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A study of the correlation between the pathogenicity of viruses of the tick-borne encephalitis group for animals and peculiarities of their multiplication in the organism

Second Report

Use of the fluorescent antibody method to detect an uninfective viral antigen in the organs of animals infected with the Malayan Langat virus (TR-21)

by

V. V. Pogodina and Kahn' Shi-tsze

The Malayan Langat virus (strain TR-21) is known as the virus of the tick-borne encephalitis group that is least pathogenic for man and for various experimental animals under natural conditions. In this connection, it is regarded as a candidate for use as a vaccine strain in the development of a live vaccine (4, 5) and has already been approved for use in human beings (3). The weak cerebral pathogenicity of strain TR-21 is associated with low virulence when it is introduced peripherally and weak viremia in sensitive animals and human beings (3, 8). The other aspects of the pathogenesis of infestion with the Malayan Langat virus have been studied to a very inadequate extent. The aim of the present investigation was to study the characteristics of the multiplication of strain TR-21 in sensitive animals infected subcutaneously.

Material and method

Virus. We used a variant of strain TR-21 adapted to mouse brain. The titers of the strain when inoculated into mouse brain were $10^{6.1\pm0.23}$, in hamsters, $10^{1}LD_{50}/0.03$ ml, when inoculated subcutaneously into mice, $10^{2.2\pm0.7}$, in hamsters, $10^{1}LD_{50}/0.25$ ml.

Animal inoculation. The experiments were performed on mice weighing 7-8 g from the Moscow breeding station and Syrian hamsters weighing 40-60 g. In some few experiments we used piglets 3-5 weeks old.

The mice and hamsters received the virus-containing material subcutaneously in the region of the nape of the neck in a dose of 0.25 ml.; the piglets received 1-3 ml under the skin of the thigh.

The infectivity of the virus was determined by means of cerebral titration in mice (inoculation with 0.03 ml) according to the development of experimental encephalitis in them. Each dilution was administered to 4 mice.

In order to study the characteristics of the multiplication of strain TR-21, we used the immunofluorescence method (the Coons [tr.: sp.?] direct method) as described in the first report.

Results

In mice inoculated subcutaneously with a dilution of 10^{-3}, equivalent to 1000 cerebral LD_{50}, the viral antigen was detected during the first 48 hours in the subcutaneous tissue at the site of injection and in many visceral organs, and it also appeared in various parts of the central nervous system within 3 days after inoculation. As we see from Table 1, the specific fluorescence appeared in the extraneural tissues in a definite sequence: it was first seen in the subcutaneous tissue at the site of inoculation, in the spleen, and in the regional lymph nodes (first day), later (2nd-3rd day) in the collateral lymph nodes, liver, and intestine.

A weak specific fluorescence was discovered in the subcutaneous connective tissue 6 hours after inoculation. The glow was localized largely in the intercellular space and appeared only in single cells. By the end of 24 hours, a distinct cellular localization of the viral antigen was noted, and a considerable number of cells glowed (up to 4-5 per visual field). The intensity of the fluorescence gradually increased, and it assumed the character of a distinct yellow-green glow, diffusely disseminated in the cytoplasm of the cell. The background remained dark and did not fluoresce (Fig. 1).

In the lymph nodes and spleen, the viral antigen was localized in a large number of cells, chiefly of the medullary layer. In the intestines glowing cells were seen in the stroma of the villi and fluoresence was sometimes seen in muscle fibers of the submucosal layer. Specific fluorescence was observed less commonly in the liver than in other internal organs.

A localization of the viral antigen in many cells of the extraneural tissues was also noted in the Syrian hamsters inoculated subcutaneously either with a 10^{-1} virus dilution, with which a few of the animals fell ill, or with a 10^{-3} dilution, with which none of the animals became ill. The fluorescence spread in the sequence described above and was detected as regularly as in the mouse experiments in the subcutaneous tissue at the site of the injection and in all the internal organs that were examined: lymph nodes, spleen, intestine, and liver, beginning from the 1st and up to the 5th day after inoculation (Fig. 2).

In 2 piglets inoculated subcutaneously with 5 ml of a 10% suspension of strain TR-21, the viral antigen was also discovered in various visceral organs: in the regional and mesenteric lymph nodes, liver, and intestine (Fig. 3).

The regular discovery of viral antigen by the fluorescent antibody method in the extraneural tissues of various animals appeared somewhat inconsistent with the low infective titers of the virus in the same organs. The rather distinct glow gave the impression of a considerable concentration of viral antigen in the organs, but titration of the virus in fragments of organs from these same animals did not reveal any infectious virus at all or gave only low titers: 10^{1}-10^{2} LD_{50}/0.03 ml (Table 2).

The same kind of discrepancy between the results of demonstration of the virus by the fluorescent antibody method and by its infectivity was observed in studies of various segments of the central nervous system in mice, hamsters, and piglets inoculated subcutaneously. Specific fluorescence was noted regularly, despite the fact that the doses used were nonpathogenic or only slightly pathogenic for these animals and the infectious titers in the brain tissue were extremely low: 10^{1}-$10^{1.5}$ LD_{50}/0.03 ml or absent entirely. Table 3 juxtaposes the findings as to detection of the virus by immunofluorescence and infectivity in the central nervous system in mice, hamsters, and piglets.

The unexpectedness of the results thus obtained led us to study the activity of multiplication of the TR-21 strain in the central nervous system in animals as compared with the highly virulent Khab-17 strain of the virus of Eastern tick-borne encephalitis, which has a high peripheral activity (9). A count was made of the number of glowing nerve cells in horizontal sections of mouse brains on the E-F level according to Stel'masiak's atlas (10). All experimental conditions were identical for the two strains. The mice were inoculated subcutaneously with doses equivalent to 1000 cerebral LD_{50}.

With this dose, the Khab-17 strain caused the death of 69% of the infected mice (20 out of 29), while the TR-21 strain killed only 22% (6 out of 27) or less. A comparison was made of the infective titers in the brain and the mean number of glowing nerve cells in one field of vision 3, 4, 5, and 7 days after the inoculation. The number of glowing neurons was counted without taking into consideration the cells of the microglia. In order to standardize the area of the cell count, we used a frame that was inserted into the ocular of the lateral sight of the luminescence microscope. We used an ocular with a 5 fold magnification and a frame designated for 3X magnification. The absolute dimensions of the counting area were determined with the aid of an objective micrometer with 0.01 mm divisions. With a 90X objective, this area was equal to 0.0117 sq mm. The count of the glowing nerve cells was made in 20 (sometimes 10) neighboring areas of the section demarcated by the frame described above, after which we counted the mean number of infected cells in one field of vision (in one frame).

We see from Fig. 4 that strain TR-21 infected a large number of cells, from 8 to 17-18 in a field of vision with an area of 0.01 sq mm. The same or only a slightly larger number of cells glowed in the material infected with the virulent Khab-17 strain (16-23/0.01 sq mm). At the same time, the difference in the infective titers was very great: the titer of strain Khab-17 reached $10^{4.5}$ LD_{50}/0.03 ml or more, while the titer of the TR-21 strain even at the time of the most intense fluorescence (5 days) did not exceed 10^{2} LD_{50}/0.03 ml. It should be noted that fluorescence was always absent from the control preparations.

A similar experiment performed hamsters gave similar results. The brain tissue contained almost no mouse-infecting virus (titer $<10^{1}$ LD_{50}/0.03 ml), while the number of glowing cells was substantial: 9.1± 1.4/0.01 sq mm after 2 days, 12±1.6/0.01 sq mm after 3 days, and 12.8±1.6/0.01 sq mm after 5 days.

A large number of glowing nerve cells were discovered in sections of the brain and spinal cord of piglets, with an extremely low infective virus level in the brain tissue (Fig. 5).

A morphologic study of fragments of brain and spinal cord from hamsters and piglets, performed by A. P. Savinov, did not reveal any substantial histopathologic changes.

Discussion

The pathogenesis of the infection produced by subcutaneous injection of the Malayan TR-21 strain, weakly pathogenic when inoculated intracerebrally and especially peripherally, was studied in experiments

performed on mice, hamsters and piglets.

One of the characteristics of the multiplication of this strain is its pronounced viscerotropicity. The viral antigen was regularly demonstrated by the fluorescent antibody method in a large number of cells in the subcutaneous tissue at the site of inoculation, in lymph nodes, and in various internal organs (spleen, liver, intestine). Another characteristic is its neurotropicity. Regularly penetrating into the various segments of the nervous system in the experimental animals even when small doses are used, the TR-21 strain infected almost the same number of nerve cells as did the highly virulent Khab-17 strain, that is, up to 17-18 cells/0.01 sq mm.

A third peculiarity of the multiplication of the TR-21 strain was the deviation of the indices of infection of cells (on the basis of the immunofluorescence data) and the levels of the infective virus contained in them, which were determined by cerebral titers were low, from untitratable values to 10^2 LD_{50}/0.03 ml.

These studies revealed a discrepancy among the results of the investigations of the infected animals by various methods: clinical (development of experimental encephalitis), virological (discovery of uninfectious virus in organs), and immunochemical. In those cases in which the virus was undetectable or almost undetectable by the first two methods, the fluorescent antibody method regularly revealed the presence of the viral antigen in the various tissues.

S. Ia. Gaydamovich, A. I. L'vova, and N. G. Titova studied the model of the tick-borne encephalitis virus and showed that there is a certain regularity in the dynamics of the appearance of the infective virus and various antigens in the tissue culture. The infective virus appeared at the start, the fluorescence in the cells always appeared later, and only then was it possible to detect the complement-fixing and hemagglutinating antigen in the cell extract and culture liquid (2, 7).

In our experiments with strain TR-21, we observed an inverse between the appearance of infectivity and of luminescence, so that it would appear that with the immunofluorescence method we have detected in the organs of the animals a nonspecific viral antigen, just as V. M. Zhdanov et al. (11) and A. G. Bukrinskaia (1) detected an incomplete Sendai virus in tissue culture by the use of the luminescence microscopy method.

There could be no doubt of the specificity of the glow discovered by us, in view of the agreement of the results of repeated

experiments, the absence of fluorescence in the control preparations, and the definite dynamics of the appearance of the glow. There is also some indirect evidence in support of a correct appraisal of the results obtained by us:

(a) an additional check applied in the course of the study: the absence of fluorescence in animals inoculated subcutaneously with the Fateev strain under the same experimental conditions (see First report);

(b) the satisfactory immunogenic properties of strain TR-21 in cases of subclinical infection and the absence or very limited character of viremia in the inoculated animals. Thus, strain TR-21, which was found to be nonpathogenic for sheep when administered intracerebrally and subcutaneously, gave rise to the formation of humoral immunity and resistance of the brain tissues in these animals to subsequent intracerebral inoculation with lethal dose of the Sof'in strain (5). Similar results were obtained with inoculation of goats with the TR-21 strain (6).

Our further studies will entail attemps to detect the non-infective viral antigen not only by immunofluorescence but also by other serologic methods.

A comparison of the findings obtained here with the results of a study of the peculiarities of the multiplication of the Fateev strain, as described in the First report, indicates that the weak virulence characteristic of the TR-21 and Fateev strains when they are introduced subcutaneously may be attributable to different mechanisms. In one case (Fateev strain), it is caused by the low viscerotropicity of the strain, in the other (TR-21 strain) in spite of the low virulence with peripheral inoculation, there is infection of the cells of many of the internal organs and of the central nervous system, although the viral antigen that was formed was not infective and could not be discovered by the clinical method.

Conclusions

1. The use of the fluorescent antibody method made it possible to establish the pronounced viscerotropicity and neurotropicity of the Malayan Langat virus (strain TR-21), despite its weak pathogenicity in intracerebral and particularly peripheral inoculation of various species of susceptible animals.

2. One peculiarity of the pathogenesis of the infection produced by the TR-21 strain is the disparity of the values for infected cells (according to the immunofluorescence data) and their infective virus content. The viral antigen was detected in many cells

in the extraneural tissues and central nervous system where the infective virus was entirely absent or present in only very small amounts.

English Summary

By the fluorescent antibody method a marked viscerotropy and neurotropy of the Malaya Langat virus (TR-21 strain) could be established despite a weak pathogenicity of the virus for various animals inoculated intracerebrally of peripherally. The peculiarity of the pathogenesis of infection induced by the TR-21 strain consisted in the discrepancy between infection signs in cells (according to immunofluorescence) and the content of virus infective for mice therein. In many experiments carried out with mice, hamsters and pigs, the virus antigen, though of very low infectious titres, was demonstrable in many nervous cells. It is suggested that a low virulence of some viruses of this group, with peripheral inoculation, may be due to a weak viscerotropy of a strain or to the formation of noninfectious virus.

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Table I

Dynamics of specific fluorescence in organs of mice inoculated subcutaneously with strain TR-21 (10^{-3}).

Орган	Срок обследования (в днях)						
	6 часов	1	2	3	4	5	+
Подкожная клетчатка в месте инъекции	3/3 (++.+++)	3/3(++)	3/3(++)	3/3 (++. +++)	3/3 (+.++)	3/3(++)	—
Селезенка	0/3	1/3(+)	2/3(+)	3/3(+)	3/3 (+.++)	3/3 (+.++)	+
Лимфатический узел: шейный	0/3	2/3(+)	2/3(+.++)	3/3(++)	3/3(++)	3/3(++)	—
подмышечный	0/3	3/3(+)	3/3(++)	3,3(++)	3/3(++)	3/3(+.++)	+
паховой	0/3	0/3	1/3(+)	3/3(+. ++)	3/3(++)	3/3(++)	—
Печень	0/3	0/3	1/3(+)	3/3(+)	2/3(+)	3/3(++)	+
Тонкая кишка	0/3	0/3	0/3	1/3(+)	3/3(+.++)	3/3(+)	—
Толстая »	0/3	0/3	0/3	1/3(+)	3/3(+)	3/3(++)	—
Головной мозг	0/3	0/3	0/3	2/3(+. ++)	3/3(+.++)	3/3(++.+++)	++
Спинной мозг: шейный отдел	0/3	0/3	0/3	0/3	2/3(+)	3/3(+)	+
грудной »	0/3	0/3	0/3	2/3(+)	3/3(++)	3/3(+)	+
поясничный »	0/3	0/3	0/3	2/3(+)	2/3(+)	3/3(++)	—

Обозначения: знаменатель — число обследованных органов; числитель — число органов с положительной флуоресценцией; плюсами обозначена интенсивность свечения.

Legend for Figure 1

1 - organ
2 - period of observation (in days)
3 - 6 hours
4 - Subcutaneous tissue at site of injection
5 - spleen
6 - lymph node:
    cervical
    axillary
    inguinal
7 - liver
    small intestine
    large intestine
    cerebrum
8 - spinal cord:
    cervical
    thoracic
    lumbar
9 - Designations: denominator = number of organs studied; numerator = number of organs with positive fluorescence; number of plusses indicates intensity of glow.

Table 2

Level of infective virus (in lg LD_{50}/0.03 ml) in organs of mice inoculated subcutaneously with strain TR-21 (10^{-3}).

Орган (1)	Срок обследования (в днях) (2)					
	1	2	3	4	5	7
Подкожная клетчатка в месте инъекции (3)	<1	<1	1.5	2.0	1.25	1.6
Кровь (3)	0	Не исследовали (6)	1.0	Не исследовали (6)	1.0	0
Селезенка (4)	<1	1.37	<1	<1	1.75	3.0
Лимфатический узел: (5)						
шейный	<1	1.25	1.0	<1	1.5	1.36
подмышечный	1.0	1.45	1.0	2.0	1.0	<1
паховой	<1	<1	1.33	<1	1.0	1.0
Печень (6)	0	<1	1.0	<1	<1	1.0
Тонкая кишка (7)	0	<1	<1	<1	1.32	2.0
Головной мозг (8)	0	<1	<1	<1	<1	<1
Спинной » (9)	0	1.5	1.0	<1	<1	<1

Legend: 1 - organ
2 - period of observation (in days)
3 - subcutaneous tissue at site of injection
3 - blood
4 - spleen
5 - lymph node:
    cervical
    axillary
    inguinal
6 - liver
7 - small intestine
8 - brain
9 - spinal cord

Table 3

Frequency of discovery of viral antigen and infective virus in the central nervous system in mice, hamsters, and piglets inoculated subcutaneously with sublethal doses of strain TR-21.

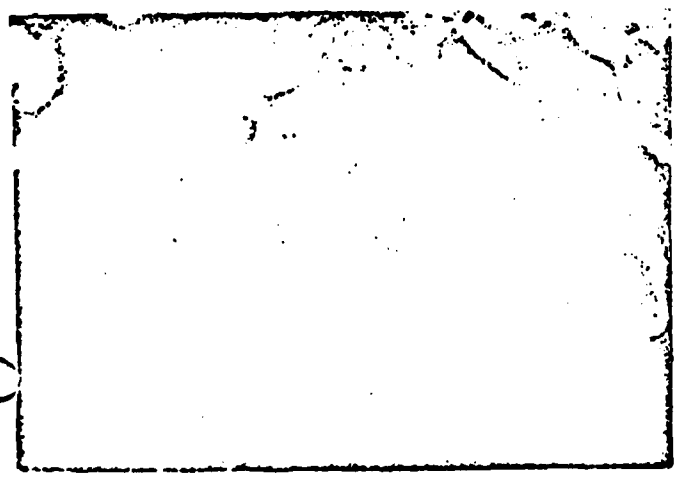
Отдел центральной нервной системы	Мыши (10^{-3})		Хомяки (10^{-3})		Поросята (10^{-1})	
	фл.	титр	фл.	титр	фл.	титр
Головной мозг	9/10	10^1—10^2	5/6	$<10^1$	3/3	0—$<10^1$
Кора	+	.	+	.	+	0
Подкорка	+	.	+	.	+	10^1
Аммонов рог	+	.	+	.	+	0
Мозжечок	+	.	+	.	—	0
Спинной мозг	9/10	10^1 — $10^{1,5}$	5/6	10^1	1/3	0—$<10^1$
Шейный отдел	+	.	+	.	+	0
Грудной »	+	.	+	.	+	$<10^1$
Поясничный »	+	.	+	.	+	0

16 Обозначения: фл. — флуоресценция; + наличие флуоресценции; — отсутствие флуоресценции; 0 — инфекционный вирус не определяется; . отдельно не исследовано; знаменатель — количество обследованных органов, числитель — число органов, содержащих вирусный антиген.

Legend:
1 - segment of central nervous system
2 - mice
3 - fl.
4 - titer
5 - hamsters
6 - piglets
7 - brain
8 - cortex
9 - subcortex
10 - horn of ammon
11 - cerebellum
12 - spinal cord
13 - cervical region
14 - thoracic region
15 - lumbar region

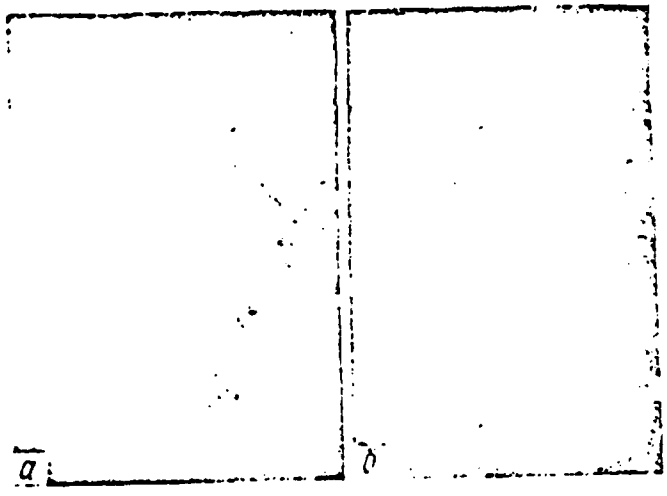
16: Designations: fl. = fluorescence; + = presence of fluorescence; - = absence of fluorescence; 0 = no infectious virus detected; . = not studied separately; denominator = number of organs studied; numerator = number of organs containing viral antigen.

Figure 1



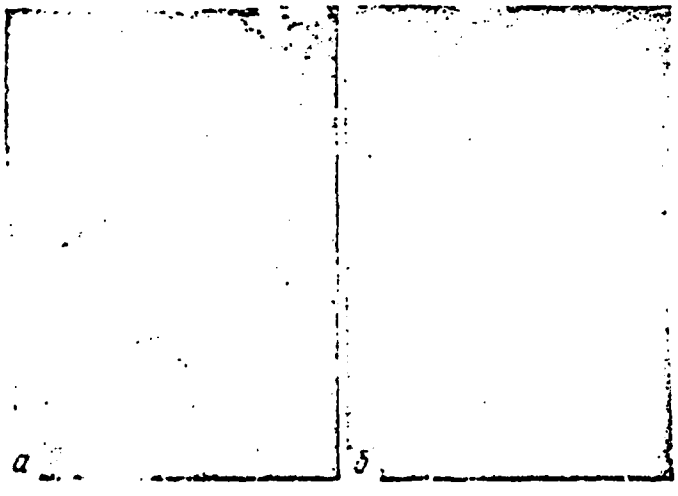
Distinct specific fluorescence in subcutaneous tissue of mouse at site of injection 3 days after inoculation with strain TR-21 (10^{-3}).

Figure 2



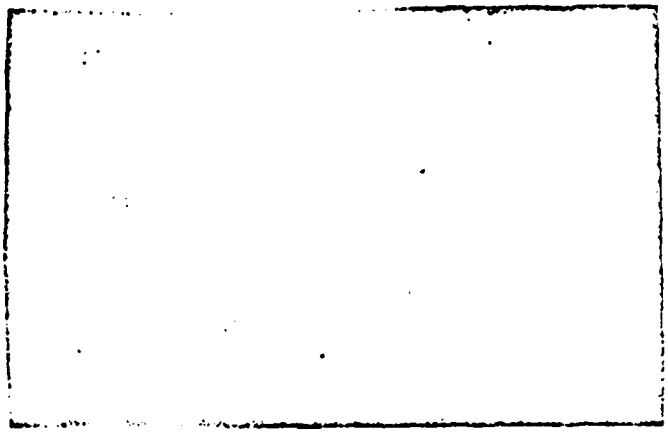
Glow of cytoplasm of cells in medullary layer of axillary lymph node of hamster 5 days after inoculation with strain TR-21 (10^{-1}). X270 (on the left); on the right, control: section of lymph node of uninfected hamster, insignificant autoluminescence.

Figure 3



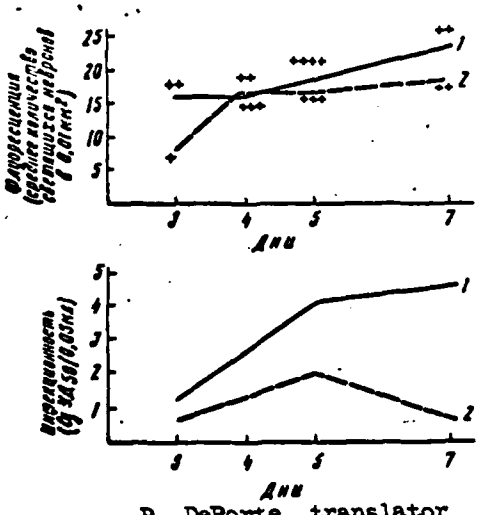
Fluorescence of cells in stroma of villus of small intestine of piglet 5 days after inoculation. X450 (on the left); on the right, control: absence of fluorescence.

Figure 4



Detection of viral antigen and infectious virus in the mouse brain after subcutaneous inoculation with the Khab-17 strain or the TR-21 strain (dose = 10^3 cerebral LD_{50})

Figure 5



Glow of nerve cells in the region of the horn of Ammon of the piglet magnif. 450X. Abscissa = days; ordinate = Fluorescence (mean number of glowing neurons per 0.01 sq mm) [above]; Infectivity (log LD_{50}/0.03 ml) [below]

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12/15/65