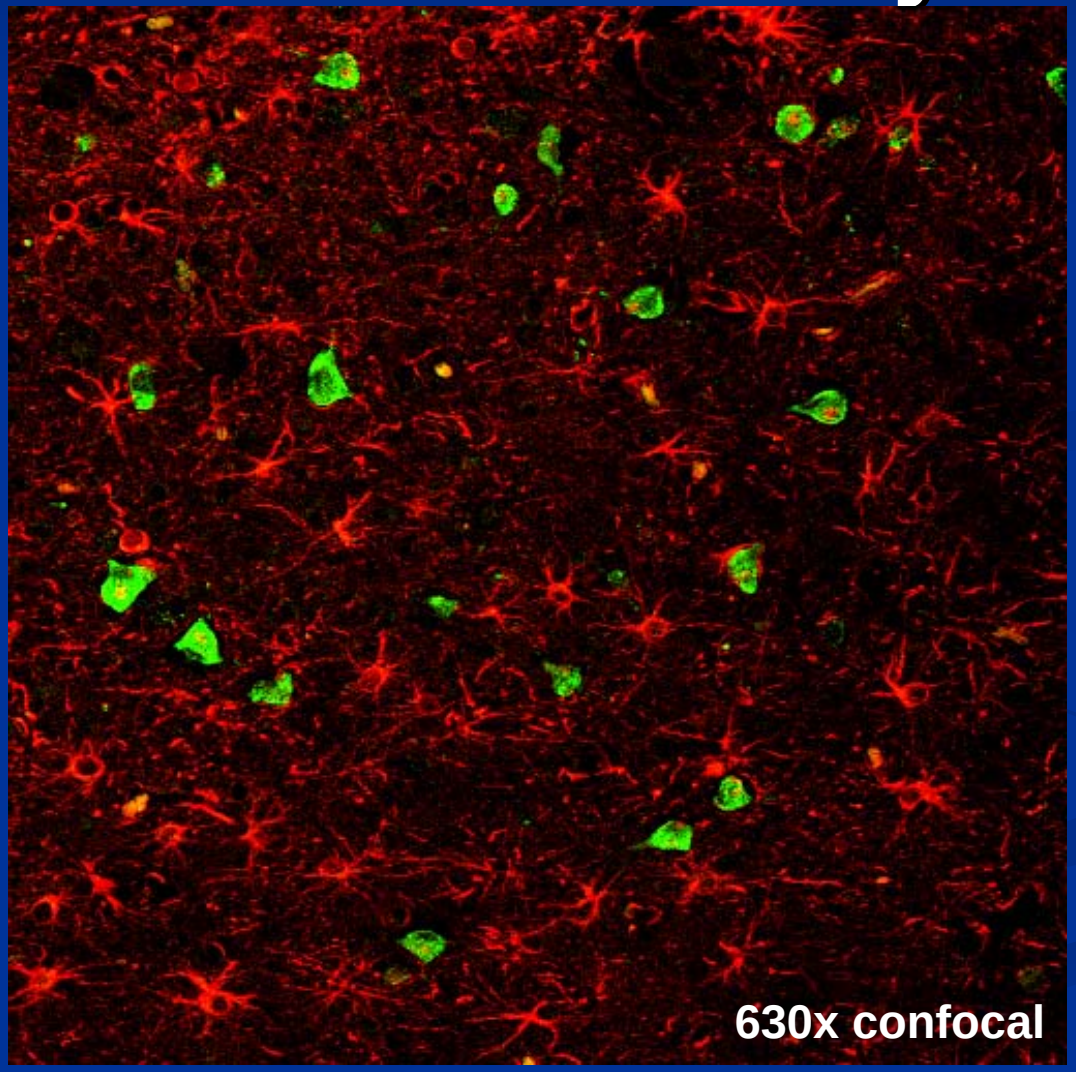
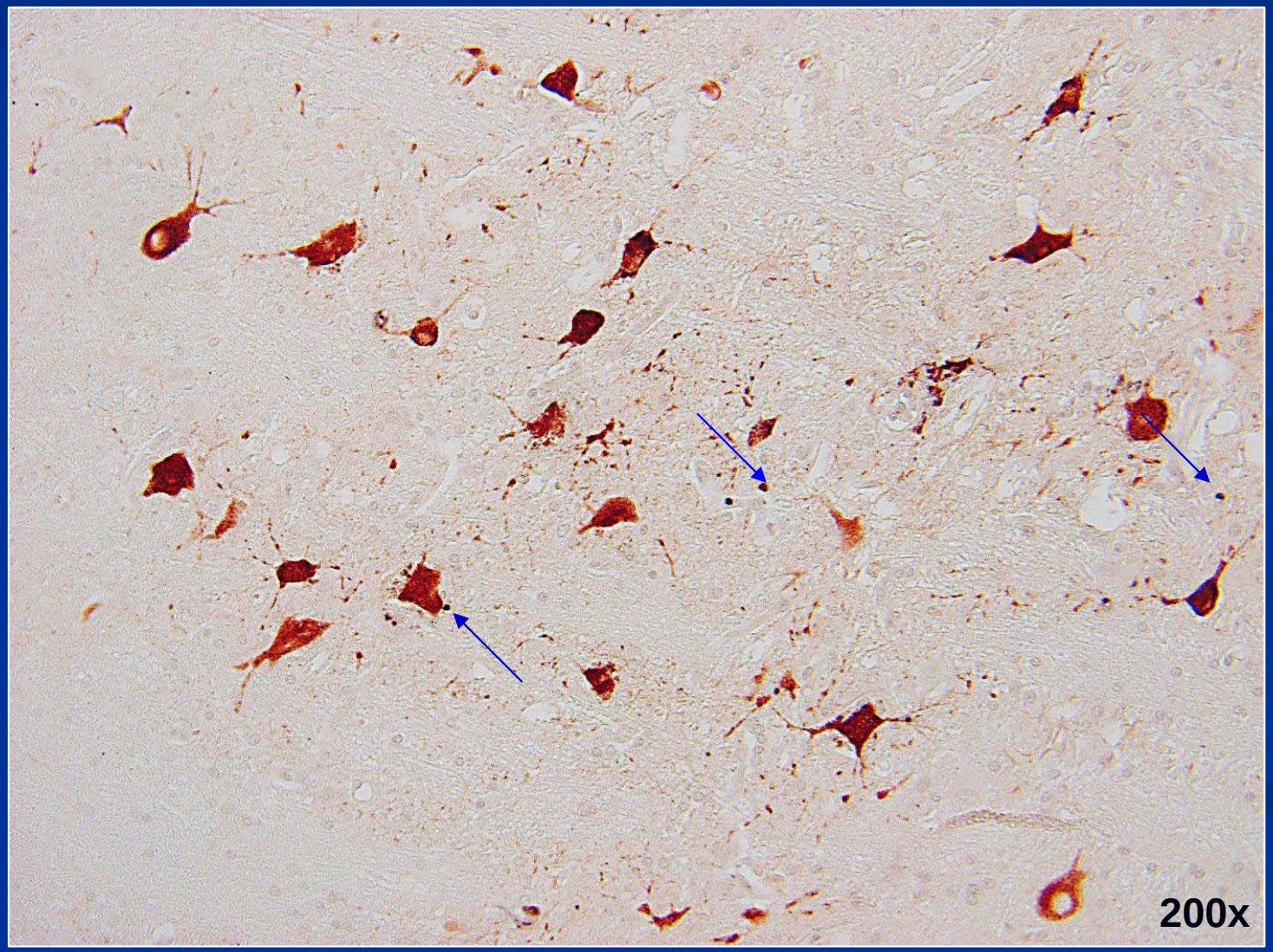


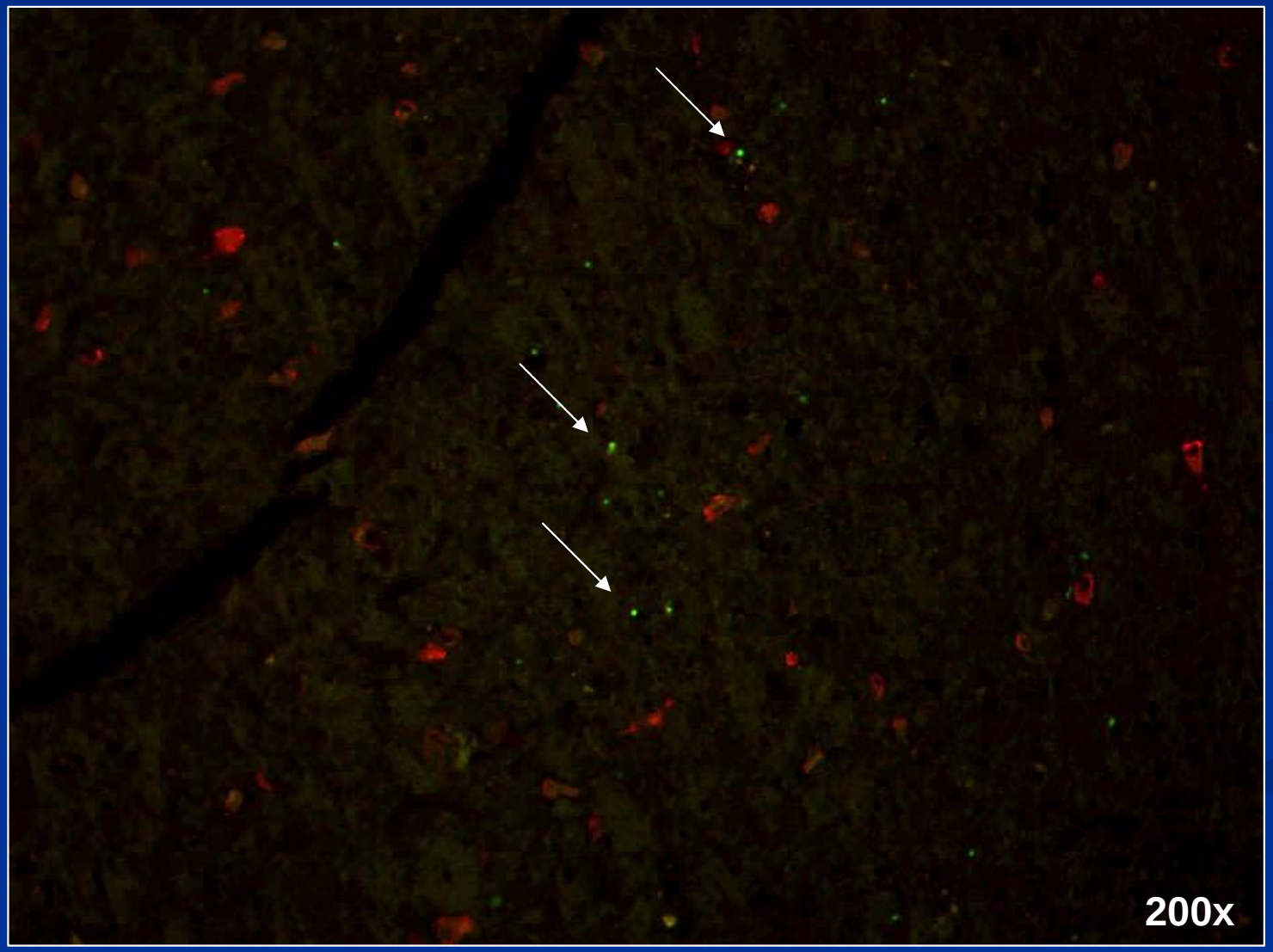
TABLE 3. Double staining with JEV antigen and TUNEL (IHC)

Monkey No.	Vet Med Access No.	JE antigen	Apoptosis
DA-352	95-013c	+	++
	95-013d	+	+
DA-378	95-014c	+	+
	95-014d	-	++
DA-413	95-016c	++	+
	95-016d	+/-	++
DA-443	95-017c	+	++
	95-017d	+	++
DA-470	94-430c	-	++
	94-430d	++	++
DA-525	94-427c	++	+
	94-427d	++	++
DA-526	94-428c	+/-	+++
	94-428d	-	+
DA-379	94-131c	++	++
	94-131d	+++	++
DA-314	94-495c	+++	++
	94-495d	+	+++
DA-322	94-498c	+++	++
	94-498d	++	++
DA-304	94-500c	+	++
	94-500d	+/-	++
DA-349	94-255c	+++	++
	94-255d	+++	++

Double staining with TUNEL (black) and JE antigen (red) in JE infected monkey brain (IHC)

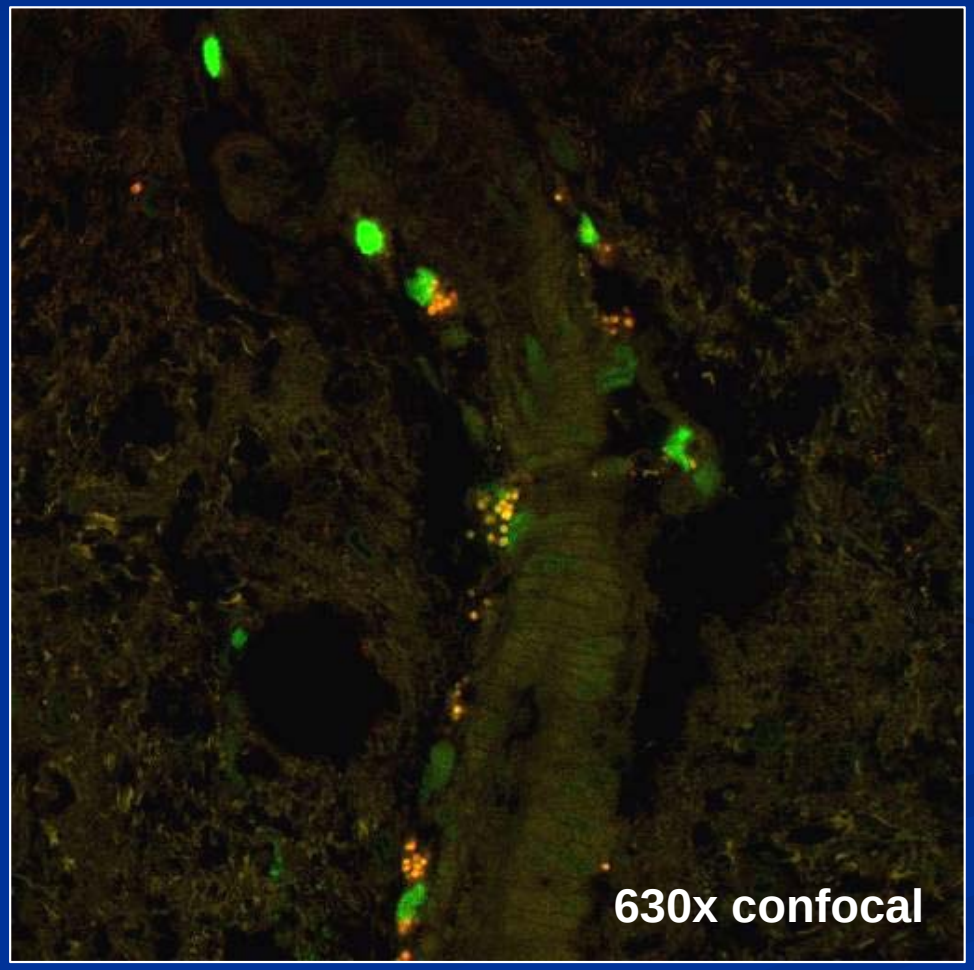


Double staining with TUNEL (green) and for JE antigen (red) in JE infected monkey brain (FA)

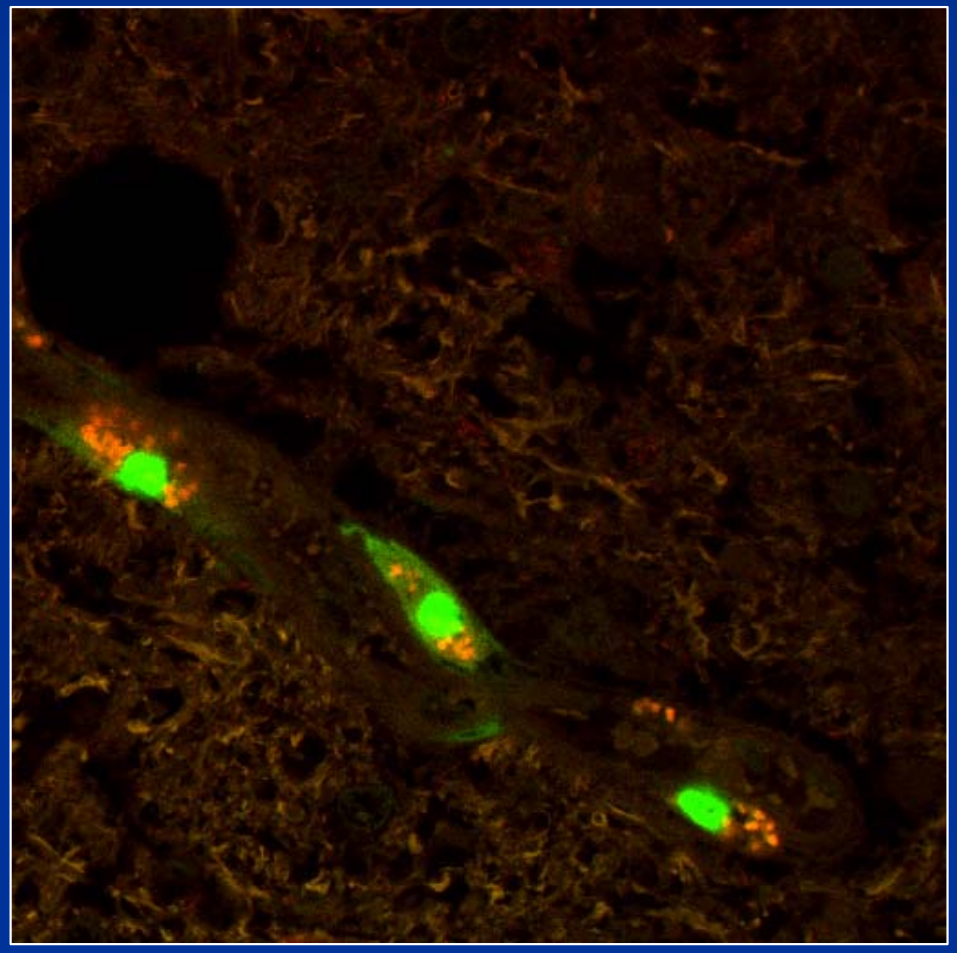


Double staining with TUNEL (green) and for JE antigen (red) in JE infected monkey brain (perivascular cell)

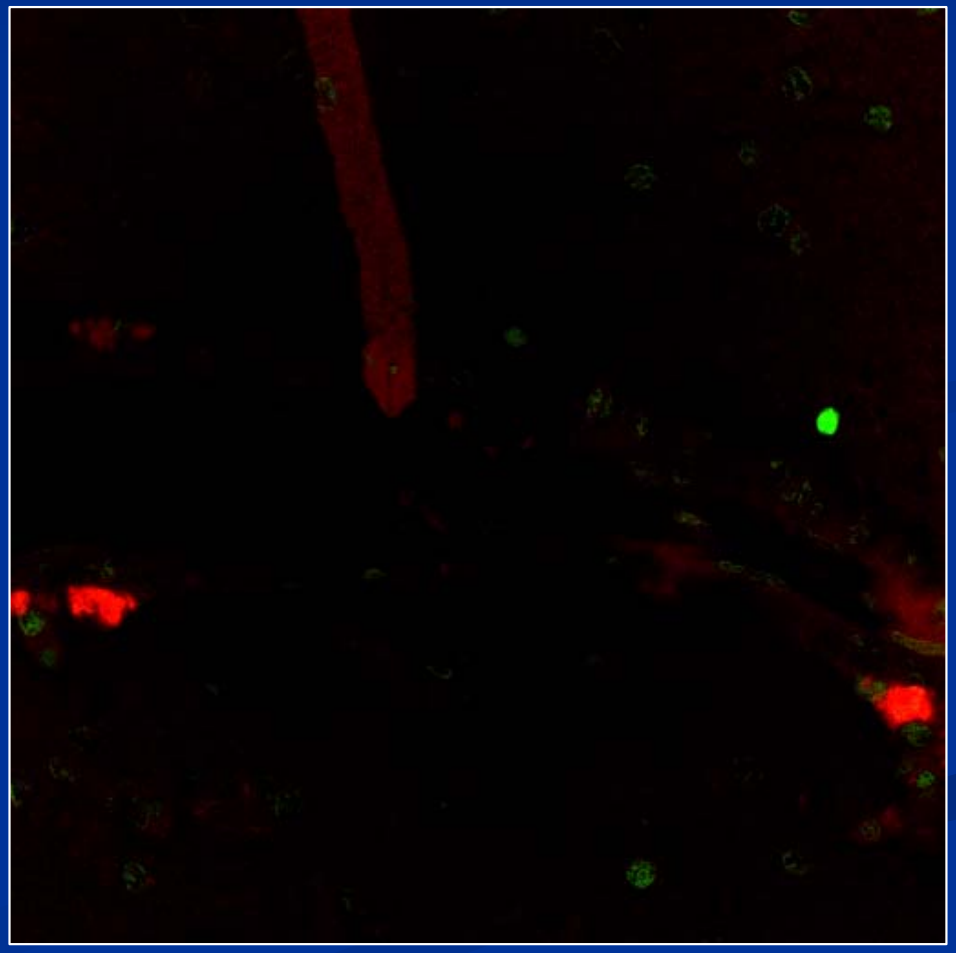
Some vessels exhibited apoptotic, JEV antigen positive perivascular cells



Double staining with TUNEL (green) and JE antigen (red) in JE infected monkey brain (perivascular cell)



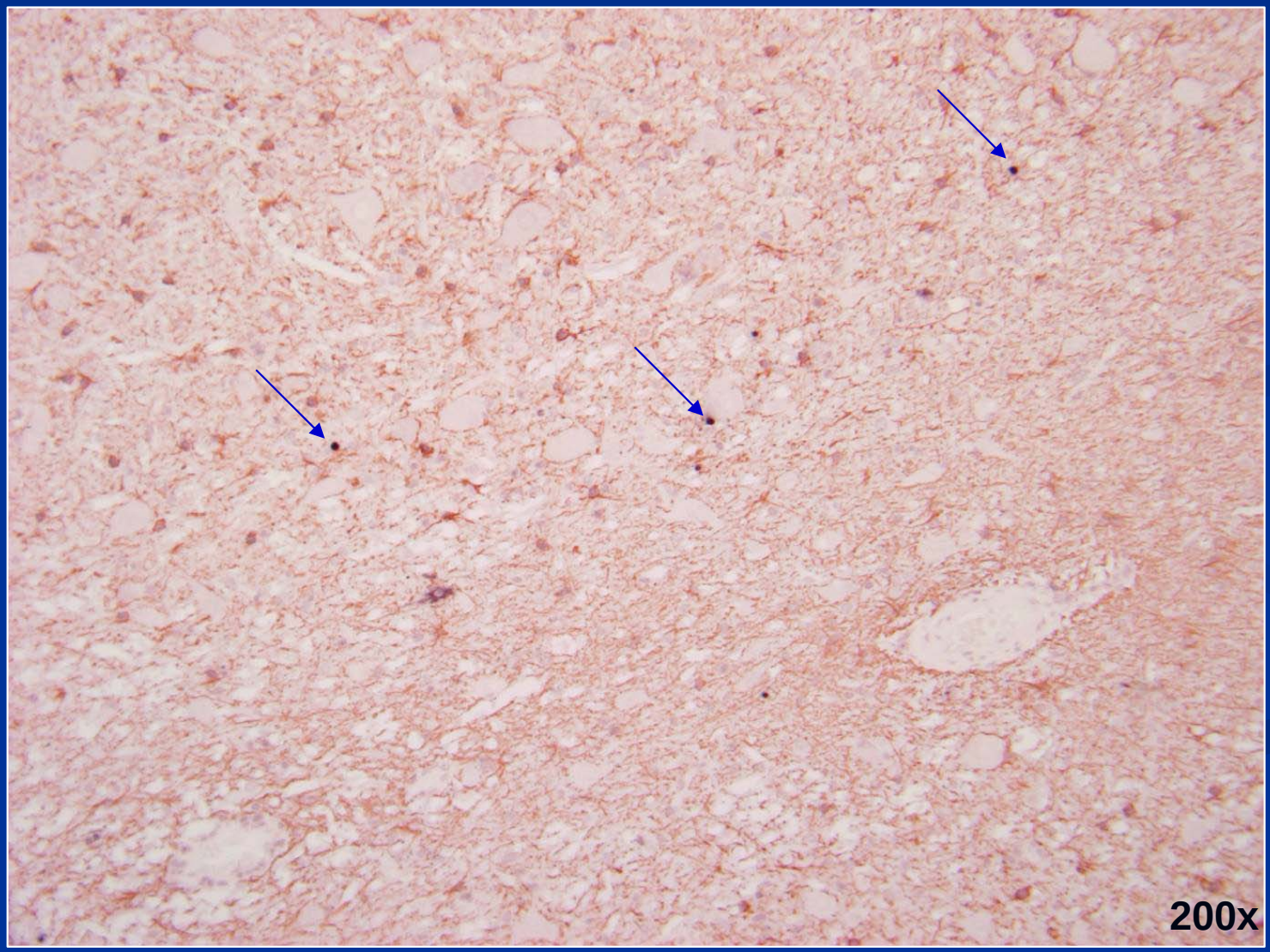
630x avi confocal



630x avi confocal

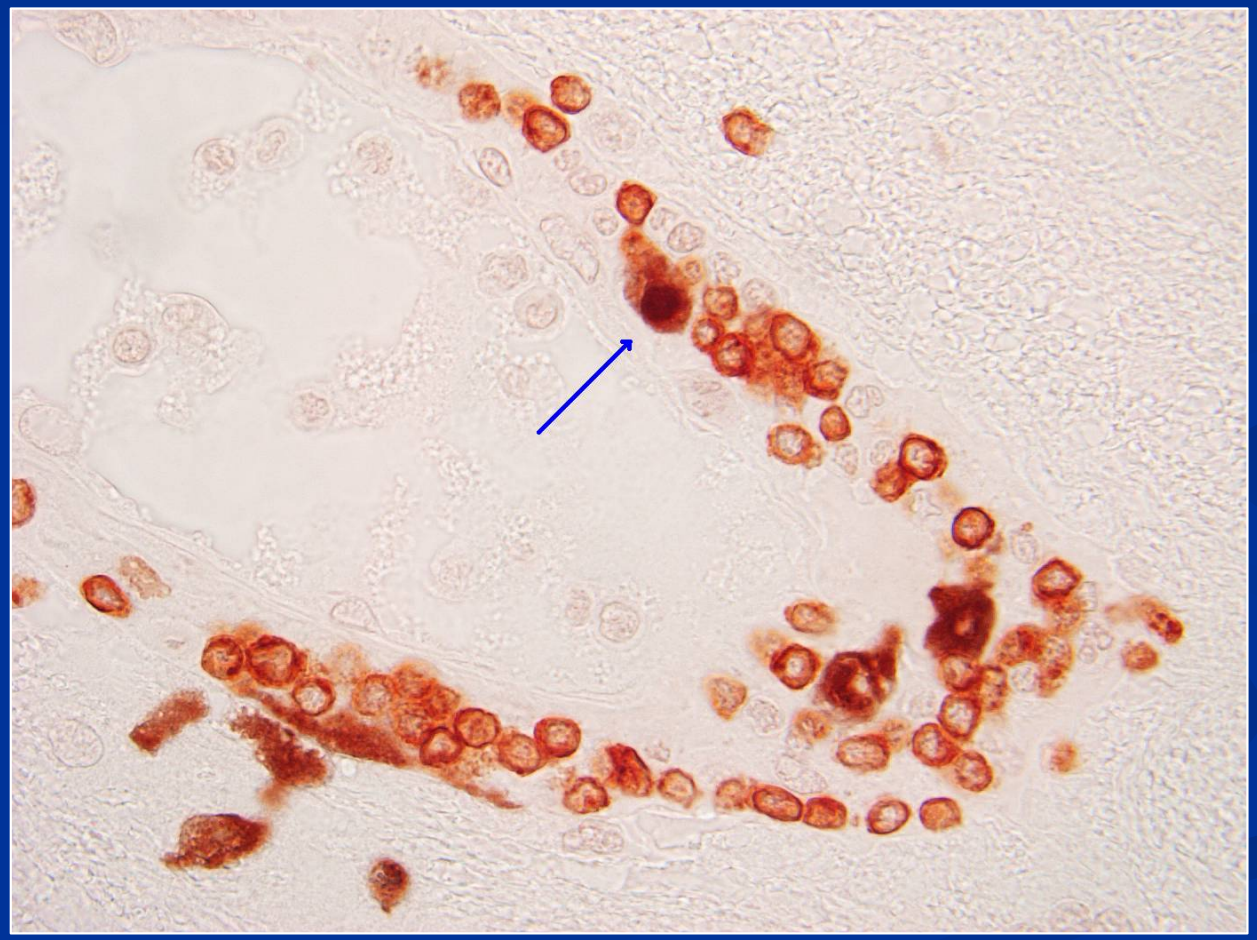
RESULT

Dual staining with TUNEL (black) and for GFAP (red) indicated that astrocytes were not apoptotic (IHC)



RESULT

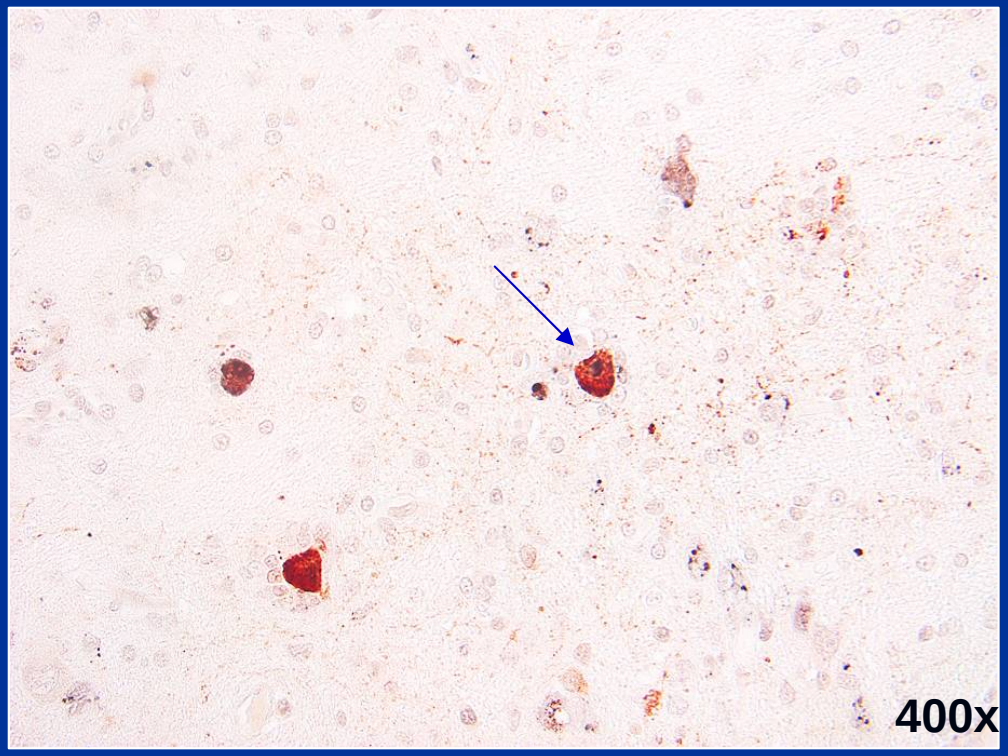
Dual staining with TUNEL and CD3 identified occasional apoptotic T cells (IHC)

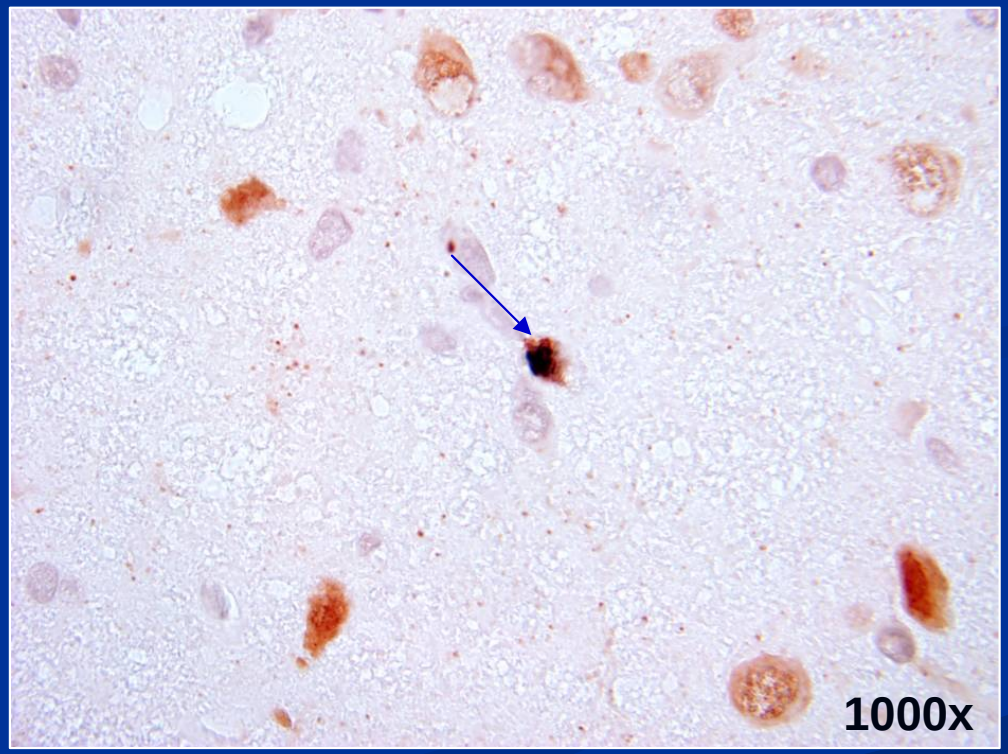


1000x

RESULT

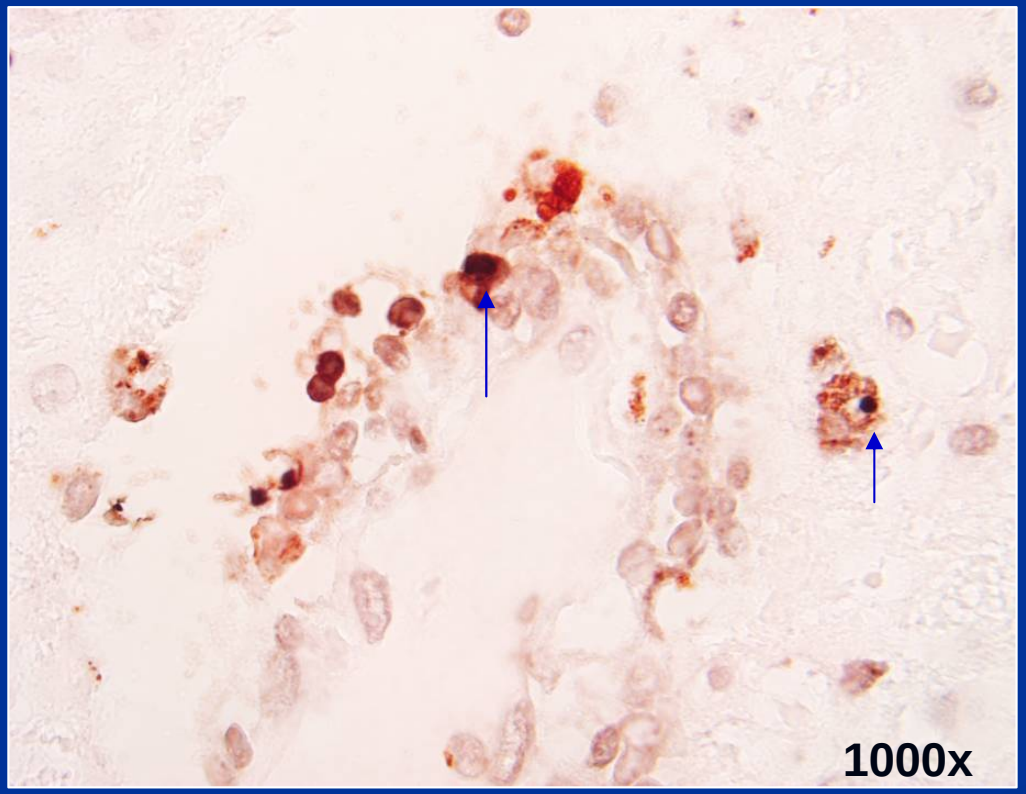
Dual staining with TUNEL and NeuN indicated that neuronal apoptosis was rare (IHC)



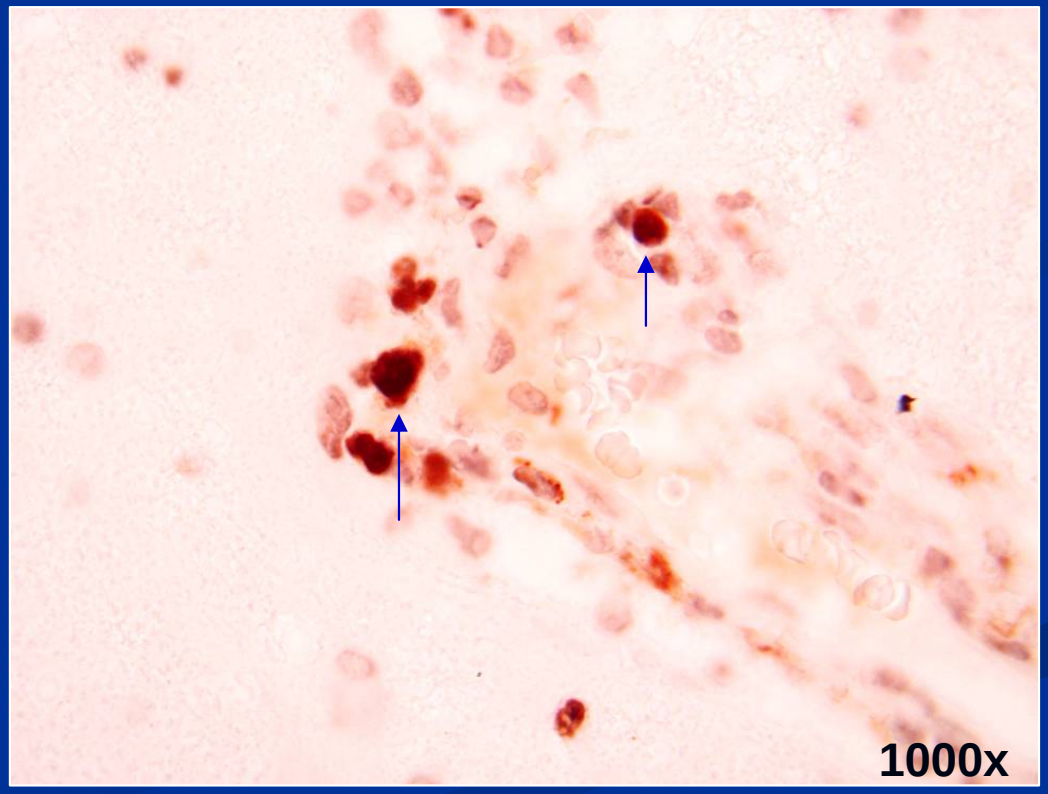


RESULT

Dual staining with TUNEL and CD68/MCA 1478 identified occasional apoptotic macrophages (IHC)



CD68



MCA1478

CONCLUSIONS

- In our preliminary study, a monkey model was used to provide insight into the mechanism of pathophysiology of JE.
- Selection of retrieval techniques, choosing primary antibodies and labeling systems are important for a successful outcome.
- JEV was detected mostly in the neuronal cell population in the macaques.

CONCLUSIONS

- Apoptosis was demonstrated mostly in uninfected cells suggesting that indirect mechanisms played a role in cellular injury (the bystander effect).
- Apoptosis was also detected in macrophages and some CD3 T cells.
- The involvement of cells located in perivascular areas is of particular interest.

CONCLUSIONS

- T cell mediated cell death could not be ruled out
- More work is needed to identify the cells undergoing apoptosis, examine the role of apoptotic-related proteins and cytokines in the regulation of apoptosis, and relate these findings to human disease.

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