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Feline Infectious Peritonitis (FIP) Virus

III. Studies on the multiplication of FIP virus in the suckling mouse

By

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With 3 figures and 3 tables

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Introduction

The successful propagation of feline infectious peritonitis (FIP) virus in the brains of one day-old laboratory mice and its demonstration by immunofluorescence and cat inoculation tests have been reported recently (6). In the present article the virus-experimental host relationship is studied in more detail.

Material and Methods

Virus

Mouse brain-adapted FIP virus (6) was used at the indicated passage levels. Seed virus was prepared by intracerebral inoculation of 5 to 20 µl. volumes of a 10 % (w/v) brain homogenate into one day-old random bred mice and harvesting after 3—5 days.

Sera

Sera from two experimentally induced FIP cases (Nrs. 17, 815), two natural FIP cases (Nrs. 342, 343) and a porcine anti-TGE immune serum (courtesy of Dr. M. Pensaert, Ghent), were employed in neutralization and immunofluorescence tests.

Infectivity titration

Serial tenfold dilutions of infected mouse brain material (10 % suspensions w/v) were inoculated intracerebrally into one day-old mice; after seven days the animals were sacrificed and their brains examined for viral antigen using the direct immunofluorescence test (IFT) on cryostat sections (6) or smear preparations (7). Dilutions were made in sterile, ice cold phosphate buffered saline, pH 7.2, containing 2 % of a normal suckling mouse brain homogenate.

Growth curve experiments

Litters of one day-old mice were pooled (about 50 animals) and injected intracerebrally with 5 µl./animal of seed virus. At one day intervals, three animals were sacrificed, their brain weight was determined, smear preparations were made for immunofluorescence tests and samples stored at — 70 °C for infectivity assay in the brains of one day-old mice.

Virus neutralization test

The virus neutralizing activity in feline and porcine sera was assayed using constant concentrations of FIP virus (15—100 mouse ID_{50} units) and serial fourfold dilutions of heat inactivated (30 min at 56 °C) serum. One-day-old mice were inoculated by intracerebral injection after the virus-serum mixtures had been incubated for 60 min at 37 °C. The infectivity of the challenge virus preparation was assayed after serial dilutions had been preincubated under neutralization test conditions. Seven days after infection the animals were sacrificed and their brains examined for viral antigen using the direct IFT.

Electron microscopy

Small pieces of FIP virus-infected suckling mouse brain (14th passage), 3 days post-infection, were fixed overnight in 4 % glutaraldehyde in Millonig's buffer (4), washed twice in the same buffer for 20 min and again fixed with 2 % buffered osmium tetroxide for 1 hr. The tissue was subsequently dehydrated by increasing concentrations of acetone and embedded in Araldite (1). Thin sections were cut on a LKB ultramicrotome III and stained for 45 min with 2 % aqueous uranyl acetate, followed by 1 min in Reynolds lead citrate. Grids were examined in a JEOL JEM-100 C electron microscope at 80 kV.

Results

Influence of mouse age and route of infection

In our initial experiments (6) one-day-old suckling mice were successfully used for the demonstration of virus multiplication by IFT. Subsequently ex-

Table 1

Age susceptibility of baby mice for FIP virus (6th mouse brain passage), after injection of about 100 MID_{50} units via the intracerebral route into litters of 8 to 12 animals

Animal age (days)	Results of IFT [a]
2	positive
4	positive
6	negative
8	positive
10	negative

[a] As evidenced by the brain smear technique (7)

periments using older animals were performed to investigate their age susceptibility. As can be seen from Table 1, mice can be infected regularly up to 4 days of age; in older animals fluorescence was seen with decreasing frequency and intensity.

Subsequently, the extent of FIP virus multiplication in intracerebrally inoculated one-day-old mice was studied by examining cryostat sections through several organs at 7 days p. i. Only cells of the central nervous system and the eye were found positive by IFT (Tab. 2).

Table 2

Organ immunofluorescence in one-day-old baby mice inoculated with FIP virus (11th mouse brain passage) via the intracerebral route

Organ \ animal no.	1	2	3	4	5	6	7	8	9	10
brain	+	+	+	+	+	+	±	+	+	+
spinal cord	+	+	+	+	+	ø	±	+	+	+
eye	ND[a]	ND	ND	+	+	ND	±	ND	+	+
heart	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø
lung	ø	ø	ND	ND	ND	ø	ø	ø	ø	ø
liver	ø	ø	ND	ø	ø	ø	ø	ø	ø	ø
spleen	ND	ND	ø	ø	ø	ø	ø	ø	ø	ø
kidney	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø
pankreas	ø	ø	ND	ø	ø	ø	ø	ø	ø	ø

[a] ND = not done, + = disctinct fluorescence, ± = few fluorescent cells, ø = no fluorescence

In addition, alternative routes of infection were studied. None of seven one-day-old mice inoculated by intranasal instillation of FIP virus material (10th mouse brain passage) showed evidence of infection when the organs listed in Table 2 were examined by the cryostat section — IFT. Intramuscular injection was also without success. One out of ten intraperitoneally inoculated baby mice showed fluorescent foci in the spinal cord.

When two adult mice were inoculated by intraperitoneal injection of 200 μl. of FIP mouse brain material (passage 11) and their organs screened seven days later, no indications for a generalized infection were obtained. In one of the animals, isolated fluorescent cells were encountered in the mesometrium seven days post infection.

Influence of time after infection on immunofluorescence

With the aim of studying the time course of appearance and disappearance of fluorescent antigen in brain cells, one-day-old mice were infected by intracerebral injection of FIP virus material (10th suckling mouse brain passage) and examined at daily intervals. The number of fluorescent cells in freshly prepared smears was scored as arbitrary units. In three experiments no antigen was found during the first three days, earliest distinct immunofluorescence being detectable at 72, 88 and 96 hours after infection, respectively. On the other hand, fluorescence was absent in mice sacrificed later than 12 days after infection (246, 248 and 288 hours p. i.). The number of fluorescent cells reached a maximum about 7 d. p. i. as shown in Fig. 1.

A mouse which had been inoculated intracerebrally at 1 day of age, was sacrificed 43 days p. i. and organ cryostat sections and smear preparations examined in parallel for fluorescent cells. No evidence for viral antigen was

found in the brain, spinal cord, retina, spleen, liver, kidney, heart, lungs, pancreas, plexus axillaris and testicles.

Single antigen-containing cells could have escaped detection by this procedure; therefore two successive brain passages were made in litters of one-day-old mice, starting from brain material of animals which had been infected 35 and 74 days previously (at 1 day of age). Baby mice harvested and examined 7 d. p. i. were consistently negative in the direct IFT.

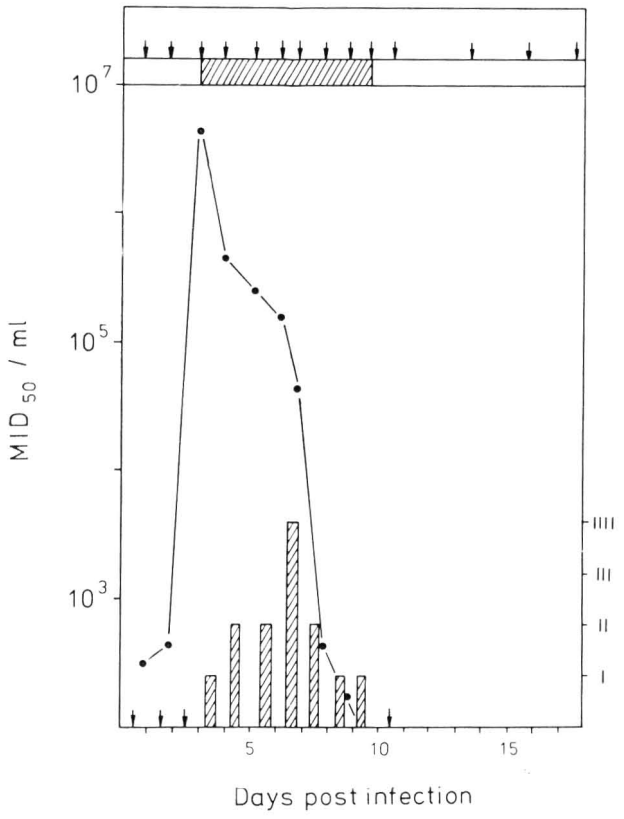


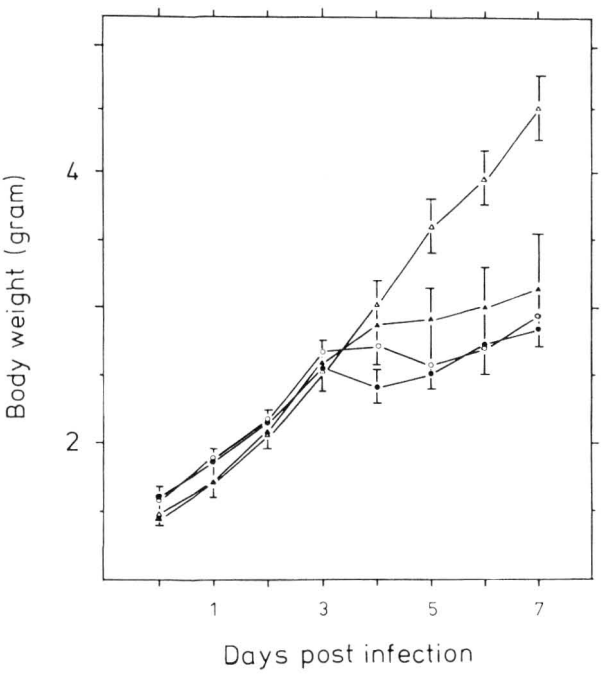
Fig. 1. Infectivity titers and immunofluorescence of FIP virus in mice infected when one day old. Note rapid decrease of infectivity (points) after day 3 post-infection. At the days indicated by arrows, immunofluorescence tests were performed on brain smears of infected mice in two independent experiments. The presence of viral antigen is indicated by the hatched areas, its quantitiy was estimated and is given in arbitrary units (right ordinate).

Growth curve of FIP virus in suckling mouse brain

Multiplication of FIP virus in one-day-old mice led to a more than 10^4 fold increase of infectivity between the 2nd and 3rd day after intracerebral inoculation (Fig. 1). A subsequent rapid decline from maximum titers exceeding 10^6 ID_{50} units/ml. to undetectable levels occurred between days 3 to 10. It can be seen in Fig. 1 that the maxima of infectivity and of immunofluorescence intensity do not coincide.

As already stated in a previous communication (6), inoculated mice did not present clinical symptoms of infection. Upon careful examination, however, a dose-dependent retardation in growth was noted in later experiments at higher passage levels (Fig. 2). It was demonstrated by immunofluorescence also that mice showing normal weight gain (10^1 in Fig. 2) contained viral antigen. Significantly, the bend in the weight curves of litters inoculated with high virus doses (10^3 and 10^4) on day 3 p. i. coincides with the infectivity peak of the viral growth curve (Fig. 1).

By electron microscopic examination of ultrathin sections through brain tissue collected 3 d. p. i. from mice that had been inoculated when one day



old, virus particles as presented in Fig. 3 were detected. They show a size, morphology and substructure compatible with coronavirions.

Fig. 2. Weight gain of suckling mice after intracerebral infection with about 10^4 (solid circles), 10^3 (open circles), 10^2 (solid triangles) and 10^1 (open triangles) intracerebral mouse ID_{50} units. Weight mean values and standard deviations have been calculated from 4 mice/inoculation dose. Viral antigen was demonstrated in all inoculated animals 7 d. p. i.

Neutralization of FIP virus infectivity

Using mouse brain adapted FIP virus, neutralizing antibodies could be demonstrated in the sera of naturally and experimentally infected cats; the sera did not neutralize TGE virus (Table 3). No antibody was found in sera from a SPF cat breeding colony (CPB TNO Zeist, The Netherlands). Neutralizing activity against FIP virus could not be demonstrated in a porcine anti-TGE virus hyperimmune serum.

Table 3

Comparative neutralization of IFT using mouse brain adapted FIP virus (11th passage) and TGE virus as antigen

	neutralization		indirect immunoflurescence, using
	of FIP virus	of TGE virus	TGE virus antigen
clinical FIP cases, field sera			
342	25[a]	< 2	320
343	≥ 400	< 2	2560
sera from experimentally infected cats			
17	10	< 4	640
815	10	ND	40
porcine anti TGE immune serum	< 10	256	ND

[a] Reciprocal of highest serum dilution resulting in complete neutralization of 15 mouse ID_{50} units

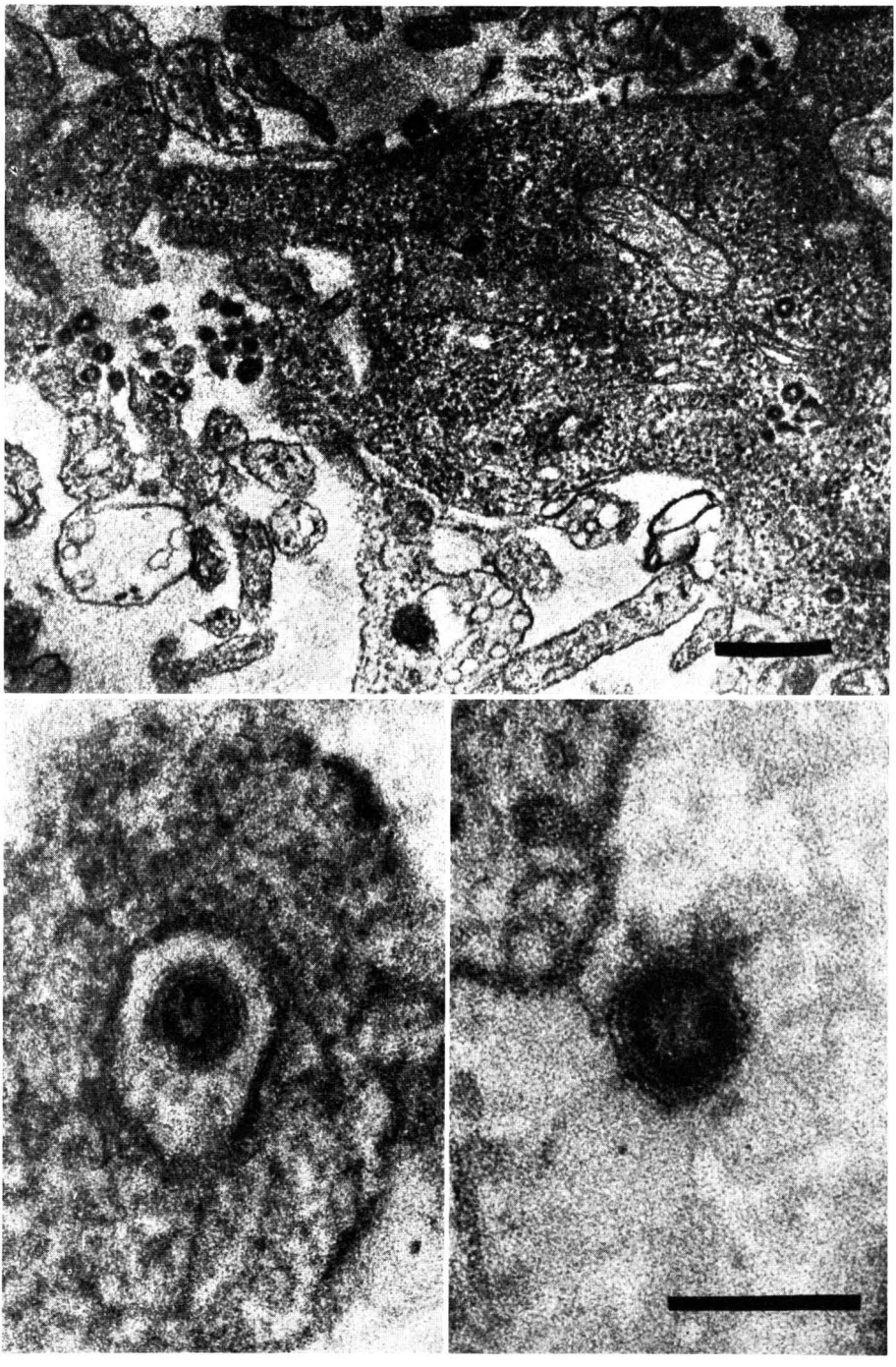


Fig. 3. Thin section electron micrographs of mouse brain cells infected with FIP virus. Note virus particles with electron-dense nucleoids in the cytoplasm and in vacuoles (above); their trilaminar unit membrane and doughnut-shaped internal structure is apparent at higher magnification (below). The bars correspond to 300 nm (above) and 100 nm (below), respectively

Discussion

From the general virologist's point of view there are some interesting features to FIP virus multiplication in the mouse: only the intracerebral route of infection is successful, infection is age-dependent and is abortive.

Although, incidentally, fluorescence has also been noticed elsewhere, cells of the central nervous system (brain, spinal cord) and the retina are the site of FIP virus replication in the mouse. It has been demonstrated that intracerebral inoculation is a highly traumatic procedure leading to the breakdown of anatomical barriers and subsequent hematogenous distribution of the inoculum throughout the body (5). Careful examination of different organs has virtually excluded their participation in virus replication which is supported by the negative results of infection by other routes. It is felt that age dependence and the abortive character of the infection are interrelated: the decrease in virus titers after day 3 p. i. (Fig. 1) is parallelled by an inability of brain tissue of older mice to support virus multiplication (Tab. 1). Immunological surveillance can be ruled out and interferon production is not considered an important factor in age-dependent resistance to viral infection (see e. g. 8). Conceivably the explanation should be sought at a cellular level. The degree of differentiation of the FIP virus-replicating cell and its physical contact with its equally permissive neighbour may determine the intra-tissue spread of infection. The central nervous system of the newborn mouse is non-myelinated and the observed decreasing susceptibility of older mice may be a reflection of "insulating" myelin formation. Although the fluorescent cells resemble neurons and glial elements, their identification in cryostat sections and smear preparations could not be made with certainty. If high enough doses are inoculated, temporary growth retardation may be observed as the only symptom of infection (Fig. 2). In suckling rats its virus specificity could be established by neutralization experiments (7).

It has been demonstrated that in the suckling mouse system infectivity of adapted virus is neutralized by feline sera from experimental and field cases of FIP. For the cat, however, these neutralizing antibodies apparently are without protective value; this observation is taken as support for the immune complex pathogenesis of FIP presented in two recent reviews (2, 3). The limited antigenic relatedness between the FIP and TGE viruses is further supported by the lack of TGE virus-neutralizing antibodies in the feline sera and of FIP virus-neutralizing antibodies in anti TGE virus hyperimmune sera (Tab. 3).

Note added in proof: As shown recently by D. J. REYNOLDS and D. J. GARWES (to be published), cats can be infected with TGE virus by the oral route. No clinical disease resulted but the virus could be isolated from the feces for up to 22 days after infection. The animals developed antibodies to TGE virus which were detected by neutralisation and indirect immunofluorescence tests. A serological response to FIP virus could only be detected after parenteral hyperimmunisation with TGE virus. The hyperimmune feline sera, however, did not neutralize FIP virus in our hands.

Summary

The highest susceptibility for infection with FIP virus (6th passage level) by the intracerebral route was observed in mice between 1 and 4 days of age, as judged from the intensity and extent of immunofluorescence in sections through the brains, spinal cord and retina; other organs were consistently negative. Inoculation by the intranasal and intramuscular routes did not

lead to virus multiplication in any organ tested; after intraperitoneal inoculation of neonate and adult mice, immunofluorescence was found only exceptionally. In litters infected intracerebrally at one day of age, distinct immunofluorescence was present between 4 and 10 days post-infection, with a maximum at 7 d. p. i. After $>$ 1 month p. i. FIP virus could no longer be detected, either in brain sections of the infected animal itself or after repeated blind passages in one-day-old mice. The growth curve of FIP virus in neonatal mice showed a maximum of infectivity at 3 d. p. i. (titers exceeding 10^6 mouse ID_{50} units/ml. of a 10 % w/v brain suspension) with a subsequent rapid decrease below the level of detection at 10 d. p. i. By electron microscopy, virus particles could be visualised in thin sections through brain material 3 d. p. i. In infected animals significant dose-dependent growth retardation was noted. Using mouse brain adapted virus, neutralizing antibodies were detected in the sera of field and experimental FIP cases; the same sera did not neutralize TGE virus. FIP virus was not neutralized by porcine anti-TGE antibodies.

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Zusammenfassung

Virus der felinen infektiösen Peritonitis (FIP)
III. Untersuchungen über die Vermehrung des FIP-Virus in der Säuglingsmaus

Zwischen dem ersten und vierten Lebenstag war bei Mäusen die größte Empfänglichkeit für eine intracerebrale Infektion mit dem FIP Virus (6. Passage) festzustellen, wie anhand der Stärke und des Ausmaßes des Immunofluoreszenz in Gehirn-, Rückenmarks- und Retinaschnitten beurteilt wurde; andere Organe waren stets negativ. Nach Inokulierung auf intranasalem und intramuskulärem Wege konnte eine Virusvermehrung in keinem Organ beobachtet werden; nur ausnahmsweise wurde eine Immunofluoreszenz nach intraperitonealer Infektion gefunden. In Würfen, die am ersten Lebenstage intracerebral inokuliert worden waren, ließ sich eine deutliche Immunofluoreszenz zwischen dem 4. und 10. Tage nach der Infektion wahrnehmen, mit einem Maximum am 7. Tage p. i. Nach einem Monat und später konnte das FIP Virus nicht mehr nachgewiesen werden, und zwar weder in Gehirnschnitten des infizierten Tieres selbst noch nach wiederholten Blindpassagen in eintägigen Mäusen. Die Vermehrungskurve des FIP Virus in neugeborenen Mäusen zeigte ein Infektiositätsmaximum am 3. Tage p. i. (mehr als 10^6 Maus-ID_{50}-Einheiten / ml einer 10 %igen Gehirnsuspension Gew./Vol.) mit einem anschließenden schnellen Abfall unter die Nachweisgrenze am 10. Tage p. i. Elektronenmikroskopisch konnten Virusteilchen in Gehirn-Ultradünnschnitten am 3. Tage p. i. gezeigt werden. Bei den infizierten Tieren wurde eine signifikante dosisabhängige Wachstumshemmung beobachtet. Mit Hilfe des an Mäusegehirn adaptierten Virus wurden neutralisierende Antikörper in den Seren experimenteller und natürlicher FIP Fälle nachgewiesen; dieselben Seren neutralisierten das TGE Virus nicht. FIP Virus wurde andererseits nicht durch gegen TGE Virus gerichtete Antikörper vom Schwein neutralisiert.

Résumé

Le virus de la péritonite infectieuse du chat (FIP)
III. Etudes de la multiplication du virus FIP chez la souris nouveau-née

La plus grande susceptibilité pour l'infection avec le virus de la péritonite infectieuse féline (FIP) (sixième passage) par la route intracérébrale est montrée chez des souris entre leur premier et quatrième jour; cette susceptibilité est jugée par l'intensité et l'extension de l'immunofluorescence en coupes de cerveaux, moelle épinière et rétine; les autres organes étaient constamment négatifs. L'inoculation intranasale et intramusculaire n'a pas résulté en multiplication du virus dans les organes testés; l'inoculation intrapéritonéale de la souris néonate et adulte n'a résulté en immunofluorescence que dans des cas exceptionnels. Le groupe des animaux infectés par voie intracérébrale le jour de leur naissance montrait immunofluorescence distincte entre 4 et 10 jours après infection, avec un maximum au 7ième jour. Un mois après infection le virus FIP ne pouvait plus être détecté, ni dans les coupes des cerveaux de l'animal infecté même ni après des passages répétés dans des souris nouveau-nées. La courbe de croissance du virus FIP chez des souris nouveau-nées montrait un maximum d'infectivité au 3ième jour après l'infection (les titres dépassant 10^6 DI_{50}/ml d'une suspension de cerveaux de 10 % (p/v), suivi par une chute rapide sous le niveau détectable (10ième jour). Par microscopie électronique des particules pouvaient être montrées en section ultrafine de cerveau le 13ième jour après l'infection. Chez les animaux infectés on a trouvé une retardation de croissance significante et dépendant de la dose appliquée. Utilisant le virus adapté on a détecté des anticorps neutralisants dans les sérums des cas de FIP spontanes et experimentaux; les mêmes sérums ne neutralisaient pas le virus de la TGE. Le virus FIP de sa part n'était pas neutralisé par des anticorps anti-TGE.

Resumen

Virus de la peritonitis infecciosa felina (FIP)
III. Investigaciones sobre la multiplicación del virus FIP en ratones recién nacidos

Ratones de unos cuatro días de edad muestran alta susceptibilidad por infecciones experimentales por inoculación intracerebral, con el virus de la peritonitis infecciosa felina (nivel 6° de pasaje). Esto se demuestra por la intensidad y la extensión de la inmunofluorescencia en secciones del cerebro, de la medula espinal y de la retina, mientras que en otros órganos también examinados de la misma manera no se percibe fluorescencia. Mediante inoculaciones por la vía intranasal e intramuscular no se consigue establecer una multiplicación viral en ninguno de los órganos examinados. Tras inoculación de ratones recién nacidos, como también de adultos, por la ruta intraperitoneal, se encuentra solo excepcionalmente inmunofluorescencia. Ratones de un día de edad, infectados por inoculación intracerebral, muestran una marcada fluorescencia entre los días 4 a 10 p. i., alcanzando un máximo a los 7 días p. i. Tras transcurso de un mes p. i. el virus de la peritonitis infecciosa felina (FIP) no puede ser más detectado, tanto en secciones cerebrales de los animales previamente infectados, como mediante pasajes repetidos por vía intracerebral de suspensiones cerebrales de animales previamente infectados en ratones susceptibles, de un día de edad. La curva de multiplicación del virus FIP en ratones recién nacidos muestra un máximo de infectividad a los 3 días p. i. (con títulos de 10^6 ID_{50} por ratón y por ml. de una suspensión cerebral al

10 % peso/volúmen) con la subsiguiente pérdida de infectividad bajo el nivel de detección a los 10 días p. i. Mediante microscopía electrónica se visualizaron partículas virales en secciones del cerebro 3 días p. i. Los animales infectados denotan retraso de crecimiento que es dependiente de la dosis aplicada. Con virus de material cerebral, de ratón infectado, fué posible detectar anticuerpos de acción neutralizante en sueros de gatos infectados por vía natural y de gatos infectados experimentalmente. Los mismos sueros no neutralizaron al virus de la gastroenteritis transmisible porcina (TGE). Y viceversa el virus FIP no pudo tampoco ser neutralizado con anticuerpos porcinos anti-TGE.

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