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VACCINIA VIRUS PERITONEAL INFECTION IN RATS OF DIFFERENT AGES

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Summary. — Rats of different age groups were inoculated intraperitoneally with 125,000 plaque-forming units (PFU) of vaccinia virus and both the cytology of the peritoneal cavity and the localization of the administered virus were studied. The absolute number of the peritoneal cells did not depend on age up to the 30th day of life; it fluctuated from 0.79 to 1.9 million cells in 1 ml of the peritoneal washing. In the older animals the number of cells considerably increased. Differential counts of the cells differed: macrophages and lymphocytes prevailed in younger rats, the number of granulocytes increased in older age groups. After the infection, a strong increase in the number of cells, especially of granulocytes, took place within the youngest age groups and, to a lesser extent, within the older ones. Macrophages from infected 3-day-old rats lost the ability to adhere to the glass. Immunofluorescence revealed visible amounts of viral material in macrophages only; the number of positive cells fell rapidly with the donor age. The infectious virus occurred both in the cells and extracellularly and its quantity decreased slowly with increasing age. On the other hand, with increasing age the ratio of the intracellular virus to the free virus progressively increased. The number of infective centers remained relatively constant up to 30 days of age.

Introduction

The adult albino rat is very resistant to the majority of viruses having any importance in medicine. In their first days of life, however, the young albino rats are very susceptible to tick-borne encephalitis (Pešek, 1956; Pešek and Dluhoš, 1954), mumps (Pospíšil and Brychtová, 1956), and herpes simplex (Satoshi and Southam, 1965) viruses. Because of these great differences in the susceptibility depending upon age, the rat represents a suitable model for studying the natural defence mechanisms that make up the resistance of adult animals.

The vaccinia virus multiplies best in the brain of a young rat, as little as one PFU being sufficient to start an infection. Some ten times greater inoculum is necessary for an infection by the peritoneal route to become detectable.

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It has been ascertained that 7 hours after peritoneal infection, it is not yet possible to detect the virus in the peritoneal washings; 24 hours after infection, the virus appears in a quantity depending upon the age of the inoculated rats. In animals up to the 7th day of life, the virus was also found after 24 hours in the kidneys and lungs whereas, in older animals, its presence was limited to the peritoneal cavity (Jandásek, 1968).

These observations support our opinion that the peritoneal cells represent one of the factors involved in the resistance of the adult rat, at least of an intraperitoneally infected rat. In the present work the role of these cells in vaccinia virus infection in rats of different ages was studied.

Materials and Methods

Media and cell cultures. Chick embryo cells were cultivated in Gey's balanced salt solution with 5% calf serum, 0.05% lactalbumin hydrolysate, 1% chick embryonal extract and 5% tris (hydroxymethyl) aminomethane (Tris) buffer (Porterfield and Allison, 1960). This was also used as a maintenance medium. Amounts of 7.5×10^6 cells gained by trypsinization of 11-day old chick embryos were cultivated in Petri dishes of 5 cm diameter.

The vaccinia virus from commercial anti-smallpox lymph was passages on chorioallantoic membranes of 10—11 day old chick embryos. Three days after infection, the membranes were harvested and ground in saline solution. The virus suspensions were centrifuged for 5 minutes at 1500 rev/min to remove cell debris and then stored at —60° C. Before each experiment the stock virus was assayed by plaque titration. The rats were infected with doses of 125,000 PFU.

Rats. Wistar rats were used. Pregnant females were supplied by the firm "Velaz", Prague. The young of several mothers were mixed together and then randomly redistributed among the mothers in numbers of 8—10 each. Before each experiment, the young rats were weighed and individuals differing by more than 20% from the average were discarded. After inoculation, they were again randomly redistributed to the mothers. All rats were very carefully examined for the presence of intestinal pathogens (viz. salmonellae) and only pathogen-free animals were used.

Peritoneal washings were obtained without any previous induction, by injecting Earle's salt solution (pH 6.5) with 5% calf serum into the peritoneal cavity of rats previously exsanguinated under light ether anaesthesia. The amount of injected solution corresponded to 20% of the animal's body-weight. Washings destined for cytological investigations were immediately mixed with 30% of calf serum. The smears were fixed in Bouin-Hollande solution. Washings intended for virological investigations were temporarily kept in an ice bath in test tubes coated with paraffin.

Cytological investigation of cells from peritoneal washings was performed after staining with methyl green and pyronin. All specimens were parallelly stained with haematoxylin and eosin. Some smears were investigated by using Sato's peroxidase reaction (Pearse, 1961) which has the great advantage of very distinctly demonstrating the cytoplasmic granules in cells of the myeloid line. The absolute cell counts were determined in a Bürker's chamber using the phase contrast microscope: by this method the admixture of red cells, unavoidable in the youngest rats examined, was clearly differentiated.

Immunofluorescent staining was performed by the indirect method according to Albrecht (1963, p. 105—107) using the contrasting staining with sulphorhodamine B conjugated with normal calf serum; this was necessary regarding to relatively strong own fluorescence of peritoneal cells themselves.

Virus assay. Virus in fractions of peritoneal washings was estimated by plaque titration. For ascertaining the quantity of the intracellular virus, the peritoneal cells were pelleted at 1000 rev/min for 10 minutes, washed with buffered salt solution and subjected to threefold freezing and thawing. The number of infected centres was estimated as described by Dougherty (1964). Immediately after spreading the infected peritoneal cells onto a monolayer in a Petri dish, the cells were covered by 0.5 ml of agar and after 30 minutes' incubation, a new layer of 4.5 ml of agar was poured over (1% Difco agar in Gey's medium buffered with $NaHCO_3$). After solidi-

ficacion of the agar, the dishes were incubated for 4 days in a moist atmosphere with 5% CO_2 and then overlaid with 1 ml of agar containing neutral red (1 : 4,000). Four hours later, the plaques were counted.

Results

Cytology of the peritoneal cavity

Using the method of washing and fixation described above, we obtained preparations where practically 100% of the cells had well preserved nuclear and cytoplasmic outlines and details. In classifying the peritoneal cells we accept the possibility of a transformation of some cell types into other types.

In classifying the cells from the peritoneal cavity we occasionally encounter cells of a transient type which can be classed only with difficulty. It is necessary, therefore, to keep in mind that each classification is, in fact, a simplification of the actual situation. In our opinion, however, by using the light microscope and simple cytological staining, the following classification of the peritoneal cells is most acceptable: 1) macrophages among which, besides the large mononuclear phagocytes, also belong all large monohistiocyte-like elements, 2) lymphocytes, 3) the neutrophilic and 4) eosinophilic granulocytes and 5) the mastocytes.

The results can be summarized as follows: In peritoneal washings from healthy animals soon after birth, macrophages and the lymphocytes prevail. With increasing age, neutrophilic granulocytes gain in number and in adult animals they amount to about 25%. The amount of the granulocytes in

Table 1. Mean numbers of cells in the peritoneal washings from rats of different ages

Age of rats (days)	Mean weight of rats (g)	Mean numbers of cells obtained from one rat (in millions per ml)	
		Normal rats	Rats 48 hr after infection
2—4	8.3	1.9	10.4
7—8	9.7	1.22	3.6
10	10.1	0.76	1.4
15	14.0	1.76	1.6
20	27.2	1.0	3.0
30	38.5	1.55	2.8
> 100	190.0	8.5	n. d.

n. d. = not determined.

rats is therefore greater than that given for mice (Dvořák *et al.*, 1966). An injection of a suspension from uninfected chorioallantoic membranes into the peritoneal cavity in a concentration as used for infection, had no effect on the total number of the cells nor on their composition. The absolute cell count in the peritoneal washings of normal rats was basically the same in the age-groups up to 1 month of life. In adult rats we found higher mean counts but also considerable individual differences. After infection of the young rats, mainly of the youngest groups, the number of the peritoneal cells increased (Table 1).

Among the cells of the same class, no morphological differences were observed in rats of all age groups. Only among the cells of the granulocyte class were the neutrophils more segmented in the older animals than in the younger individuals: in the latter there occurred also juvenile, non-segmented forms of neutrophils. The granulocytes were significantly more numerous in the infected animals of all age groups than in the controls (Fig. 1). With the exception of a greater segmentation of the neutrophil nuclei, the infection

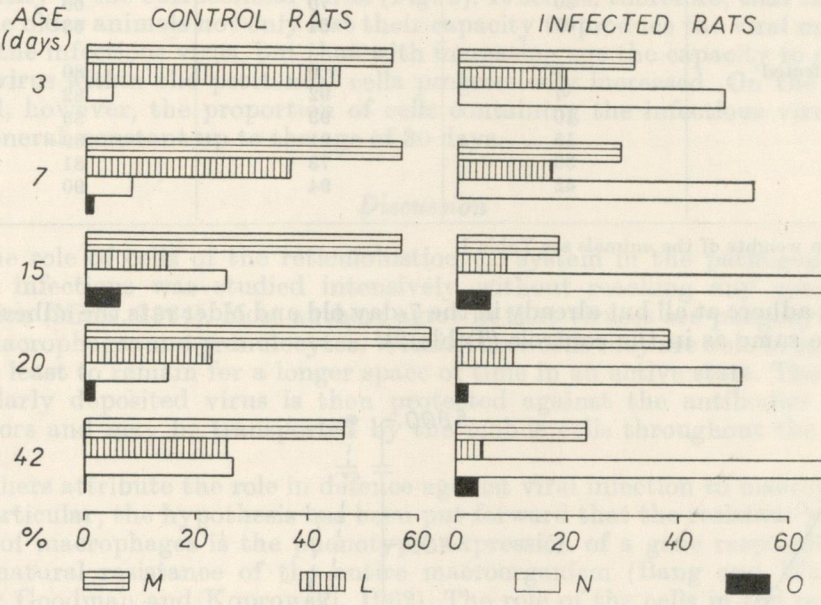

Fig. 1.

Composition of cell population in peritoneal washings from rats
M — macrophages; L — lymphocytes, N — neutrophil granulocytes; O — other cells (mast cells and eosinophils)

with vaccinia virus had no effect on the appearance of the peritoneal cells. Further we studied the influence of the infection on the ability of the peritoneal cells to adhere to the glass. The normal peritoneal cells from the macrophage-class adhere permanently to the glass; the granulocytes also adhere but detach after 24 hours; and the lymphocytes and the other cells, unless engulfed by the macrophages, either do not adhere at all or they detach after some hours (Bennet, 1965). In accordance with this we found that the number of cells adhering to the glass corresponded to the number of macrophages plus granulocytes. We calculated the number of the adherent cells after one hour of incubation at 37° C, by gently shaking the tubes with the peritoneal cells; the not adherent cells were then counted in a Bürker's chamber. After infection with vaccinia virus, the cells from 3-day old rats

Table 2. Adherence of rat peritoneal cells to glass

Rats	Age of rats (days)	Per cent of	
		adherent cells	macrophages plus granulocytes
Normal	3	62	58
	7	69	64
	30	70	66
	> 100	64	63
Infected	3	0	80
	7	92	82
	10	93	89
	15	91	89
	30	73	81
	42	94	90

For mean weights of the animals see Table 1.

did not adhere at all but already in the 7-day old and older rats the adherence was the same as in the controls (Table 2).

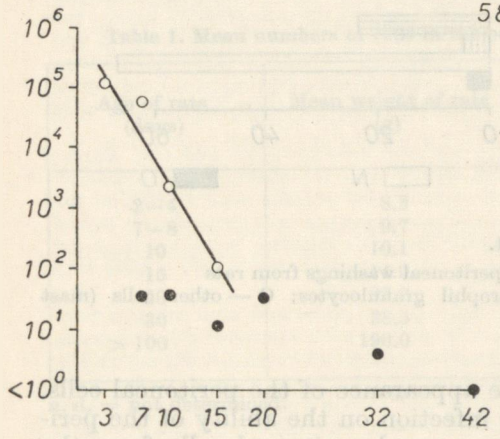

Fig. 2.

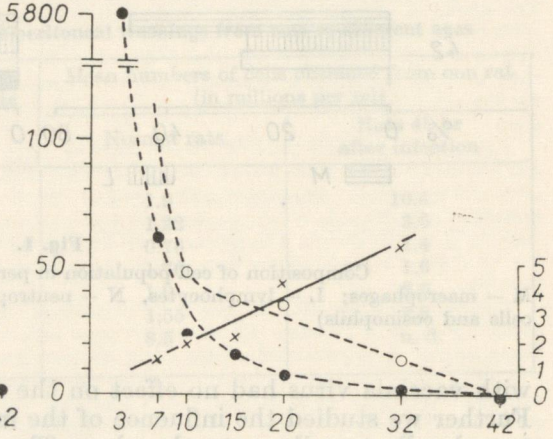

Fig. 3.

Fig. 2.

Quantity of vaccinia virus in peritoneal cells from rats of different ages
Abscissa: age of rats in days: ordinate: number of cells, fluorescent (○) or containing infectious virus (●), per million.

Fig. 3.

Distribution of vaccinia virus in peritoneal washings from rats of different ages
Abscissa: age of rats in days; left ordinate: concentration of cell-bound (○- - - -○) and extracellular (●——●) virus in PFU/0.2 ml; right ordinate: the ratio of cell-bound to extracelular virus (×——×)

Distribution of the virus in the peritoneal washings

Immunofluorescence revealed the presence of viral antigen only in cells with non-segmented nuclei, these most likely being macrophages. The number of cells containing visible viral material decreased strongly with increasing age of the infected rats. In the washings from 15-day and older rats, no cells with fluorescent material were found (Fig. 2). The quantity of the cell-bound virus diminished with increasing age but more slowly then did the quantity of the extracellular virus (Fig. 3). It seems, therefore, that the cells of the older animals not only lost their capacity to produce the viral material and the infectious virus, but that with increasing age the capacity to confine the virus within the peritoneal cells progressively increased. On the other hand, however, the proportion of cells containing the infectious virus was in general, constant up to the age of 30 days.

Discussion

The role of cells of the reticulohistiocytic system in the pathogenesis of viral infections was studied intensively without reaching any conclusive opinion (Mims, 1964). Most authors agree in that viruses are phagocytizable by macrophages and granulocytes. Within these cells they are able to multiply or at least to remain for a longer space of time in an active state. The intracellularly deposited virus is then protected against the antibodies or inhibitors and may be transported by the mobile cells throughout the host's body.

Others attribute the role in defence against viral infection to macrophages in particular; the hypothesis has been put forward that the resistant population of macrophages is the phenotypic expression of a gene responsible for the natural resistance of the entire macroorganism (Bang and Warwick, 1960; Goodman and Koprowski, 1962). The role of the cells in the reticulohistiocytic system in the production of interferon has been emphasized by a number of authors (Gresser, 1961); Glasgow and Habel, 1963; Lackovič and Borecký, 1965).

Poxviruses are taken up by the reticulohistiocytic cellls very intensively in the liver (Mims, 1959), lungs (Roberts, 1962; Daneš *et al.*, 1968), and also in the lymph nodes draining the site of virus inoculation (Brykina *et al.*, 1967). The role played by phagocytosis as a defence mechanism in the organism is, in the case of the poxviruses — the same as in other viruses, open to doubt. Subrahmanyan (1968), however, found no differences in susceptibility to the ectromelia virus between the macrophages of young mice and of adult ones. On the other hand, it was observed that the blockade of the reticulohistiocytic system brings about a viraemia otherwise undemonstrable (Hoens *et al.*, 1926; Goldman, 1928) and, in contrast to this, the agents inducing the proliferation of macrophages prevent the spread of vaccinia infection from the skin (Ledingham, 1927).

It is unlikely that the reticulohistiocytic cells are the only system responsible for the resistance of the rat to vaccinia infection, because the adult rat is resistant even after the inoculation of virus into the brain where the

reticulohistiocytic system is represented only by small number of cells. Nevertheless, after peritoneal infection it must be assumed that the peritoneal cells play a significant role in the resistance of adult rats by localization of the infectious process into the peritoneal cavity.

The present results have demonstrated the presence of the virus both within the peritoneal cells and extracellularly 48 hours after inoculation of 125,000 PFU. The quantity of both the free and cell-bound virus diminished with increasing age. The intracellular virus level continuously rose, this being proof of the increasing virus-fixing ability of the peritoneal cells in older animals. A similar phenomenon was observed in vitro by Johnson (1964) with herpes simplex virus. The composition of the peritoneal cell population in the younger groups differs from that in older animals, mainly in the scantiness of the granulocytes. According to the immunofluorescent examination the macrophages harbour the virus, i.e., they contain the virus in such a quantity as to be visualized. This finding does not exclude the possible role of the granulocytes in the fixation and liquidation of the virus. It is impossible to decide whether the viral material observed in the macrophages is the consequence of phagocytosis of a large quantity of the virus, or of incomplete reproduction. At all events, there is a striking discrepancy between the discovery of the relatively high percentage of cells containing viral material on the one hand and the low percentage of cells containing the infectious virus on the other. The level of the virus in the peritoneal cells is very low, like in rat organs, including organs from rats immediately before death due to vaccinia infection. The same findings were reached by Spies (1960), who examined the multiplication of vaccinia virus in different kinds of tissue culture. In his experiments the infected L cells amounted to about only 1% of cells exposed to high concentration of the virus. Spies (1960) determined the number of infected cells by plaque titration without the agar overlay. The possibility cannot be excluded that, by this technique, he may have obtained values higher then those corresponding to reality.

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