STUDIES ON THE MECHANISM OF ACTION OF RILEY VIRUS

II. Action of Substances Affecting the Reticuloendothelial System on the Level of Viraemia

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Riley's plasma enzyme-elevating virus (1) has been shown to reduce temporarily the rate at which infected mice clear intravenously injected carbon particles from their blood (2, 3). In an accompanying paper (4) reasons are given for believing that a reduced activity of the reticuloendothelial system (RES) is probably the major factor producing the elevated plasma enzyme levels which are characteristic of Riley virus–infected mice. In support of this view it has been shown that intravenously injected lactate dehydrogenase (LDH) is cleared from the plasma at a reduced rate in Riley virus–infected mice (2, 3), and that substances which alter the activity of the RES, as judged by the rate of carbon clearance, affect the levels of certain plasma enzymes in Riley virus–infected mice (4). The mechanism by which the activity of the RES is altered by Riley virus is not clear. The present study was undertaken to determine whether agents which affect the activity of the RES alter the titre of circulating virus, which is a remarkably constant feature of Riley virus infection. A preliminary report of this work has been published (5).

Materials and Methods

Animals.—Randomly bred albino mice of the Parkes strain were used.

Test Materials.—Methods of administration and dosage of carbon, zymosan, thorotrast, and stilboestrol were as described previously (4). In one experiment stilboestrol was given as an implant: a 15 mg tablet of stilboestrol (implantin; Burroughs, Wellcome and Co., Inc., Tuckahoe, New York) was inserted under the skin at the back of the neck.

Blood Samples.—Serial samples were obtained by retro-orbital (6) or tail-bleeding, as previously described (7).

Riley Virus.—The virus used was the same as that described in an accompanying paper (4). For one experiment, a partially purified preparation was made from the pooled plasma of mice infected 24 hours previously with stock Riley virus. The virus was sedimented at 150,000 *g* for 60 minutes, and resuspended in one-tenth the volume of phosphate-buffered saline (dulbecco solution A) containing 0.5 per cent gelatin. The resulting preparation had an infectivity titre of $10^{9.5}$ ID_{50} per ml.

Virus Titration.—Blood samples were diluted 1 in 100 in Hanks' salt solution containing

0.5 per cent gelatin, blood cells removed by centrifugation, and 10-fold dilutions, based on the original volume of blood, prepared in the same diluent. One ml volumes were injected intraperitoneally into groups of mice which were caged individually. The test mice were bled after 3 days, and their plasma LDH activity estimated as described below. The number of mice infected at each dilution was determined, and the dose which infected 50 per cent of the mice (ID_{50}) was calculated by the method of Thompson (8) and expressed on the basis of 1 ml of blood. The accuracy of the titration depends on the number of mice used. Never fewer than 2, and in most experiments 3, mice were used at each dilution. In one experiment, where greater accuracy was required, groups of 6 mice were used. Using 2 mice at each dilution the standard error was about $10^{0.8}$, with groups of 3 it was about $10^{0.6}$, and with groups of 6 it was between $10^{0.3}$ and $10^{0.4}$.

Qualitative Estimation of Plasma LDH.—For estimation of plasma LDH activity, 0.05 ml of blood was diluted 1 in 12 with phosphate-buffered saline containing 1 international unit of heparin per ml, and the cells removed by centrifugation. Thus the plasma was diluted approximately 1 in 24 assuming the packed cell volume to be 50 per cent. Plasma LDH activity was estimated by a modification of the depression plate technique described by Kelly and Greiff (9). This test was performed in plastic haemagglutination trays warmed to 37°C. Into each depression was pipetted 0.1 ml of standard pyruvate substrate (Sigma No. 500L-1) containing 1 mg reduced nicotinamide-adenine-dinucleotide (C. F. Boehringer and Soehne, Mannheim, Germany) per ml. Then 0.05 ml of each plasma sample under test, diluted 1 in 24, was added in turn to one of the depressions and stirred. The plasma samples were added at quarter minute intervals. The plate was incubated at 37°C, and exactly 30 minutes after adding the plasma to each depression 0.1 ml of colour reagent (200 mg 2:4 dinitrophenylhydrazine and 85 ml concentrated hydrochloric acid, diluted to 1 litre in distilled water and stored in the dark at + 4°C) was added. Incubation was continued for 10 minutes, and 0.2 ml 2 N sodium hydroxide was then pipetted into each depression. A blank (phosphate-buffered saline in plasma) and a control plasma with LDH activity of 1500 IU per ml, were included on each plate. Plasma samples with an LDH activity of below 800 IU per ml give an intense brown colour, while those with an activity over 1200 IU per ml are colourless. By the 3rd day after Riley virus infection the plasma LDH activity is always over 1500 IU per ml, whereas the normal level is always below 800 IU per ml (10).

RESULTS

Viraemia in Riley Virus-Infected Mice.—The level of viraemia in 2 mice was determined at intervals following the intraperitoneal injection of 1 ml of the stock Riley virus preparation ($10^7 ID_{50}$). Titrations were performed using 3 mice at each 10-fold dilution. The maximum virus titre was attained by 24 hours from the time of injection, after which there was a fairly rapid fall during the next 48 to 72 hours (Fig. 1). Subsequently the titre fell more slowly to a relatively stable level between 10^5 and $10^6 ID_{50}$ per ml of blood. The shape of the blood virus titre curve (Fig. 1) suggested that the action of various substances on the level of viraemia could most easily be determined by measuring their effect on either the peak virus titre 24 hours after infection, or on the stable level of viraemia 10 days or more after infection.

Effect of Stimulation or Blockade of the RES on the Level of Viraemia 24 hours After Infection.—Pairs of male mice were treated with stilboestrol, carbon, zymosan, or thorotrast before the injection of Riley virus. The effects of these

treatments on RES function as measured by carbon clearance rates is described in a previous paper (4). Stilboestrol was given either as an implant or as a course of 6 daily subcutaneous injections, and the virus was injected 72 hours after the implant or 48 hours after the last injection. Carbon and zymosan were given intravenously and thorotrast intraperitoneally at intervals before virus injection as shown in Table I. Two control and 2 experimental mice were injected with virus at the same time. Table I shows the mean virus titres obtained 24 hours

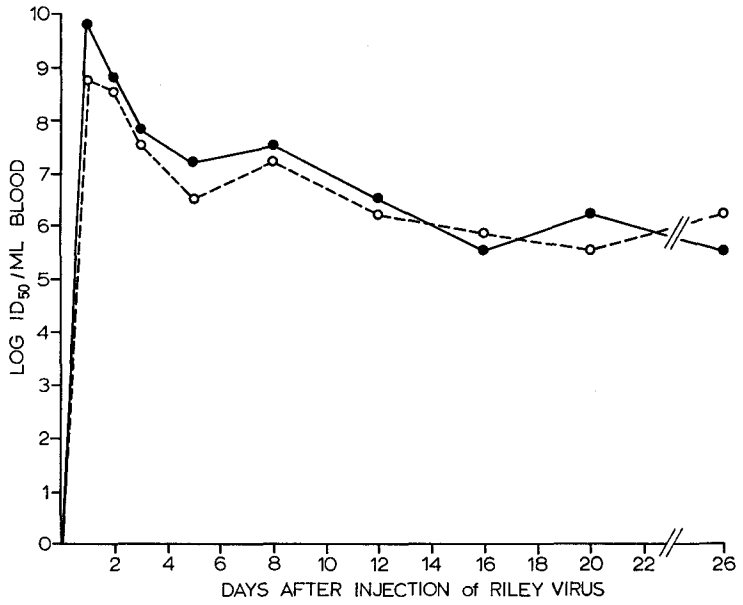

FIG. 1. Virus titre in the plasma of 2 mice following the intraperitoneal injection of $10^7 ID_{50}$ of Riley virus.

after injection in the test and control mice. None of the test substances had any significant effect on the 24 hour virus titre in the blood.

Effect of Stimulation or Blockade of the RES on the Stable Level of Viraemia.— Pairs of male mice which had been injected with Riley virus more than 10 days previously were bled before, and at intervals after, treatment with thorotrast, zymosan, or stilboestrol, to determine the titre of circulating virus. Virus titrations were performed using 2 mice at each 10-fold dilution.

Thorotrast was given as a single intraperitoneal injection in a dose of either 1.0 ml or 0.5 ml per mouse. As shown in Fig. 2 the blood virus titre rose 24 hours after thorotrast administration. The small dose produced a 10-fold rise and the larger dose a more than 100-fold rise. In the latter case the effect lasted for at least 48 hours.

TABLE I

The Effect of Various Substances on the Blood Virus Titre 24 Hours After the Injection of Riley Virus

Test substances	Interval between injections of test substances and virus	Expected effect on RES*	Level of viraemia 24 hrs. after virus injection ($Log_{10}ID_{50}$ per ml blood)	
			Control	Test
	hrs.			
Stilboestrol				
(Implant)	72	Stimulation	9.5‡	8.5
(6 daily injections)	48	Stimulation	8.5	8.5
Carbon	1	Blockade	10.0	10.5
Zymosan	½	Blockade	9.0	10.0
	3	Blockade	9.0	10.0
	20	Stimulation	10.0	9.5
Thorotrast	1	Blockade	8.5	9.5
	18	Blockade	9.5	9.5

* See Table I in accompanying paper (4).

‡ Titrations performed using 2 mice at each 10-fold dilution. Values are the mean plasma virus titres of 2 mice.

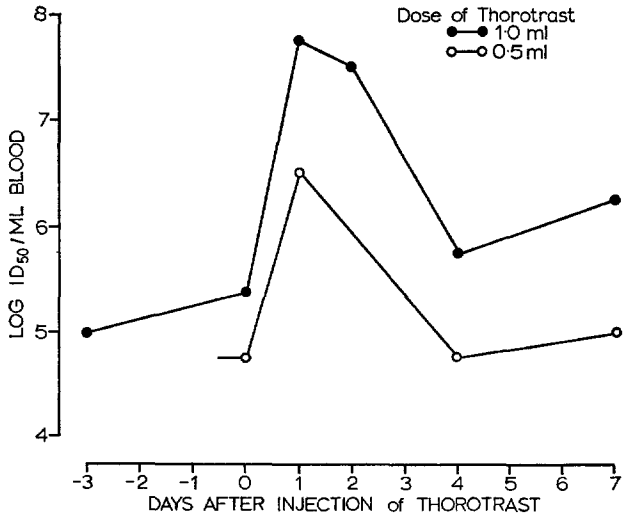

FIG. 2. Mean blood virus titre in 2 pairs of mice injected intraperitoneally with either 1.0 ml or 0.5 ml of thorotrast per mouse 10 days after infection with Riley virus.

Zymosan was injected intravenously into 2 mice as a single dose of 10 mg per 100 gm body weight. Fig. 3 shows the mean blood virus titres in these mice at intervals after injection. Zymosan did not cause as large a rise in the mean blood virus titre as thorotrast, and the effect was not maintained for much more than 24 hours. By 48 hours after injection the level of viraemia had fallen to a value below the starting level.

Stilboestrol was administered as a course of 6 daily subcutaneous injections of 0.1 mg in 0.1 ml of arachis oil per mouse. The effect of this course of injections

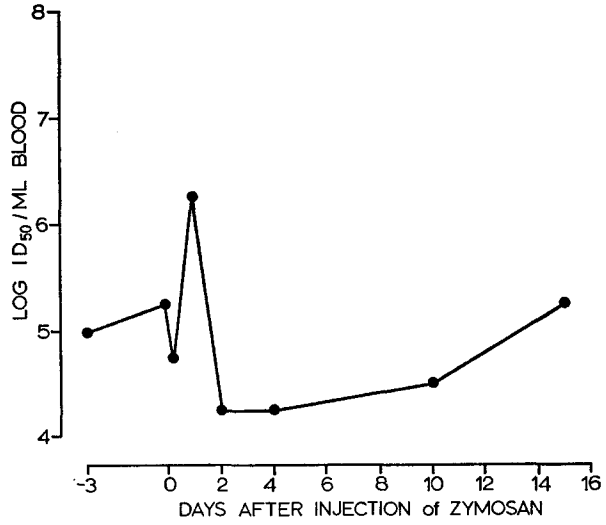

FIG. 3. Mean blood virus titre in 2 mice injected intravenously with zymosan (10 mg/100 gm body weight, as a suspension in normal saline) 10 days after infection with Riley virus.

on the mean blood virus titre is shown in Fig. 4. There was no rise in virus titre at 24 hours as occurred after the injection of thorotrast or zymosan. However, stilboestrol appeared to depress the level of viraemia slightly as did zymosan 48 hours after injection.

The Rate of Clearance of Riley Virus from the Plasma.—It was of interest to know how rapidly Riley virus particles are removed from the circulation after injection, and whether the rate of clearance is different in mice previously infected with the virus. It has been shown that Riley virus-infected mice clear carbon particles and enzymes from the blood more slowly than normal mice (2, 3). The clearance rate of injected Riley virus particles cannot be measured in uninfected mice strictly speaking, since the inoculum itself will initiate infection. However, as no new virus particles are released into the plasma for 5 to 6 hours after infection (11, 12) the clearance rate measured during this period in

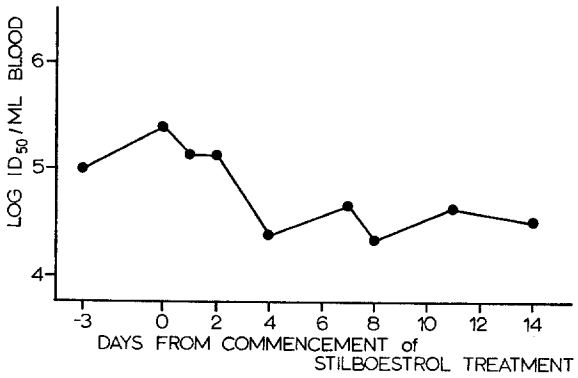

FIG. 4. Mean blood virus titre in 2 mice receiving a course of subcutaneous injections of stilboestrol. Six injections were given on days 0 to 5, each consisting of 0.1 mg stilboestrol/mouse, dissolved in 0.1 ml arachis oil.

TABLE II

Clearance from the Plasma of Intravenously Injected Riley Virus in Previously Uninfected Mice, and in Mice Infected with Riley Virus for 3 Weeks

Mouse No.	Previous Riley virus infection	Plasma virus titre ($log_{10}ID_{50}$/ml)*	
		2 minutes after virus injection	3 hours after virus injection
1	—	8.5	7.3
2	—	8.0	7.2
3	—	8.5	7.0
4	—	8.5	7.0
Mean		8.4	7.2
5	+	7.7	6.5
6	+	8.3	6.3
7	+	7.8	7.0
8	+	8.5	5.9
Mean		8.2	6.2

* Titrations performed using 6 mice at each 10-fold dilution.

previously uninfected mice will be of injected virus only. Four uninfected mice, and 4 mice infected with Riley virus 3 weeks previously, were each injected intravenously with 0.1 ml of a purified virus preparation which contained $10^{9 \cdot 5}ID_{50}$ per ml of virus. The mice were bled 2 minutes and 3 hours after the virus injection, and the blood virus titre determined using 6 test mice at each 10-fold dilution of blood. The results are given in Table II. Since the dose

of virus injected was large in comparison with the level of previously circulating virus in the infected mice it is not surprising that the blood virus titre in the two groups was virtually the same 2 minutes after injection. After 3 hours the mice infected 3 weeks previously had cleared rather more of the injected virus from their circulation than the previously uninfected mice. In fact the number of virus particles cleared by the previously infected mice must have been greater than appeared since in these mice new virus was entering the circulation throughout the experiment.

DISCUSSION

One of the most interesting features of Riley virus infection in mice is the persistent viraemia. The changes in the blood virus titre following the injection of Riley virus shown in Fig. 1 are similar to those reported previously (11, 13, 14) from other laboratories using different strains of virus.

The rapid rise in virus titre during the first 24 hours after infection is probably terminated by the action of interferon, as high titres of interferon have been found in the plasma at this time (15). However other factors, such as the death of virus-producing cells and a scarcity of further cells susceptible to infection, may be involved. It seems unlikely that the RES plays any major role in limiting the peak level of viraemia, since the administration of RES blocking or stimulating agents before virus injection produced no detectable change in the peak level of viraemia.

It appears likely that in the later stages of the infection phagocytosis of virus particles by the RES is a factor in determining the relatively stable level of viraemia. In support of this, it was found that the administration of RES blocking or stimulating agents affected the level of viraemia in Riley virus–infected mice. That the effect of thorotrast, zymosan, and stilboestrol on virus titres is mediated by the RES is supported by the close correlation observed between their effects on virus titres and on the rate of carbon clearance (4). Thorotrast was a more active RES-blocking agent than zymosan as judged by the rate of carbon clearance, and it produced a larger rise in blood virus titre. Zymosan blocked carbon clearance only during the first 24 hours, after which it stimulated clearance; and it was during the latter period, 48 hours after zymosan injection, that the virus titre fell below the pretreatment level. Stilboestrol stimulated carbon clearance, and it produced a small but prolonged fall in virus titre. Similar results have been reported in two other virus infections. Blockade of the RES with thorotrast was found to increase the level of viraemia in mice infected with Semliki forest virus (16), and to decrease the rate at which injected Newcastle disease virus disappeared from the blood of mice (17).

Although in Riley virus-infected mice there is evidence of a reduced capability to clear LDH and, in the first few days of infection, injected carbon particles also (2, 3), we found no evidence of reduced clearance of injected Riley

virus particles 21 days after infection. In fact, the short half-life of injected virus in both infected and previously uninfected mice suggests that the virus has a high turnover rate in the plasma. It is possible that such active and prolonged clearance of virus reduces the capacity of certain elements of the RES to clear some plasma enzymes, resulting in the high level of these enzymes by which Riley virus infection is recognised.

The viraemic phase in many infections is terminated by the appearances of circulating neutralizing antibodies (18). However there are a few infections, such as murine lymphocytic choriomeningitis (19), equine infectious anaemia (20), hog cholera (21), and infectious hepatitis, both human (22) and murine (23), in which a persistent viraemia has been described in certain circumstances. In Riley virus–infected mice antibodies in the plasma have not been reported, and in some at least of the virus infections the persistence of the viraemia depends on the failure of antibody formation. Where infection occurs before or soon after birth a state of immunological paralysis may be induced. This appears to be the mechanism involved in the case of mice neonatally infected with lymphocytic choriomeningitis (24). On the other hand, in those cases where mature animals are infected another explanation must be sought. It may be that the virus is enclosed in a non-antigenic coat, or is circulating as a free infective nucleic acid without, for some reason, being inactivated by nuclease (25). However although antibodies to Riley virus have not been reported in mice, they have been produced in rats (26). Assuming that the virus is antigenic in mice also, the failure of Riley virus–infected mice to clear their plasma of circulating virus by antibody formation might be explained on the theory that there are two types of Riley virus particle, only one of which is antigenic. In support of this theory evidence has recently been published that infective virus exists in the plasma in two forms, which differ in certain physicochemical properties (27, 28). An alternative explanation would postulate immunological paralysis by excess of antigen due to the very rapid replication of the virus. At present the evidence is not adequate to decide between the various possibilities.

SUMMARY

The level of viraemia was determined in serial blood samples obtained from 2 mice after the injection of Riley virus. The plasma virus titre rose rapidly to a peak value of 10^9 to 10^{10} ID_{50} per ml by 24 hours after infection, and then fell slowly to a level of 10^5 to $10^6 ID_{50}$ per ml by the 10th day after infection, where it remained relatively stable.

Neither blockade of the RES with thorotrast, zymosan, or carbon, nor stimulation of the RES with stilboestrol or zymosan, before the injection of Riley virus, produced any observable alteration in the level of viraemia attained 24 hours after infection. However 10 days or more after infection with Riley virus blockade of the RES with thorotrast caused a transitory rise, and stimulation

of the RES with stilboestrol caused a slight but prolonged fall, in the level of viraemia. Zymosan injection at this period of infection caused an initial rise, followed by a fall, in the level of viraemia; these changes correlated with the initial period of blockade and the subsequent period of stimulation of the RES observed in carbon clearance studies.

The clearance of injected Riley virus particles from the plasma over a period of 3 hours after injection was measured in previously uninfected mice and mice which had been infected with Riley virus for 3 weeks. The mice which had been infected 3 weeks before the test cleared rather more of the injected virus than the previously uninfected mice.

It is concluded that although the activity of the RES affects, and may determine, the level of viraemia, the permanence of the viraemia in Riley virus–infected mice does not appear to be due to a failure of the RES to clear virus particles from the plasma.

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