

EXHIBIT D

Lodish Decl. in Support of Opposition to Roche's Motion for Summary Judgment of Invalidity for Double Patenting Over Claim 10 of the '016 Patent

Hepatic clearance of intact and desialylated erythropoietin

DANIEL W. BRIGGS, JAMES W. FISHER, AND WILLIAM J. GEORGE

Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana 70112

BRIGGS, DANIEL W., JAMES W. FISHER, AND WILLIAM J. GEORGE. *Hepatic clearance of intact and desialylated erythropoietin.* Am. J. Physiol. 227(6): 1385-1388. 1974.—Following desialylation by neuraminidase, erythropoietin (ESF) is inactive *in vivo* but retains its activity when assayed in the *in vitro* bone marrow culture. This loss of erythropoietic activity *in vivo* has been attributed to a rapid clearance of desialylated ESF (dESF) from the circulation of the assay animal, although an increased rate of clearance of dESF has not been demonstrated. In this study, rat livers were perfused with diluted rat blood containing either ESF or dESF obtained from hypoxic rats. ESF or dESF content of the perfusates was determined by measuring the stimulation of ^{59}Fe incorporation into heme in rabbit marrow cultures (*in vitro*). Perfusates containing dESF showed a rapid decline in erythropoietic activity after only 15 min of perfusion, whereas the activity of perfusates containing intact ESF remained elevated for several hours of perfusion. Desialylated, but not intact, orosomucoid prevented the loss of erythropoietic activity of dESF during liver perfusion. These results indicate that desialylation of ESF causes its rapid hepatic clearance from the circulation and that desialylated orosomucoid is able to competitively inhibit this process. These results also suggest that the mechanism of ESF catabolism by the liver is similar to that reported for certain other plasma glycoproteins.

neuraminidase; glycoprotein; metabolism; sialic acids; orosomucoid

MANY STUDIES HAVE BEEN CONDUCTED which implicate the liver as the site of inactivation of erythropoietin (erythropoietic stimulating factor, ESF). Employing isolated organ perfusions, Burke and Morse (3) demonstrated that normal rat livers reduced the ESF content of the perfusates, whereas carbon tetrachloride-damaged livers were unable to cause reductions in ESF levels. Roh et al. (15) perfused dog livers or hindlimbs with blood containing high titers of ESF and found that only the livers decreased blood levels of ESF. Briggs et al. (2) found that the hepatic inactivation of ESF *in vitro* was correlated with the activity of lysosomal enzymes.

Since ESF is a plasma glycoprotein, its method of catabolism might be similar to that of other plasma glycoproteins. Morell et al. (12, 13) after desialylating several plasma glycoproteins with neuraminidase, demonstrated that many of these proteins were rapidly cleared from the circulation and were concentrated within the hepatic parenchymal cells. Desialylated ceruloplasmin was found to be extensively degraded by the lysosomal fraction of hepatocytes following hepatic clearance from the circulation (7).

It has been demonstrated that Cohn Fraction VI from mammalian plasma contains low levels of neuraminidase (19). It is possible, therefore, that desialylation within the circulation, followed by hepatic uptake and further catabolism within the lysosomes, may be a physiologic mechanism for the metabolism of certain plasma glycoproteins.

Many investigators have reported that neuraminidase inactivates ESF *in vivo* (10, 16-18, 21); however, ESF desialylated with neuraminidase retains its activity *in vitro* in marrow cultures (5, 11). The loss of erythropoietic activity *in vivo* has been postulated to be due to the rapid clearance of desialylated ESF from the circulation of the assay animals before it can exert its effects upon target cells in the marrow (6, 11). However, it has not been demonstrated that desialylated ESF is removed from the circulation more rapidly than is the intact glycoprotein. Also, the role of the liver in this process has not been well defined.

The present studies were conducted to investigate the clearance of intact and desialylated ESF by the liver and to evaluate the effects of competitive inhibitors on this process.

MATERIALS AND METHODS

Both an *in vivo* and an *in vitro* assay for erythropoietin were employed in these studies. The *in vivo* exhypoxic polycythemic mouse assay (2, 4), which measures ^{59}Fe incorporation in RBC, can detect intact erythropoietin but not erythropoietin desialylated (dESF) with neuraminidase (10, 18). The *in vitro* rabbit bone marrow culture (8, 9), which determines ^{59}Fe incorporation into heme, can detect both ESF and dESF (5). For this assay, the femoral bone marrow was removed from male New Zealand albino rabbits and suspended in 20% new-born calf serum and 80% NCTC-109 medium. Each marrow culture plate contained 2×10^6 viable nucleated cells in 2 ml. The culture plates were stimulated with 20 μl of a test sample or a standard preparation of human urinary ESF and were incubated at 37°C in 95% air, 5% CO_2 , and 95% humidity. After 29 h, 0.5 μCi of ^{59}Fe was added to each plate and the incubation was continued for an additional 16 h. The cells were lysed with Drabkin's solution and the acidified heme was extracted with cyclohexanone.

Serum from hypoxic rats (HRS) was employed as the source of ESF in these studies. HRS was obtained by exposing male Sprague-Dawley rats to 0.42 atm pressure for 18 h in a hypobaric chamber. Upon removal from the chamber the rats were immediately anesthetized with ether and exsanguinated via the abdominal aorta. HRS was obtained

from the blood of several rats, pooled, and assayed for ESF in the polycythemic mouse assay. One milliliter of HRS was found to contain approximately 1 U of ESF. Desialylation of ESF was accomplished by incubating 15 ml HRS with 67.5 U of *Vibrio cholera* neuraminidase (General Biochemicals) at 37°C for 2 h in a Dubnoff metabolic shaker.

Aliquots of several concentrations of intact HRS or desialylated HRS were assayed for erythropoietic activity in both the *in vivo* polycythemic mouse assay and the *in vitro* marrow assay in order to determine whether the incubation of HRS with neuraminidase was desialylating ESF and thereby inactivating it in the *in vivo* assay system. Neuraminidase was also tested alone at several concentrations in both assay systems in order to determine any direct effects that this enzyme itself might have on ^{59}Fe incorporation into newly formed red cells.

Orosomucoid (ORM) was obtained from human plasma by the method of Weimer et al. (20) and was desialylated by incubating 50 mg of the freeze-dried glycoprotein for 24 h at 37°C with 150 U neuraminidase in a total volume of 1.3 ml (12).

The hepatic perfusion medium employed in these experiments was a modification of that described by Brauer et al. (1). Bovine serum albumin 2.3 g, 37.5 mg NaHCO_3 , 460 mg NaCl , 21 mg KCl , and 6 mg CaCl_2 in a total volume of 50 ml distilled water was combined with 5 ml of 0.1 M phosphate buffer (pH 7.4). Thirty milliliters of heparinized whole-rat blood were then added and the total mixture was equilibrated at 37°C with a humidified gas mixture (95% O_2 , 5% CO_2) in an organ perfusion apparatus. The medium was maintained at pH 7.4 by addition of 8% NaHCO_3 solution, as required, throughout the perfusion period.

Livers for perfusion were obtained from male Sprague-Dawley rats (225 g) anesthetized with ether. The abdominal cavity was opened and the bile duct was cannulated with PE-10 tubing. The portal vein was cannulated with PE-100 tubing and the liver was immediately flushed with 1–2 ml heparinized saline. The liver with diaphragm attached was then quickly excised and placed in the perfusion apparatus. Hepatic perfusion via the portal vein was begun immediately at a pressure of 20–22 cmH_2O . This resulted in a flow rate through the liver of 10–12 ml/min.

Each liver was allowed to equilibrate for 10–15 min until oxygen consumption and bile production were evident. Following an initial sampling of the perfusate for use as a control, 15 ml of HRS or dHRS containing 15 U ESF (or dESF) were added to the perfusion medium. This addition occurred over a 3-min period, and an additional 5 min was allowed to ensure equilibration of the ESF throughout the perfusion medium. During certain experiments, ORM or desialylated ORM (dORM) was also added to the perfusate, either at the time of the addition of dHRS or 1 h later. Perfusate samples were taken at various times following the addition of ESF to the perfusion medium. These samples were centrifuged (2,000 $\times g$, 10 min) and the supernatants decanted and frozen until assayed for ESF or dESF in the *in vitro* rabbit bone marrow assay.

Liver viability was assessed by monitoring O_2 consumption and bile production throughout the perfusion period (15). Oxygen consumption was calculated from hepatic blood flow, hemoglobin content of the perfusate, and the

arterial-venous oxygen differences, which were determined with a blood-gas analyzer (Instrumentation Laboratory, Inc., model 113). Arterial and venous Pco_2 levels, pH, and hematocrit were also monitored. Following perfusion, the livers were sectioned and compared histologically to non-perfused livers for evidence of sinusoidal congestion or cellular degeneration.

RESULTS

A comparison of the *in vivo* and *in vitro* assay systems for erythropoietin indicates that HRS had significant activity in both assays. Although dHRS was without significant erythropoietic activity in the polycythemic mouse assay, its activity was equal to or greater than that of HRS when assayed in the marrow culture (Fig. 1). Neuraminidase alone had no significant effect on ^{59}Fe incorporation in either assay system.

Control experiments were conducted in which HRS or dHRS was added to the perfusion medium and circulated for 6 h through the perfusion system with no liver present. The erythropoietic activity of these perfusates remained constant throughout the 6 h of perfusion. There was no significant difference in erythropoietic activity between perfusates containing HRS and those containing dHRS.

Figure 2 depicts two representative experiments in which livers were perfused with either HRS, dHRS, or with normal rat serum (NRS) from animals not exposed to hypoxia. At various time intervals, perfusate samples were removed from the system and assayed for erythropoietic activity in the marrow culture. The perfusate containing NRS did not possess significant erythropoietic activity at any time (*experiment I*). However, the perfusates containing intact HRS exhibited significant increases in erythropoietic activity as early as 5 min following HRS addition and retained this activity for at least 4 h (*experiments I and II*). The activity of the perfusates containing dHRS also was significantly increased at the 5-min period, but this activity declined significantly after only 15 min of perfusion and continued to decrease during the remainder of the perfusion period.

Addition of a single 50-mg dose of dORM to the perfusion medium at the same time as the dHRS addition main-

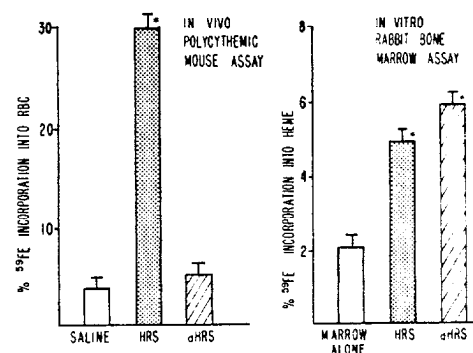


FIG. 1. Erythropoietic activity of HRS and dHRS *in vivo* and *in vitro* represented as ^{59}Fe incorporation into RBC or into heme. Bars represent mean \pm SE of 5 mice or 4 marrow culture plates. Asterisks denote significant difference ($P < 0.05$) from mice injected with saline or from marrow cells incubated alone. This graph represents 1 of 4 similar experiments.

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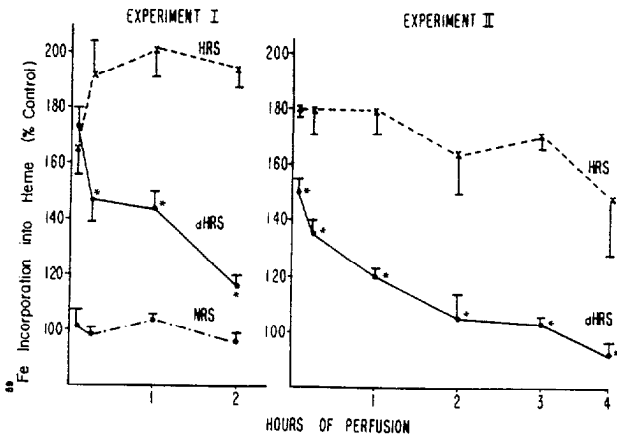


FIG. 2. Effect of desialylation upon hepatic clearance of HRS. Each point represents mean \pm SE of 3 marrow plates. Asterisks denote significant decreases ($P < 0.05$) in erythropoietic activity of dHRS when compared to HRS after a similar time of perfusion. This graph represents 2 of 5 similar experiments.

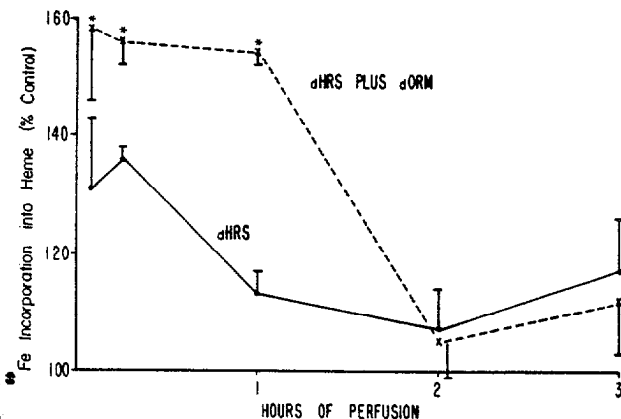


FIG. 3. Effect of a single dose of dORM on hepatic clearance of dHRS. Each point represents mean \pm SE of 3 marrow plates. Asterisks denote significant increases ($P < 0.05$) in erythropoietic activity of "dHRS plus dORM" when compared to "dHRS alone" after a similar time of perfusion.

tained, for at least 1 h, a level of erythropoietic activity which was significantly greater than that observed after addition of dHRS alone (Fig. 3). After 2 h of perfusion, the activity of the perfusate containing dORM plus dHRS had declined to a level equal to that of the perfusate containing dHRS alone.

In an additional series of experiments, a second 50-mg dose of dORM was added to the perfusion medium after 1 h of perfusion. When this additional dose was given, the activity of the dHRS-dORM perfusate remained elevated throughout the entire perfusion period (Fig. 4). When two doses of "intact" ORM were substituted for dORM, the perfusate did not maintain elevated levels of erythropoietic activity, but instead the activity declined in a manner similar to that observed with dHRS alone (Fig. 4).

A series of experiments was also conducted in order to determine whether ORM or dORM was directly enhancing the stimulatory effect of HRS or dHRS on ^{59}Fe incorpora-

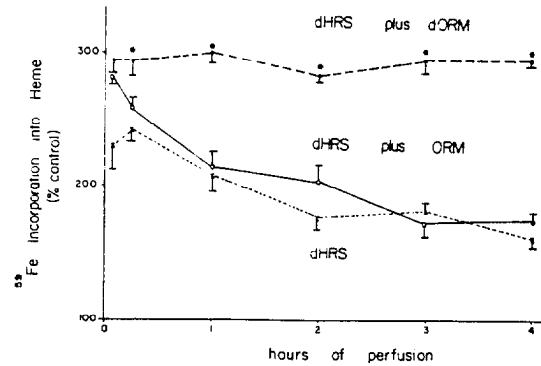


FIG. 4. Effects of 2 doses of ORM or dORM on hepatic clearance of dHRS. Each point represents mean \pm SE of 3 marrow plates. Asterisks denote significant increases ($P < 0.05$) in erythropoietic activity of "dHRS plus dORM" when compared to "dHRS alone" after a similar time of perfusion.

tion in the marrow culture. Neither ORM nor dORM significantly affected the erythropoietic activity of HRS or dHRS in this assay system.

DISCUSSION

Following incubation with neuraminidase, HRS lost its erythropoietic activity in the in vivo mouse assay but retained its activity in the in vitro marrow assay (Fig. 1). This indicates that incubation with neuraminidase results in the desialylation of ESF (6, 11). Neuraminidase alone did not stimulate ^{59}Fe incorporation into the heme of the marrow cells. Therefore, the slightly increased activity of dHRS over HRS seen in our marrow cultures (Fig. 1) appears to be a direct result of the dHRS acting upon the marrow. It is possible that desialylation increases the potency of erythropoietin by enabling it to bind more easily to receptors on the marrow cell surface or to more easily gain access to the cell interior. Since neuraminidase desialylates other glycoproteins besides ESF, it is not possible to exclude a role for other desialylated glycoproteins in the enhanced erythropoietic activity of dHRS seen with the marrow culture assay.

Goldwasser et al. (6) reported a threefold increase in the activity of dESF over intact ESF when these substances were compared in dilute concentrations in the marrow assay. Our results do not support this finding, since after dilution in the perfusion medium, HRS and dHRS exhibited comparable erythropoietic potency in the marrow cultures. However, both our source of ESF and our assay methods differed from those employed by these investigators and this may explain the difference in the results.

As shown in Figs. 2-4, the initial activities of the perfusates containing dHRS alone were usually lower than the initial activities of intact HRS or of dHRS plus dORM. Since the initial samples were taken after 5 min of perfusion, hepatic clearance of the dESF added to the medium may have already begun, thus resulting in the lower erythropoietic activity.

Our results are in agreement with the proposal that ESF may be rapidly cleared by the liver following desialylation (6). Intact ESF appears to be removed at a much slower

rate than the desialylated substance (Fig. 2). Since neuraminidase exists in normal mammalian plasma (19), it is possible that desialylation may be an early step in the clearance of ESF and other glycoproteins from the circulation.

Our data do not provide direct evidence that dESF enters the interior of the hepatic cells. However, since a single dose of dORM could sustain the erythropoietic activity of the perfusate only temporarily (Fig. 3), it is probably that dORM was being preferentially cleared by the liver and thereby competitively inhibiting the uptake of dESF. Once dORM was removed from the perfusate, dESF could be rapidly cleared, resulting in the observed decline in erythropoietic activity to control levels. The results of repeated administration of dORM (Fig. 4), together with the studies of Morell et al. (12) using dORM, also suggest that desialylated ESF is not merely being bound to some extracellular receptor. The physiological significance of this competitive inhibition in the hepatic clearance of dESF is further supported by the report of Goldwasser et al. (6) that dORM can maintain the erythropoietic activity of dESF *in vivo*.

The sustained erythropoietic activity of the perfusates containing intact HRS and the inability of intact ORM to prevent the loss of activity of perfusates containing dHRS indicate a degree of specificity of hepatic receptors for desialylated glycoproteins. These findings are in agreement with earlier studies (13) which reported that rapid clearance of desialylated ceruloplasmin occurred only when intact galactose residues of this protein were exposed. Lukowsky and Painter (11) and Goldwasser et al. (6) have recently

shown that oxidation of dESF with galactose oxidase restores its erythropoietic activity *in vivo*. This suggests that dESF and desialylated ceruloplasmin may both bind to a common receptor site for galactose. Pricer and Ashwell (14) have found that the plasma membranes of the liver are major loci of binding for circulating glycoproteins. They also reported that the presence of sialic acids on circulating glycoproteins was incompatible with binding to these membranes, thus suggesting that desialylation may be a prerequisite for clearance.

The present studies clearly show that the erythropoietic activity of dHRS declines rapidly during hepatic perfusion, while the activity of intact HRS remains elevated for several hours. The dORM, but not the intact ORM, effectively prevents this decline in the activity of dHRS, possibly by competitively inhibiting hepatic binding and uptake. These results provide evidence that desialylated erythropoietin is cleared from the circulation much more rapidly than is the intact molecule and that the liver may be the organ responsible for this clearance.

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