Comparison of a Lactate Dehydrogenase Elevating Viruslike Agent and Eperythrozoon Coccoides.* (29286)

VERNON RILEY, J. D. LOVELESS AND M. A. FITZMAURICE
Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Rye, N. Y.

Recent reports(1,2) have suggested that the abnormal plasma lactate dehydrogenase (LDH) elevations observed in the blood plasma of mice, with or without transplanted tumors, might be caused by a Bartonella type organism, *Eperythrozoon coccoides* (EC), rather than the viruslike agent previously described in this connection(3-16). The data of this report confirm the original observations(3,4) and further demonstrate a clear distinction in respect to the LDH elevating capacity of these two dissimilar biological entities. Various differences in host enzymic and other responses are illustrated.

One of the obstacles heretofore preventing a decisive study of the specific effects of these entities has been the apparently universal contamination of EC preparations with the "LDH-agent." It was therefore necessary to separate the two infectious factors and to insure pure cultures of each prior to a systematic study of their properties and host effects.

Material and methods. Eperythrozoon coccoides (EC). The original culture of this organism was obtained from Dr. John Nelson of Rockefeller Institute. The contaminating LDH elevating agent was removed by passage of the doubly infected whole mouse blood in weanling rats since EC will survive for long periods in the rat while the "LDH-agent" disappears in about 5 days. The agent-free EC was then passed in pre-tested Swiss ICR/Ha male mice at weekly intervals. Whole blood, harvested by the orbital bleeding procedure(17), was used as a standard EC source.

LDH elevating agent. This viruslike entity was originally derived from a mouse bearing an Ehrlich carcinoma, and has been carried in this laboratory by weekly passage for about 2 years. Pooled mouse blood plasma having an infectivity titer of approximately 10^{10} ID_{50} units/ml provided the stock agent preparation for these studies(3, 7). Its freedom from EC was assured by oxophenarsine treatment of the host and test passage in splenectomized mice(2).

Mice. All mice employed were Swiss ICR/Ha male weanlings, 18-22 g, which had been pre-tested for absence of interfering agents.

LDH assay. The enzyme lactate dehydrogenase (LDH) was measured by determining rate of oxidation of DPNH in presence of excess pyruvate at pH 7.4. The details of this semi-micro procedure employing a recording spectrophotometer have been reported(7). Enzyme activity is expressed in conventional Wroblewski units, although these may be easily converted to International Units by dividing them by 2 to give μ moles of substrate utilized per liter per minute, or by 2,000 to give μ moles of DPNH oxidized per ml per minute(18).

Hematocrits. Anemia was followed by measuring the percent of packed red cells in whole mouse blood following centrifugation for 20 minutes at 2,000 RPM (1,000 $\times$ *g*), employing horizontal head No. 253 in the International refrigerated centrifuge. The mouse blood was centrifuged directly in the disposable heparinized collection pipettes and the percent hematocrit was determined by use of a specially designed chart that permits the packed RBC readings to be made independent of the amount of blood collected(17,19). The resulting plasma was harvested for enzyme and agent assay.

OPA. (oxophenarsine hydrochloride; or, 3 amino-4-hydroxyphenyl-arsine oxide hydrochloride; or 2-amino-4-arsenosophenol hydrochloride) was obtained in sealed, dated ampoules from Parke, Davis & Co. as Mapharsen. The solution for injection was freshly prepared by addition of sterile distilled water so that 0.1 ml contained 0.5 mg of OPA.

* These investigations were partially supported by the Nat. Cancer Inst., P.H.S. Grant CA-03192. The authors are also indebted to Dr. C. Chester Stock for multiple forms of support.

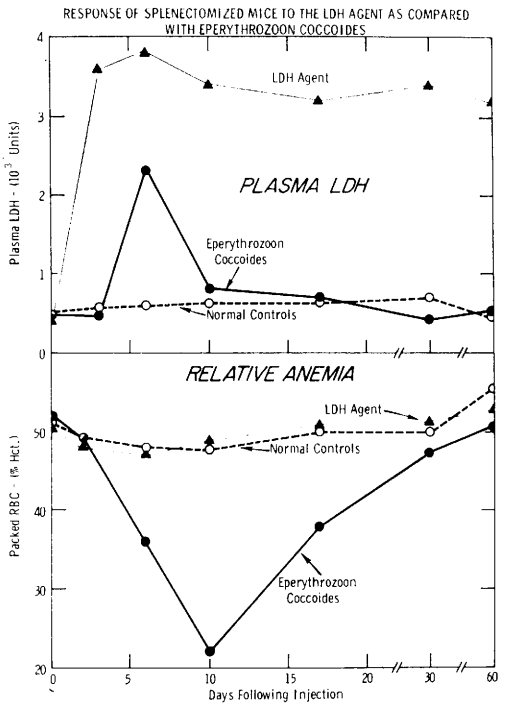

FIG. 1. Comparison of plasma lactate dehydrogenase (LDH) response of mice receiving either a pure culture of *Eperythrozoon coccoides* (EC) or an LDH elevating viruslike agent. Illustration of the differences in induction of anemia by the 2 infectious agents is shown in lower chart. Magnitude and time of occurrence of peaks are a function of the original concentration of EC.

This solution was injected subcutaneously at a dose of 25 mg/kg of mouse, or 0.5 mg per 20 g mouse.

Results. Fig. 1 illustrates the differences in plasma LDH and anemia induced in splenectomized mice by a pure culture of *Eperythrozoon coccoides* as compared with a pure preparation of the LDH elevating agent. The term "pure" is used here in the limited sense of freedom from interfering LDH elevating entities. The plasma LDH of the mice receiving the "LDH-agent" invariably rises 5- to 10-fold within 48 to 72 hours and remains at this elevated level during the entire lifetime of the host. In contrast, EC may cause no elevation, slight elevation, or a moderate elevation, depending upon the maximum titer of the EC infection and whether the host is splenectomized or not. However, in any instance of EC-induced elevation, the plasma LDH level always returns to normal, usually within 10 to 14 days. In our experience, any apparent exceptions to this are due to contamination by an LDH elevating agent or to the non-specific influence of a growing tumor(5).

Although the LDH elevating agent appears to cause a slight reduction in the host hematocrit during the first few days following injection into normal or splenectomized mice, it is insignificant compared with the overt anemia induced by EC. Fig. 1 demonstrates this host anemia as expressed by the reduction in percentage of packed red cells of the peripheral blood. The contrast in host hematocrit response to EC, as compared with that of animals injected with the "LDH-agent" is typical also of other related parameters such as RBC count and hemoglobin. A point of particular interest is the lack of correlation between degree of anemia produced by the 2 agents and resulting plasma LDH elevation. This indicates that the 2 biological entities produce LDH elevation by different mechanisms. The EC-destroyed erythrocytes appear to be the logical source of the EC-induced plasma LDH elevation. Support for this interpretation was also obtained by inducing a similar transitory rise in plasma LDH following destruction of a comparable red cell mass *in vivo* by phenylhydrazine injections. The cellular source of the higher and sustained elevation induced by the "LDH-agent" is still uncertain, although here too, erythrocytes have not been exonerated.

Arison *et al*(1,2) reported a reduction in plasma LDH of EC-infected normal and tumor-bearing animals by treatment with OPA. Since the arsenical compound destroys EC, it was assumed that the elevation was due to this organism rather than the "LDH-agent." To determine whether OPA could destroy, inhibit, or modify the "LDH-agent" and its capacity to elevate plasma LDH, normal and agent-infected mice were treated with OPA as prescribed(2). Table I illustrates the results. It was found that OPA does not eliminate the agent, nor does it reduce the agent-induced plasma LDH elevation. On the contrary, OPA causes a temporary increase in plasma LDH of both nor-

TABLE I. Response of Plasma Lactate Dehydrogenase in Normal and "LDH Agent"-Infected Mice Following Injection of Oxophenarsine (OPA).

Conditions	Plasma LDH*					
Hr post-injection	0†	1	24	48	96	168
Normal controls	440	450	400	400	500	540
" mice treated with OPA‡	500	1,300	2,300	600	600	500
LDH agent-infected controls§	3,400	3,200	3,600	3,600	3,500	3,500
" " " mice treated with OPA§	3,500	4,400	13,500	12,000	5,400	3,600

* Expressed as conventional Wroblewski units per ml of plasma, based upon 0.001 OD reduction per min. of DPNH (at 340 mμ) per ml of plasma. Each value represents average of 5 mice. Differences shown are typical of those obtained in other similar independent experiments.

† Baseline PLDH determined prior to OPA injection.

‡ Oxophenarsine injected subcutaneously at 25 mg/kg. This duplicated the recommended 0.5 mg dose per 20 g mouse(2).

§ Mice infected with "LDH-elevating agent" 26 days prior to OPA injection.

TABLE II. Synergism Between *Eperythrozoon coccoides* (EC) and an LDH Elevating Virus-like Agent as Expressed by Host Enzyme Elevation.

	Plasma LDH*					
	Intact mice			Splenectomized mice		
Day	2	7	14	2	7	14
Controls	400	400	400	700	850	620
"LDH-agent" only	2,000	3,600	3,000	2,200	3,100	3,200
EC only†	2,100	1,100	790	20,600	850	600
EC + "LDH-agent"	7,100	4,500	3,000	102,000	5,800	4,400

* Plasma LDH determined on day 2, 7, and 14 following intraperitoneal injections.

† Whole mouse blood containing a high concentration of EC obtained by repeated short term passage.

mal and agent-infected hosts. It may be noted that the OPA-induced LDH increase was substantially more in the infected mice than with the treated controls, although the relative increase was somewhat greater in the non-infected animals. A tentative explanation for this enzymic effect might be agent-induced sensitization of some host cellular component to OPA insult. These, and related experiments, establish that the original plasma LDH elevations reported(3,4) are not subject to elimination by arsenical treatment, and therefore are not primarily due to *Eperythrozoon coccoides*.

Table II illustrates one aspect of the enzymic synergistic reaction which occurs when the LDH agent and EC are present together in the mouse host. It may be seen that the substantial increase in plasma LDH exceeds the additive effects of the two agents individually at this point in the experiment. It is presumably this synergism which accounts for Arison's observation of LDH increase and its subsequent reduction by treatment with an EC-destroying arsenical(2). Such treatment would return the host plasma LDH level to the virus-induced plateau but not to a normal host level. The baseline values, and the OPA treated mouse LDH levels listed by Arison(2) appear to be in the elevated range found with the agent infection(3,7).

Discussion. The LDH elevating characteristics of *Eperythrozoon coccoides* and the LDH elevating viruslike agent are conspicuously different and readily distinguished when studied as separate entities. The studies of Arison *et al* (1,2) were apparently complicated by the unsuspected presence of the "LDH-agent." When the LDH values listed in their Tables(2) are converted to conventional Wroblewski units, the "control" mice appear to have typical elevated plasma LDH levels usually associated with agent infection. Their observation that after one week the plasma LDH activity of all tumor-bearing mice increased, whether EC infection was present or not, also suggests the typical be-

havior expected of tumors infected with the "LDH-agent"(3,4,5).

Addition of EC to such mice, either by injection or tumor implantation, has been shown to produce a further reaction in respect to increased plasma LDH. Such secondary elevation is sensitive to arsenical treatment since this reduces or eliminates *Eperythrozoon coccoides*. This readily accounts for Arison's observed decrease in plasma LDH level(2), although as indicated in Table I, such OPA treatment does not influence the stabilized "LDH virus"-induced level.

Among the various differences which distinguish the 2 agents are their titration characteristics. High titer "LDH-agent"-containing plasma can be diluted to 10^{-9} or more with continued infectious activity, while 10^{-5} or 10^{-6} is the maximum observed dilution permissible with retention of EC infectivity. This serves also as an additional method for obtaining the "LDH-agent" free from EC. Although EC may pass through a 0.3 μ Millipore filter(2), we have not observed its passage through a 50 mμ filter which readily passes the "LDH-agent."

In addition to the acute anemia induced by EC, it also causes a conspicuous enlargement of the spleen. The "LDH-agent" does not, by itself, participate significantly in either of these abnormalities, although it is capable of augmenting such effects when present in conjunction with EC(6).

Summarizing the differences observed between *Eperythrozoon coccoides* and the LDH elevating agent, it may be shown that their filtration characteristics are quite distinct, their dilution endpoints are separated by several logs, and the agent is not affected adversely by arsenicals although EC is inhibited or destroyed. Plasma LDH elevation induced by the agent never returns to normal, although that induced by EC invariably does. EC causes gross anemia in the host whereas the agent does not. Splenectomy alters both degree of anemia and LDH elevation when EC is present, but splenectomy has little influence where the agent only is involved. EC also survives for long periods in the rat while the LDH agent does not. Giemsa-stained EC is visible in the light microscope in contrast to the agent which is, of course, too small to be detected. By one or several of these criteria, distinction of these two biological entities and their recognition in a host is relatively simple.

Summary. *Eperythrozoon coccoides* (EC), a Bartonella-like organism existing as a widespread latent infection in rodents, has been separated from other known infectious contaminants so that its influence on plasma LDH (lactate dehydrogenase) elevation and other pathological properties could be studied and compared with those of an LDH elevating viruslike agent. Distinct differences in their LDH elevating characteristics were demonstrated. The viruslike agent induces a high and sustained elevation while that produced by EC is usually unremarkable in magnitude in normal intact animals, and is invariably transitory. In combination, the two infectious entities act synergistically to produce higher plasma enzyme elevations than their additive separate effects. Recent suggestions that EC may be responsible for the LDH elevations originally reported appear to be in error.

1. Arison, R. N., Cassara, J. A., Shonk, C. E., *Fed. Proc.*, 1963, v22, 602.

2. ———, PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 497.

3. Riley, V., Lilly, F., Huerto, E., Bardell, D., *Science,* 1960, v132, 545.

4. Riley, V., *ibid.,* 1961, v134, 666.

5. ———, in *Control mechanism in respiration and fermentation,* Ronald Press, 1963, 211-244.

6. ———, *N. Y. State J. Med.,* 1963, v63, 1523.

7. ———, in The pigment cell: *Ann. N. Y. Acad. Sci.,* 1963, v100, 762.

8. Riley, V., Wroblewski, F., *Science,* 1960, v132, 151.

9. Bailey, J. M., Stearman, M., Clough, J., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 148.

10. Burgess, A. E., Sylven, B., *Cancer Res.,* 1963, v23, 714.

11. Crispens, C. G., Jr., *J. Nat. Cancer Inst.,* 1963, v30, 361.

12. Mundy, L., Williams, P. C., *Science,* 1961, v134, 834.

13. Notkins, A. L., Greenfield, R. E., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 988.

14. Plageman, P. G. W., Gregory, K. F., Swim, H. E., Chan, K. K. W., *Can. J. Microbiol.,* 1963, v9, 75.

15. Rowson, K. E. E., Adams, D. H., Salaman, M. H., *Acta: Unio Internationalis Contra Cancrum,* 1963, v19, 404.
16. Yaffee, D., *Cancer Res.,* 1961, v22, 573.
17. Riley, V., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 751.
18. King, E. J., Campbell, D. M., *Clin. Chim. Acta,* 1961, v6, 301.
19. Riley, V., Valles, W. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 341.

Received February 24, 1964. P.S.E.B.M., 1964, v116.

Variation of Alkaline Phosphatase Activity Among Cells of Inducible and Constitutive Strains of Human Fibroblasts.* (29287)

GEORGE M. MARTIN (Introduced by Earl P. Benditt)
Department of Pathology, University of Washington School of Medicine, Seattle

Investigators concerned with enzyme induction in microorganisms commonly assume that all of the cells in a genetically homogeneous population behave similarly. Evidence is presented here that this may not be true for an instance of enzyme induction in cultures of diploid human fibroblasts.

Cox and Pontecorvo(1) reported differences among established strains of human fibroblasts with respect to the activity of alkaline phosphatase of lysates of the mass cultures. Under the conditions of their experiments, most strains had no measurable alkaline phosphatase activity unless permitted to grow in the presence of small amounts of a substrate of that enzyme. Two more unusual types were noted: "constitutive" strains which demonstrated relatively high enzyme activity and "non-inducible" strains, which had no measurable enzyme activity even after treatment with substrate.

Methods. Histochemical stains for alkaline phosphatase were originally performed (on coverslip monolayers) for the purpose of conveniently classifying strains with respect to their enzyme activity when grown with or without an inducing substrate (1 mM phenyl phosphate). The diploid strains, of typical "fibroblast" morphology, had been established for periods of 2-10 months by explant or trypsinization techniques and were grown in Waymouth's medium with 10% calf serum. Cultures for pleuropneumonia-like organisms were negative. A modification of the method of Fredricsson(2) was employed for the alkaline phosphatase stains. Fredricsson discovered that diffusion of enzyme could be greatly diminished when the Gomori technique was performed in an incubation medium of 40% acetone; only slight inhibition of enzyme activity resulted. Our experience confirmed this; aqueous incubations yielded a more diffuse precipitation of calcium phosphate, with less sharp localization and considerably more nuclear staining, generally accepted as an artifact(3). Fixation in formalin (at 4°C, pH 7.0 for 15 min) did not prevent this diffusion, which was quite evident when aqueous incubations were carried out in the presence of di Na p-NO_2 phenyl PO_4; even after the coverslip was removed from the incubation medium, enzymic hydrolysis continued at a rapid linear rate (as measured by the optical density of p-NO_2 phenol at 410 mμ). Markedly improved preparations resulted even with final concentrations of acetone of only 16%; the higher concentration recommended by Fredricsson was less preferable because a variable degree of precipitation of substrate was observed. A second modification was a doubling of the calcium concentration, designed to favor the "capture reaction" (precipitation of the enzymically liberated phosphate as calcium phosphate). Finally, in order to prevent resolubilization of the precipitated phosphate, all rinses subsequent to the enzyme incubation were in 40% acetone buffered with 10 mM 2 amino-2-methyl 1-propanol-HCl, pH 9-10. Controls

* Supported in part by U.S.P.H.S. grants. A portion of this work was done under a USPHS fellowship in the Genetics Dept., Glasgow University, Scotland.