STUDIES ON THE MECHANISM OF ACTION OF RILEY VIRUS

I. Action of Substances Affecting the Reticuloendothelial System on Plasma Enzyme Levels in Mice

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It was recently suggested that the increases in plasma enzyme activity following infection of mice with Riley virus (1) might be due to inhibition of the clearance of enzymes from the blood plasma (2). Preliminary studies confirmed that the plasma clearance of intravenously injected lactate dehydrogenase (LDH) was greatly impaired in Riley virus–infected mice, and also showed that blood clearance of colloidal carbon, a measure of reticuloendothelial activity, was temporarily depressed concurrently with the peak plasma virus titre (3, 4). There is some evidence that enzymes are removed from the plasma by the reticuloendothelial system (RES) (5). We have therefore studied the effects of various agents known to stimulate or depress RES function on plasma enzyme levels in normal or Riley virus–infected mice. A preliminary report of this work has been published (6).

Materials and Methods

Animals.—Randomly bred albino male mice of the Parkes strain were used at 6 to 8 weeks of age.

Injections and Sampling.—Intravenous injections were made *via* the dorsal vein of the penis and serial blood samples taken by retro-orbital bleeding (7), or by tail-bleeding as previously described (8). In some cases mice were bled out from the brachial artery under ether anaesthesia (2).

Test Materials.—Colloidal carbon (pelikan ink, Günther Wagner, Hanover) was centrifuged at 1200 *g* for 15 minutes, and the supernatant (7 ml) mixed with 3 ml of 3 per cent gelatin, neutralized with ammonium hydroxide. This carbon-gelatin suspension was further diluted 1:3 in normal saline just before injection. The carbon concentration in the suspension injected was 16 mg/ml. Zymosan (Fleishmann's type A from fresh yeast, obtained from Koch-Light Laboratories Ltd., Colnbrook, England) was injected as a 10 mg/ml suspension in normal saline. Thorotrast (Testagar and Co., Detroit) consisted of a 24 to 26 per cent suspension of colloidal thorium dioxide, stabilised in 25 per cent dextrin. Stilboestrol (British Drug Houses Ltd., Poole, England) was injected as a 1 mg/ml solution in arachis oil. Cholesterol oleate, (British Drug Houses, Ltd.) 100 mg/ml, was prepared for injection as an emulsion by mixing with 1 per cent tween 20 (Koch-Light Laboratories Ltd.) in 5 per cent dextrose water, according to Stuart and Davidson (9).

Irradiation.—Mice were treated with whole body x-irradiation, 2000 roentgen, unanaesthetized, in a chamber made of 1 mm thick aluminium, using a 0.5 mm copper plus 1.0 mm aluminium filter, 1.1 mm copper half value thickness (H.V.T.) The voltage used was 220 kv peak and the applicator size was 20 × 20 cm.

Determinations.—Lactate dehydrogenase (LDH) and alanine transaminase (ALT) were determined by the spectrophotometric procedures of Wroblewski and LaDue (10, 11), using a Unicam S.P. 500 spectrophotometer. Reduced nicotinamide-adenine dinucleotide ($NADH_2$) for these assays was obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Phosphoglucose isomerase (PGI) was determined by the method of Parr, described in detail elsewhere (2). Aldolase (ALD) was determined by the colorimetric procedure of Sibley and Lehninger (12). All enzyme activities are expressed in International Units (IU) per ml of plasma (13).

Blood carbon concentration was determined by diluting 0.02 ml blood samples in 3 ml of 0.1 per cent sodium carbonate solution and measuring the optical density of the solution at 660 mμ in an EEL 'spectra' absorptiometer, Evans Electroselenium Ltd., Halstead, England. A standard curve of optical density at 660 mμ was prepared from carbon suspensions containing 1 to 20 μg carbon per ml, and used to convert optical density readings of blood samples to mg carbon per ml blood.

Riley Virus.—Our strain of Riley virus was originally obtained (8) from the plasma of a mouse bearing the transplantable sarcoma 37. The virus has since been maintained in this laboratory by occasional passage in mice. The stock virus preparation used consisted of heparinised plasma from mice infected 24 hours previously, diluted 1 in 100 with Hanks' saline containing 0.5 per cent gelatin. The preparation was filtered through an 02 selas filter and stored in sealed ampoules at −20°C. Mice were inoculated intraperitoneally with this virus preparation, which had an infectivity titre of $10^7 ID_{50}$/ml.

RESULTS

Effect of Various Treatments on Reticuloendothelial Activity.—Groups of mice were treated in various ways in order to alter the activity of their reticuloendothelial system. The effect of the treatment was assessed by measuring the rate of clearance from the blood of injected colloidal carbon-gelatin suspension on randomly selected mice from each group. The results are shown in Table I. Untreated control mice were similarly studied concomitantly with mice from experimental groups, and the results used to establish mean control values.

An injection of carbon caused only a slight reduction in the rate at which a second dose of carbon was cleared 1 to 4 hours later. Cholesterol oleate and thorotrast both caused reductions in carbon clearance rate over the first 1 to 2 days. Zymosan caused an initial reduction in carbon clearance rate at 1 to 4 hours, but by 24 hours the rate had returned to normal, and by 2 days was greatly stimulated. A single injection of stilboestrol caused a marked stimulation in carbon clearance rate 1 and 2 days after injection, with a return to normal by the 7th day. A course of six daily injections caused a more prolonged stimulation of carbon clearance rate, lasting for at least a week after the first injection. X-Irradiation at a high dose level, lethal within 3 to 4 days, caused a slight increase in carbon clearance rate on the 1st and 2nd days after treatment.

Effect of Alteration of RES Function on the Plasma LDH Level in Normal or

Riley Virus–Infected Mice.—Following infection of mice with Riley virus the plasma LDH level rises within 2 days to reach a peak level of about 10 times the normal by 3 to 4 days. Subsequently an approximately steady level of about 8

TABLE I

Effect of Various Treatments on the Rate of Carbon Clearance in Mice

Treatment	Dose	Time after commencement of treatment	Mean rate of carbon clearance (m* ± SE)	No. Mice
Control			11.7 ± 0.7	22
Carbon	16 mg/100 gm body weight i.v.	1 hr.	8.8 ± 1.3	4
		4 hrs.	9.4 ± 1.3	5
Cholesterol oleate	100 mg/100 gm body weight i.v.	1 hr.	7.6 ± 0.6	3
		4 hrs.	4.1 ± 0.4	3
		2 days	4.4 ± 0.3	4
		14 days	11.6 ± 1.1	5
Stilboestrol	0.1 mg/mouse s.c.	1 day	17.6 ± 2.4	4
		2 days	25.3 ± 2.0	4
		7 days	11.2 ± 1.4	5
	0.1 mg/mouse/day, 6 days treatment s.c.	7 days	66.3 ± 8.9	6
Thorotrast	0.5 ml/mouse i.p.	1 hr.	5.2 ± 0.9	3
		1 day	3.8 ± 0.9	5
		7 days	9.7 ± 1.3	3
Zymosan	10 mg/100 gm body weight i.v.	1 hr.	9.6 ± 0.5	3
		4 hrs.	7.7 ± 0.4	4
		1 day	10.7 ± 4.0	3
		2 days	30.5 ± 6.7	4
X-irradiation	2000 r whole body	1 day	15.5 ± 1.3	4
		2 days	14.5 ± 1.7	3

Values expressed as means ± standard error of mean.

* m = slope of line of best fit of the carbon clearance readings.

times normal is maintained indefinitely (2, 8) . The effects of alterations in RES function on the initial rise in plasma LDH level, and on the later steady-state level, were studied in separate experiments.

Initial Rise of Plasma LDH Level.—Groups of uninfected mice were treated with either thorotrast, 0.4 ml per mouse intraperitoneally, or an equivalent

volume of normal saline intraperitoneally, and 18 hours later half the animals in each group were inoculated intraperitoneally with 0.5 ml stock Riley virus (Fig. 1). The plasma LDH level was measured at intervals over the next 14 days. Thorotrast treatment produced a marked rise in plasma LDH activity at 24 hours, and subsequently the plasma LDH level in the animals given thorotrast alone remained above that in the control animals. The rise in plasma LDH activity in the Riley virus–infected animals was considerably greater in the thorotrast-treated group (Fig. 1).

The effect of stimulation of the RES on the initial rise in plasma LDH level

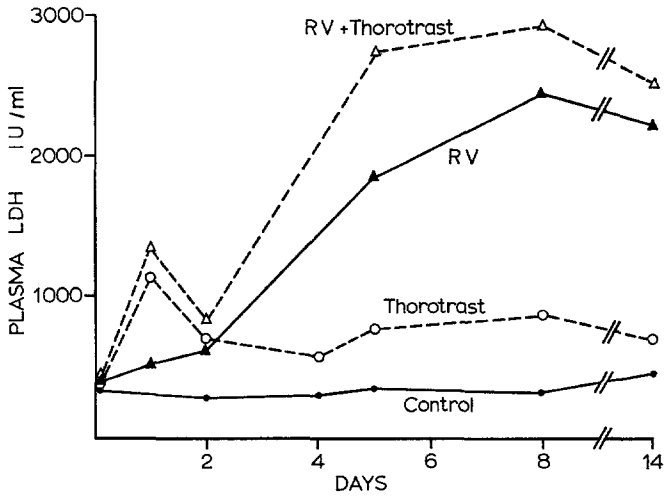

FIG. 1. Changes in plasma LDH level in groups of mice injected intraperitoneally with either 0.4 ml normal saline on day 0 (●——●), 0.4 ml normal saline on day 0 followed by 0.5 ml Riley virus (RV) 18 hours later (▲——▲), 0.4 ml thorotrast on day 0 (○ – – ○), or 0.4 ml thorotrast on day 0 followed by 0.5 ml Riley virus 18 hours later (△ – – △). Each point represents a mean value obtained from 3 to 6 mice.

following Riley virus infection was studied using stilboestrol treatment. Two groups of mice were injected subcutaneously once daily for 6 days with 0.1 ml of stilboestrol in arachis oil, 1 mg/ml, or 0.1 ml arachis oil only (controls). On the 8th day half the animals in each group were inoculated intraperitoneally with 1 ml stock Riley virus. Plasma LDH levels were studied in all four groups of animals over the following 8 days (Fig. 2). In the non-infected animals, the plasma LDH level was slightly lower in the stilboestrol-treated group. The rise in plasma LDH activity after Riley virus infection was, however, markedly depressed from 2 days after infection in the stilboestrol-treated group.

It has been reported that in mice, moderately high doses of whole-body irradiation (600 r), whilst interfering with the ability of the RES to respond to the

stimulating effect of agents such as zymosan, have no effect on the ability of the RES to clear colloidal particles from the blood (14). In the course of an investigation of the effects of x-irradiation on the replication of Riley virus (15) we studied the effect of rapidly lethal doses of x-rays (2000 r) on plasma LDH levels in normal and infected mice.

Two groups of 6 mice were treated with whole-body x-irradiation (2000 r), one group receiving an intraperitoneal inoculation of 1 ml stock Riley virus 24 hours later. An untreated group, and one receiving Riley virus alone, were also

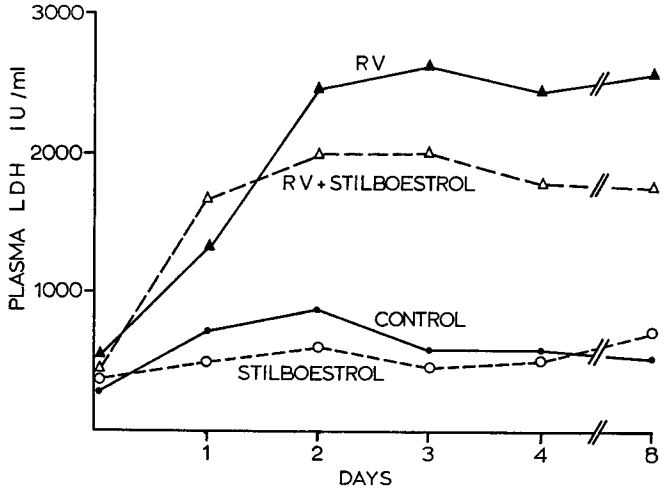

FIG. 2. Changes in plasma LDH level in groups of mice pretreated with arachis oil (●——●), pretreated with arachis oil and injected with 1 ml Riley virus (RV) on day 0 (▲——▲), pretreated with stilboestrol (○ – – ○), and pretreated with stilboestrol and injected with 1 ml Riley virus on day 0 (△ – – △). Pretreatment consisted of six daily subcutaneous injections of 0.1 ml arachis oil, or 0.1 mg stilboestrol in 0.1 ml arachis oil, commencing 1 week before virus injection. Each point represents a mean value obtained from 3 or 4 mice.

studied. Plasma LDH levels were determined up to 72 hours after irradiation. Plasma LDH activity was unaltered by x-irradiation in either the normal or Riley virus–infected groups (Fig. 3). Carbon clearance studies revealed no effect of this dose of x-irradiation on the function of the reticuloendothelial system.

Steady-State Level of Plasma LDH.—Groups of mice, either uninfected, or infected with 1 ml stock Riley virus at least 5 days previously, were treated with zymosan, cholesterol oleate, or thorotrast, to induce RES blockade, or with stilboestrol to induce RES stimulation. Plasma LDH levels were determined at intervals.

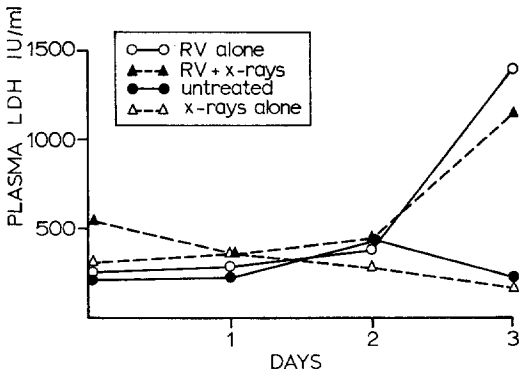

FIG. 3. Changes in plasma LDH level in groups of mice treated with 2000 r whole-body x-irradiation on day 0, and/or an injection of 1 ml Riley virus on day 1. Each point represents a mean value obtained from 4 to 6 mice.

Zymosan, 10 mg/100 gm body weight intravenously, cholesterol oleate, 100 mg/100 gm body weight intravenously, and thorotrast, 0.5 ml per mouse intraperitoneally, all caused increases in plasma LDH level over the first 6 hours after administration in both normal and Riley virus–infected mice (Figs. 4–6). Plasma LDH reached a peak level at 4 hours after zymosan administration, and returned to normal within 1 to 3 days. After cholesterol oleate or thorotrast administration the plasma LDH level rose steadily during the first 6 hours, and remained elevated for at least 24 hours.

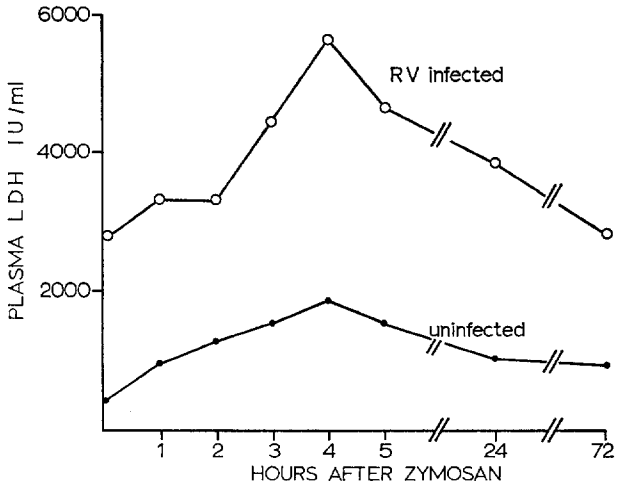

FIG. 4. Plasma LDH levels in mice uninfected or Riley virus–infected (60 days previously), following intravenous injection of zymosan 10 mg/100 gm body weight, as a suspension in normal saline. Each point represents a mean value obtained from 4 mice.

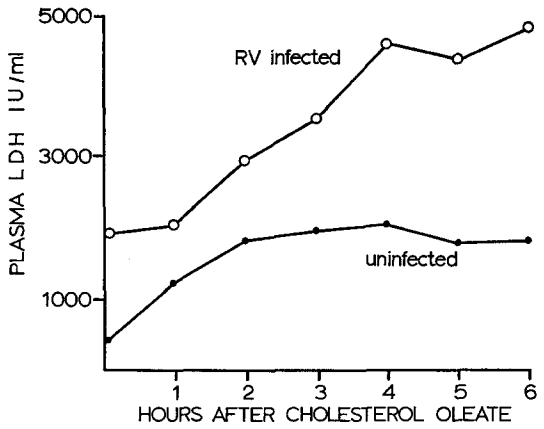

FIG. 5. Plasma LDH levels in uninfected mice or Riley virus-infected mice (14 days previously), following intravenous injection of cholesterol oleate, 100 mg/100 gm body weight, as an emulsion in 1 per cent tween 20. Each point represents a mean value obtained from 4 mice.

Stimulation of the RES was induced in normal and Riley virus-infected mice by subcutaneous injections of 0.1 mg stilboestrol, dissolved in 0.1 ml arachis oil. At first a series of six daily injections was given, which induced a very marked acceleration of blood carbon clearance when measured on the 7th day after treatment commenced (Table I). Plasma LDH levels, studied at daily intervals during such a course of stilboestrol treatment, fell markedly within 1 to 2 days and remained below the pretreatment level for several days (Fig. 7). As a rapid decrease in plasma LDH level occurred after only one or two injections we studied the effect of a single stilboestrol injection on plasma LDH levels. This produced a marked acceleration in carbon clearance rate within 1 to 2 days, followed by a return to normal at 1 week (Table I). Plasma LDH activity fell after this treatment, returning to normal by about 1 week in uninfected

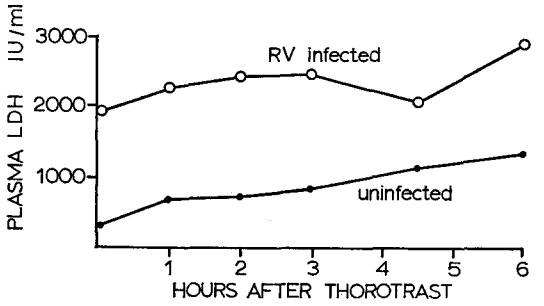

FIG. 6. Plasma LDH levels in uninfected mice or Riley virus-infected mice (5 days previously), following intraperitoneal injection of thorotrast (0.5 ml per mouse). Each point represents a mean value obtained from 4 mice.

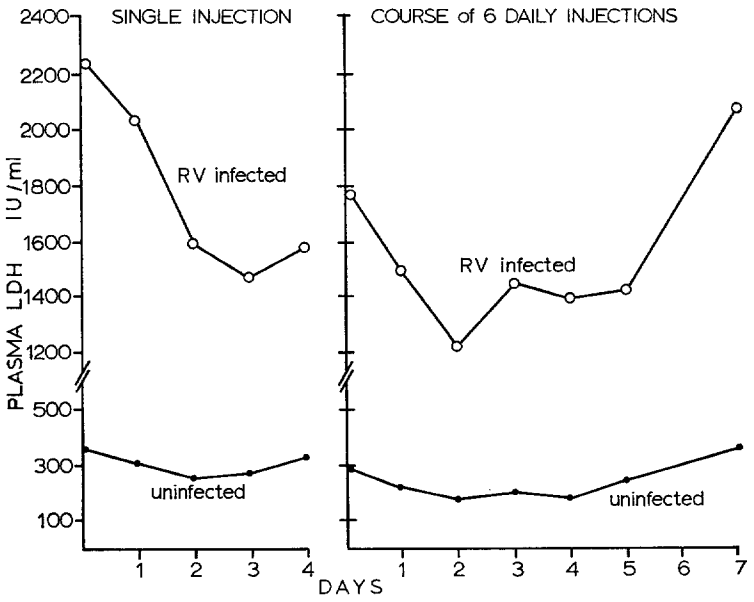

FIG. 7. Plasma LDH levels in uninfected mice or Riley virus-infected mice (7 to 14 days previously), following a single subcutaneous injection of stilboestrol (0.1 mg in 0.1 ml arachis oil), or during a course of six such injections, given on days 0 to 5. Each point represents a mean value obtained from 4 to 6 mice.

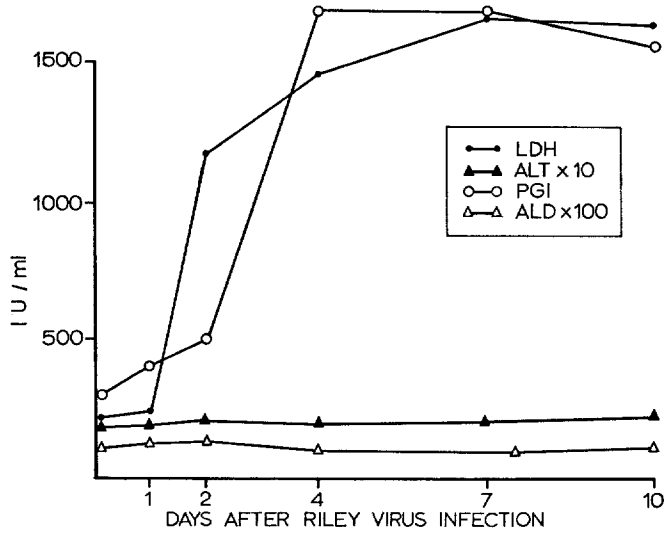

FIG. 8. Changes in plasma LDH, ALT, PGI, and ALD levels in mice up to 10 days after infection with Riley virus. Each point represents a mean value obtained from 3 to 6 mice.

controls, but in infected mice it remained below pretreatment level for several days (Fig. 7).

Plasma Enzyme Pattern after Treatment with RES-Blocking Agents or Infection with Riley Virus.—The previous experiments showed that increases in plasma LDH level can be induced in mice by blockade of the RES, and that the effects of Riley virus on the plasma LDH level are accentuated by blockade, and diminished by stimulation, of the RES.

However, Riley virus infection in mice results not only in a rise of plasma LDH level, but in a specific pattern of plasma enzyme elevations, several en-

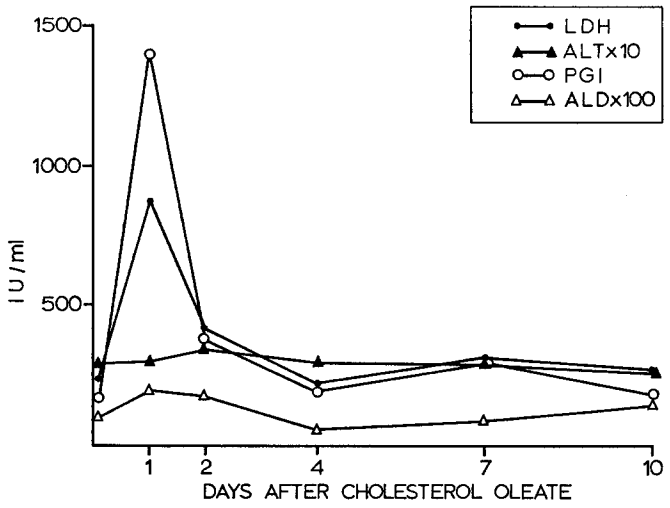

FIG. 9. Changes in plasma LDH, ALT, PGI, and ALD levels in mice up to 10 days after intravenous injection of 100 mg/100 gm body weight of cholesterol oleate emulsion in 1 per cent tween 20. Each point represents a mean value obtained from 5 mice, except the values for day 0, which are from 10 mice.

zymes being increased in activity, whereas several others are unaffected (16–18). We therefore compared the levels of activity of four plasma enzymes, alanine transaminase (ALT), aldolase (ALD), LDH, and phosphoglucose isomerase (PGI), after Riley virus infection or blockade of the RES with either cholesterol oleate or thorotrast. Since these assays required larger quantities of plasma than serial sampling would permit, groups of mice were bled out at intervals. Fig. 8 shows the changes in level of these four plasma enzymes in groups of mice up to 10 days after intraperitoneal inoculation with 0.5 ml stock Riley virus. Both LDH and PGI activity rose on the 1st or 2nd day after inoculation, and remained at a high level for the period of study. ALD and ALT activities showed no changes during this period.

The effects of cholesterol oleate injection on the plasma levels of LDH, PGI,

ALT, and ALD in uninfected mice were studied in a number of groups, each comprising 5 cholesterol oleate treated (100 mg/100 gm body weight intravenous) and 2 untreated mice; one group was bled out at various times up to 10 days after treatment. The values for untreated mice were pooled. One day after cholesterol oleate injection the plasma levels of both LDH and PGI were increased 3- to 5-fold (Fig. 9), returning to normal by 2 to 4 days after cholesterol oleate injection. The levels of both ALT and ALD remained within normal limits.

In the next experiment, the plasma levels of LDH, PGI, ALT, and ALD were studied in uninfected mice after thorotrast administration. Groups of 6 thoro-

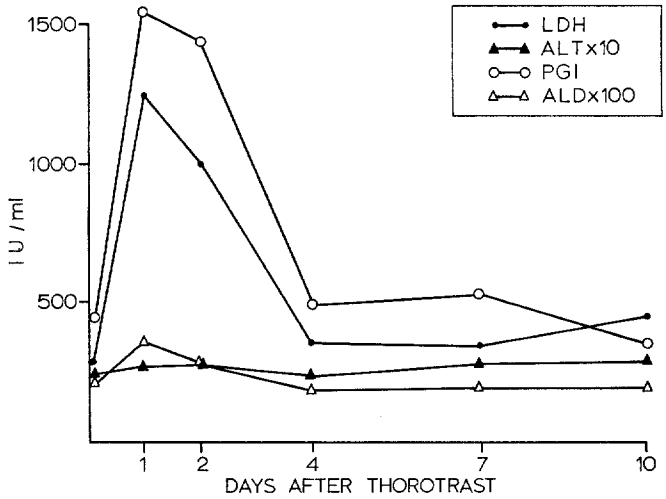

FIG. 10. Change in plasma LDH, ALT, PGI, and ALD levels in mice up to 10 days after intraperitoneal injection of 0.5 ml thorotrast per mouse. Each point represents a mean value obtained from 6 mice, except the values for day 0, which are from 10 mice.

trast treated (0.5 ml intraperitoneal), and 2 untreated, mice were bled out at intervals up to 10 days. The values for untreated mice were pooled. One day after thorotrast injection the plasma levels of both LDH and PGI were increased 3- to 5-fold (Fig. 10). The levels of these enzymes had returned to normal by 2 to 4 days after thorotrast injection, when reticuloendothelial activity, as assessed by carbon clearance, had also returned to normal (Table I).

In contrast, the levels of ALT and ALD remained within normal limits after thorotrast injection, apart from a small rise in ALD on day 1, which was just significant ($t = 3.12$; $P < 0.02$).

DISCUSSION

The term "reticuloendothelial system" is used for the purpose of this study to describe those body cells having in common the property of phagocytosis of

injected carbon particles. By "stimulation" or "blockade" of the mouse RES we mean a condition in which there is an increased or decreased rate of removal from the blood of injected colloidal carbon gel suspension. In general our findings regarding the effects of the drugs used on carbon clearance rates in mice agree well with previous reports. The blockading effects of cholesterol oleate were described by Stuart (19), and thorotrast has been widely used as a blockading agent (20–23). Zymosan has generally been used as a stimulating agent (24–26), but has also been shown to cause an initial period of blockade (27), as described here. The stimulating effect of stilboestrol has been described by Nicol and coworkers (28), and our results are in agreement with their observations. Benacerraf and coworkers found that whole-body irradiation (850 r to rats, 600 r to mice) had no effect on carbon clearance rate in these animals (14), and Di Luzio has reported no change in phagocytic function in rats studied up to 3 days after 1040 r whole-body x-irradiation, as assessed by blood colloidal radioactive gold clearance (29). However, it has been reported that a whole-body dose of 2500 r caused an increased clearance rate of labelled chromium phosphate in rats (30). Our results showed a slightly increased rate of carbon clearance in mice after 2000 r, agreeing with the latter observation.

The results reported here show that increased plasma LDH levels result from injection of substances causing blockade of the RES in normal or Riley virus–infected mice. On the other hand, stimulation of the RES by stilboestrol treatment decreases the plasma LDH level of both normal and infected mice, and greatly lessens the early rise of plasma LDH activity following Riley virus infection. Supralethal doses of whole-body x-irradiation, producing only slight stimulation of phagocytosis, cause no change in plasma LDH level, or in the rise in plasma LDH up to 2 days after Riley virus infection. These facts support the contention put forward by Wakim and Fleisher (5) that the RES plays a major part in controlling plasma enzyme levels by clearing endogenous enzymes from the blood. Such a role of the reticuloendothelial system has also been suggested for proteins generally, since it was found that disappearance of labelled protein from the blood of rats was depressed by blockade with thorotrast (31).

The evidence presented here, and that of Wakim and Fleisher (5), is open to the criticism that the rise in plasma LDH following RES blockade might be due in part to tissue damage, resulting in accelerated release of tissue enzymes into the plasma. Arterial lesions have been observed in rabbits injected intravenously with carbon particles or thorotrast, but they did not develop until several days after injection (23). The dextrin associated with preparations of thorotrast, such as that used by us, has been shown to cause some degree of mast cell damage in the rat (32). However, it is difficult to explain the fall in plasma LDH level following injection with RES-stimulating agents on these grounds. Moreover, the levels of two common tissue enzymes, aldolase and alanine transaminase, remained unaltered after thorotrast or cholesterol oleate injection, in contrast to the increases observed in LDH and phosphoglucose isomerase. It is

difficult to reconcile this finding with any suggestion that generalised tissue damage is responsible for the enzyme increases following RES blockade.

The clearance rates of different enzymes vary widely, and there is no apparent correlation with molecular weight (33), even different isoenzymes being cleared at vastly differing rates (34, 35). These differences may or may not be due to clearance by different elements of the RES, but in any event offer some explanation for the specific nature of the enzyme increases following RES blockade.

Riley virus infection in mice causes a similar pattern of plasma enzyme increases to that caused by RES blockade. These increases correlate with reductions in plasma enzyme clearance rates in infected mice. The clearances of at least four enzymes, the levels of which rise in infected mice, are blocked during infection, whereas clearances of at least two other enzymes, the levels of which do not rise in the plasma of infected mice, are normal (3, 4, 6, 36). Recently we have shown that in the case of LDH, impaired clearance in infected mice occurs with the LDH-5 but not the LDH-1 isoenzyme (37).

The mechanism by which the Riley virus impairs the normal clearance mechanism for certain enzymes may be related to the persistent high level of viraemia characteristic of this infection. Circulating virus particles are cleared from the plasma by the reticuloendothelial system (38), and there may be competitive inhibition of plasma enzyme clearance by Riley virus particles. However, at least one other virus infection, the Moloney leukaemia, in which there is a viraemia of moderate duration, causes no changes in plasma enzyme levels before the development of the disease (2). Thus a prolonged viraemia does not by itself appear to cause changes in plasma enzyme level.

SUMMARY

Plasma LDH levels were determined in normal and Riley virus–infected mice following treatment with various drugs known to alter the activity of the RES. The rise in plasma LDH level after Riley virus infection was considerably enhanced by previous treatment with thorotrast (to produce blockade of the RES), and decreased by previous treatment with stilboestrol (to stimulate the RES).

A dose of 2000 r whole-body x-irradiation, lethal within 3 to 4 days, did not alter the phagocytic activity of the RES, and was without effect on plasma LDH activity in normal mice, or on the rise in plasma LDH level following infection with Riley virus.

Blockade of the RES with cholesterol oleate, thorotrast, or zymosan, resulted in a 2- to 3-fold rise in plasma LDH level within a few hours. The level returned to normal by 1 to 3 days. Stimulation of the RES with stilboestrol resulted in a decrease in plasma LDH level by 1 to 2 days in both normal and infected mice, with a return to normal by about a week.

Blockade of the RES in uninfected mice with thorotrast or cholesterol oleate, besides increasing the plasma LDH level caused a rise in plasma phosphoglucose isomerase level, but no significant alterations in plasma aldolase or alanine transaminase levels, studied up to 10 days. Riley virus causes a similar pattern of enzyme elevation. It is suggested that the increased levels of certain plasma enzymes in Riley virus–infected mice may be due to competitive inhibition by virus particles of plasma enzyme clearance by the RES.

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