PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 159, 359-363 (1978)

The Effect of Glucocorticoid Antagonizing Factor on Hepatoma Cells (40348)

K. J. GOODRUM[1] AND L. J. BERRY

Department of Microbiology, The University of Texas, Austin, Texas 78712

Endotoxin poisoned animals are refractory to hydrocortisone induced glucose synthesis and glycogen deposition in the liver (1). Endotoxin also inhibits the hydrocortisone induced synthesis of several hepatic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK), one of the key enzymes in gluconeogenesis (2). The cortisol antagonist in endotoxin-poisoned animals, glucocorticoid antagonizing factor (GAF), is a heat and trypsin sensitive serum borne factor believed to be released by the poisoned host's macrophages (3).

In vivo studies of GAF are difficult, and its precise quantitation has not been possible. To overcome problems inherent in animal studies, an *in vitro* system involving cultured minimal deviation Reuber H35 rat hepatoma cells has been adapted for study of the endotoxin–cortisol antagonism. These cells are responsive to corticosteroids and retain fully inducible PEPCK activity (4) even when endotoxin is added directly to the culture. However, when serum from endotoxemic animals or the supernatant fluid from a poisoned macrophage culture is added to the hepatoma cells sufficient GAF is present to block cortisol induced PEPCK synthesis. Thus, hepatoma cells are suitable for the assay of the endotoxin induced cortisol antagonist, GAF. The present report makes the usefulness of these cells evident.

Materials and methods. Animals. Specific pathogen free CD1 mice of both sexes 8–10 weeks old were employed. They were given food and water *ad libitum.*

Enzyme induction. PEPCK induction was initiated in mice by injecting subcutaneously (sc) into the interscapular region one mg of hydrocortisone acetate (cortisol, Sigma Chemical Co., St. Louis, MO) suspended in 0.2 ml of sterile saline containing 0.0025% Tween-80 (Sigma). PEPCK synthesis was also induced by two ip injections at 90 min intervals of 500 μg N^6-$O^{2'}$-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid (dibutyryl cyclic AMP, Sigma) plus 1 mg theophylline (Sigma) dissolved in sterile saline.

Endotoxin. Endotoxin extracted from *Salmonella typhimurium,* SR11, by the method of Westphal and Jann (5) was dissolved in sterile nonpyrogenic saline (Travenol Labs, Deerfield, IL) for injection.

Cell culture conditions. A cloned line (KRC7) of Reuber H35 cells derived from the H35 rat hepatoma (6, 7) were obtained from Dr. W. D. Wicks, Department of Pharmacology, University of Colorado Medical Center, Denver. Experimental cultures were grown as monolayers in 60 × 15 mm glass petri plates in Dulbecco's modified Eagle's medium (KC Biological, Inc., Lenexa, KS) containing 5% fetal bovine serum, 5% calf serum, 50 μg/ml streptomycin and 75 units/ml penicillin G. Stock cultures were passaged by trypsinization every week. All cultures were grown in a humidified incubator at 37° in an atmosphere of 5% CO_2-95% air. The medium was changed once on day 4 and cells were used when confluent, i.e., after 7–8 days of subculture (200,000 cell inoculum per plate).

Experimental conditions for cell culture. When cells were used experimentally, Dulbecco's medium was replaced with serum-free medium of the same formulation and left overnight (ca. 12 hr). At this time, fresh serumless medium was added along with inducers and inhibitors that were dissolved in the same serum-free medium. The final volume of medium was 5 ml/plate. Stock solutions of hydrocortisone-21-sodium succinate, N^6,$O^{2'}$-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid, and theophylline were added to cultures to give final concentrations of 1 μ*M*, 0.5 m*M*, and 1 m*M*, respectively.

Enzyme assays. Enzyme activity was measured in the cytosol fraction of H35 cells. Hepatoma monolayers were washed with sa-

[1] Present address: Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514.

0037-9727/78/1593-0359$01.00/0

line and suspended in 1.0 ml of 0.15 *M* KCl, 0.001 M EDTA, pH 7.6. Cells were fractured by three cycles of freeze-thawing in a dry ice-acetone bath and the cytosol fraction was isolated by centrifugation for 20 min at 20,000*g* at 4°. PEPCK activity was measured by the $NaH^{14}CO_3$ fixation assay as described by Ballard and Hanson (8). The cytosol activity of tyrosine aminotransferase (TAT) was determined by the method of Diamondstone (9). Protein concentration was determined by the method of Lowry *et al.* (10). Hepatic PEPCK activity was determined by the method of Phillips and Berry (11).

Collection of serum. Serum from endotoxin treated mice was collected 2 hr after iv injection of 50 μg endotoxin, then filtered through 0.45 μm filters (Millipore Corp., Bedford, MA) and stored at −20°.

Reticuloendothelial system activation. Mice were primed for serum GAF production by pretreatment with Zymosan-A (Sigma). Priming of mice consisted of 3 iv injections, the first of 0.5 mg and the others of 1.0 mg zymosan given on consecutive days. Serum was collected 48 hr after the last zymosan dose.

Leucocyte preparations. Peritoneal exudate cells (PEC) were collected four days after ip injection of 3 ml of NIH thioglycollate broth (Difco Lab, Detroit, MI) by peritoneal washing with 3 ml of Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS), pH 7.4. Cells were centrifuged (500*g*, 15 min) and resuspended in serumless Dulbecco's medium for culture at 37° in 5% CO_2-95% air. Nonadherent cells were removed by washing after 2 hr incubation. Adherent cells were incubated further in fresh media. Cell viability was determined by dye exclusion of the vital dye, trypan blue. Cell numbers were determined by direct count in a hemacytometer (American Optical Corp., Buffalo, NY).

Collection of conditioned medium from macrophage cultures. Adherent cells from mouse PEC were cultured in serumless media with or without 10 μg/ml endotoxin for 24 hr. The supernatant fluid was collected and concentrated 10× by ultrafiltration (Millipore Immersible Molecular Separator). Remaining salts and small molecules were removed by elution of the concentrate through Bio-gel P-6 (Bio-Rad Lab., Richmond, CA). Protein rich fractions were pooled and reconcentrated to the original concentrate volume. Concentrates were filter sterilized through 0.45 μm filters and stored at −20°.

Statistics. Statistical significance between means was determined by the rank-sum test of White (12).

Results. The inhibition by endotoxin of cortisol induced PEPCK synthesis is believed to be a mediated effect (1, 3). Direct evidence for this hypothesis is presented in Table I which shows that endotoxin has no inhibitory effect on induced PEPCK synthesis in cultured hepatoma cells exposed to either hydrocortisone or to dibutyryl cyclic AMP.

GAF-rich serum from zymosan treated endotoxin-challenged mice (ZES) when injected into endotoxin tolerant mice inhibits PEPCK induction (13). A similar response is seen in hepatoma cells (Table II). Addition of this serum to a final concentration of 2% in the culture medium totally blocks cortisol induced PEPCK synthesis but has no effect on induction of the enzyme by dibutyryl cyclic AMP. Similar results were obtained with hepatoma cells when rat serum was the source of GAF. ZES does not significantly inhibit TAT synthesis *in vitro* (Table II) nor does endotoxin inhibit cortisol induced TAT synthesis *in vivo.*

Normal mouse serum possesses some background inhibitory activity and produces a small reduction in cortisol induced enzyme synthesis. This is seen from the data in Table III. Serum from normal mice given endotoxin

TABLE I. INDUCTION OF PEPCK IN ENDOTOXIN TREATED HEPATOMA CELLS.

	PEPCK Activity ± SEM[a]		
	Inducer added to medium		
Treatment	None	1 μ*M* Hydrocortisone	0.5 m*M* Dibutyryl cyclic AMP + 1.0 m*M* theophylline
None	40 ± 2 (6)[b]	96 ± 2 (6)	90 ± 2 (6)
Endotoxin 10 μg/ml	41 ± 2 (6)	94 ± 2 (6)	88 ± 2 (6)

[a] Mean activity as units (nmoles $NaH^{14}CO_3$ fixed/min) per mg protein ± SE of the mean for 8-hr induction period.

[b] Number of samples.

TABLE II. INDUCTION OF PEPCK AND TAT IN HEPATOMA CELLS EXPOSED TO SERUM WITH GAF ACTIVITY.

Inducer added to medium	PEPCK activity[a]		TAT activity[b]	
	Control cells	ZES treated[c] cells	Control cells	ZES treated[c] cells
None	39 ± 2 (6)[d]	41 ± 1 (6)	36 ± 2 (6)	140 ± 9 (6)
Hydrocortisone 1 μM	75 ± 3 (6)	39 ± 1 (6)	276 ± 10 (6)	249 ± 9 (6)
Dibutyryl cyclic AMP 0.5 mM + theophylline, 1 mM	71 ± 5 (6)	72 ± 5 (6)	—	—

[a] Mean activity as units (nmoles $NaH^{14}CO_3$ fixed/min) per mg protein ± SE of the mean for 8-hr induction period.

[b] Mean activity as units (μg p-hydroxyphenylpyruvate formed/10 min) per mg protein ± SE of the mean for 8-hr induction period.

[c] Zymosan primed mice challenged with endotoxin, 2 hr serum added to 2% (v/v) in medium.

[d] Number of samples.

TABLE III. INDUCTION OF PEPCK IN HEPATOMA CELLS EXPOSED TO NORMAL SERUM AND SERUM FROM ENDOTOXIN POISONED MICE.

Additions to medium	PEPCK activity[a]	
	Control	1 μM hydrocortisone
None	57 ± 8 (6)[b]	105 ± 8 (6)
Normal mouse serum (0.1 ml)	—	85 ± 6 (6)
Endotoxin serum[c] (0.1 ml)	—	70 ± 9 (6)

[a] Mean activity as units (nmoles $NaH^{14}CO_3$ fixed/min) per mg protein ± SE of the mean for 8 hr induction.

[b] Number of samples.

[c] Serum collected 2 hr post 50 μg endotoxin iv.

TABLE IV. PEPCK INDUCTION BY DIBUTYRYL CYCLIC AMP IN ENDOTOXIN POISONED MICE.

Treatment	PEPCK activity[a]	
	Cortisol treated[b] mice	Dibutyryl cyclic AMP treated[c] mice
Control	224 ± 7 (7)[e]	206 ± 12 (6)
Endotoxin[d]	111 ± 12 (7)	207 ± 15 (6)

[a] Activity expressed as μmoles PEP/g dry wt liver/6 min ± SE of the mean. Assays performed 4 hr after inducer injection.

[b] 1 mg sc.

[c] 2 doses of 500 μg ip dibutyryl cyclic AMP + 1 mg theophylline.

[d] 200 μg ip 6 hr prior to enzyme induction.

[e] Number of mice.

is markedly more inhibitory than normal serum but is less inhibitory than ZES (Tables II and III). Base levels of PEPCK are not significantly affected by endotoxin or serum addition over the 8-hr incubation period. Basal TAT activity was elevated after addition of serum from endotoxin poisoned mice. Altered serum insulin levels may account for this effect (4). Neither endotoxin nor serum samples were cytotoxic for the hepatoma cells for the duration of the experiments.

Dibutyryl cyclic AMP induced PEPCK synthesis is unaltered in both hepatoma cells exposed to ZES and in mice poisoned with endotoxin (Table IV).

Figure I demonstrates that GAF-rich serum (ZES) diluted step-wise produces progressively less inhibition of PEPCK in H35 cells. Thus a 50% inhibitory dose (ID_{50}) of serum can be determined as the amount that produces 50% inhibition of control PEPCK induction by hydrocortisone. Serum pools were titrated accordingly for GAF activity with the results shown in Table V. Normal mouse serum contains a titer of from 2–8 ID_{50}'s. Endotoxin challenge increases the titer to 13, while serum from zymosan primed mice has an average titer of 28.

As little as 0.025 ml (0.5%) of GAF-rich serum consistently produced a significant inhibition of PEPCK induction in hepatoma cells. Injection of at least 10 times this much serum is required to detect enzyme inhibition in mice (13).

Hepatoma cells were used to confirm *in vivo* experiments (3) showing production of GAF by macrophages. Supernatant fluids from adherent mouse PEC were collected 4 and 24 hr after the addition of endotoxin to macrophage cultures. Concentrated fluids

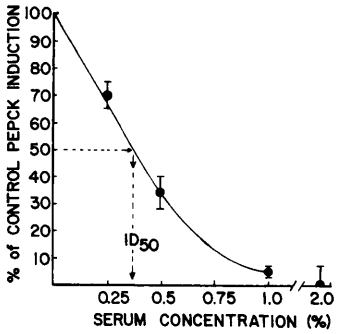

FIG. 1. Titration of serum glucocorticoid antagonizing activity in hepatoma cells. Serum was collected from zymosan pretreated mice 2 hr after challenge with 50 μg iv endotoxin. The serum pool was diluted in culture medium in a stepwise manner and added to hepatoma cultures. Values represent PEPCK activity expressed as a percentage of control (no serum addition) induction after 8 hr exposure to 1 μ*M* hydrocortisone. Each point represents the mean for four observations ± SE of the mean.

TABLE V. TITERS OF GAF IN MOUSE SERUM.

Source of serum[a]	Titer ID_{50}/ml[b]
Normal mice:	
untreated	6 ± 2 (5)[d]
endotoxin treated, 50 μg iv, (ES)	13 ± 1 (6)
Zymosan treated mice[c]:	
untreated	6 ± 1 (3)
endotoxin treated, 50 μg iv, (ZES)	28 ± 4 (7)

[a] Serum collected at 2 hr after endotoxin.

[b] ID_{50} = amount of serum inhibiting control PEPCK induction by 50%.

[c] Zymosan treated mice received 0.5 mg, 1.0 mg, and 1.0 mg of zymosan iv on days 4, 3, and 2 prior to endotoxin challenge.

[d] Number of serum pools titered.

from both unpoisoned and poisoned macrophages significantly inhibited PEPCK induction in hepatoma cells (Table VI). Fluids from unpoisoned cells may inhibit induction because of GAF release as a result of physical manipulation of the cells or because endotoxin contaminated the glassware. It is significant that the inhibition seen with the macrophage product is specific for PEPCK since TAT remains inducible in hepatoma cells exposed to macrophage supernatant fluids.

Discussion. Endotoxin suppresses cortisol induced enzyme synthesis by stimulating a secondary inhibitor, GAF. Cortisol induced PEPCK synthesis in cultured hepatoma cells has now been found to be a valuable assay

TABLE VI. EFFECT OF MACROPHAGE CULTURE SUPERNATANTS ON PEPCK AND TAT INDUCTION IN HEPATOMA CELLS.

Additions to medium	PEPCK Activity ± SEM[a]	TAT Activity ± SEM[b]
Controls:		
None	39 ± 3 (6)[d]	61 ± 3 (6)
1 μ*M* Hydrocortisone	80 ± 4 (6)	554 ± 46 (6)
1 μ*M* Hydrocortisone + Macrophage supernate from:		
4 hr untreated cells[c]	58 ± 1 (6)	529 ± 28 (6)
4 hr endotoxin treated cells[c]	51 ± 3 (6)	495 ± 19 (6)
24 hr untreated cells	64 ± 3 (6)	628 ± 34 (6)
24 hr endotoxin treated cells	50 ± 1 (6)	580 ± 27 (6)

[a] Mean activity as units (nmoles $NaH^{14}CO_3$ fixed/min) per mg ± SE of the mean for 8 hr induction.

[b] Mean activity as units (μg *p*-hydroxyphenylpyruvate formed/10 min) per mg protein ± SE of the mean for 8 hr induction.

[c] Adherent mouse PEC cultured with or without 10 μg/ml endotoxin. 10× concentrated and desalted supernate from 1.27×10^7 cells added to 10% (v/v) concentration on hepatoma cells.

[d] Number of samples.

for GAF activity. Particular advantages of this *in vitro* assay over *in vivo* assays include insensitivity to endotoxin and detection of 5–10 times less GAF than that detectable by hepatic enzyme responses in mice.

GAF as assayed in hepatoma cells is specific for cortisol induced PEPCK synthesis since it has no effect on dibutyryl cyclic AMP induced PEPCK synthesis. Cortisol is thought to induce PEPCK synthesis by stimulating DNA transcription and production of new messenger RNA, while dibutyryl cyclic AMP is believed to stimulate translational steps of PEPCK synthesis (4). GAF, therefore must block production of new specific messenger RNA but not alter the translation of existing messenger RNA. GAF does not appear to block cellular entry of cortisol since TAT remains fully inducible by cortisol. Interaction of GAF with specific cortisol receptors or receptor sites for hormone-receptor complexes in the nucleus has not been examined. Results with the hepatoma system indicate that GAF lacks species specificity between mice and rats.

Until now, assays for GAF were possible

only *in vivo* and no satisfactory dose response could be achieved (13). Hence, precise quantitation of GAF was impossible. The ability to quantitate serum GAF by titration in hepatoma cells provides a valuable tool for analyzing the responsiveness of various animals to endotoxin. It is significant that zymosan treated mice show higher GAF titers than normal mice following endotoxin challenge. Zymosan and other agents which produce hepatosplenomegaly sensitize to endotoxin lethality (14) and to rapid hypoglycemic shock. If GAF reduces gluconeogenesis by blocking PEPCK synthesis and possibly that of other enzymes in the gluconeogenic pathway, then animals sensitized to the lethal effects of endotoxin should have elevated GAF responses as zymosan treated mice do. The detection of GAF activity in culture medium from adherent mouse peritoneal cells confirms the proposed lymphoreticular source of GAF (3, 13).

The presence of GAF-like activity in normal serum may indicate a role for GAF as a normal metabolic and immunologic regulator. Adrenal cortical steroids are powerful inhibitors of immune responses so that their modulation could be advantageous under conditions of stress (i.e., infection) when endogenous cortisol is released. Cultured hepatoma cells have potential uses for assay of GAF production in animals following infection or endotoxin poisoning; quantitative assay for the purification of GAF; and qualitative analysis of the mechanism of cortisol antagonism.

Summary. Glucocorticoid antagonizing factor, GAF, from cultured macrophages and in serum of endotoxemic mice blocks cortisol induction of phosphoenolpyruvate carboxykinase in Reuber H35 rat hepatoma cells. Direct endotoxin treatment of hepatoma cells was not inhibitory. Dibutyryl cyclic AMP induced enzyme synthesis and cortisol induced synthesis of tyrosine aminotransferase were not affected by GAF. Phosphoenolpyruvate carboxykinase induction by cortisol in hepatoma cells could be used to quantitate levels of GAF in serum. This assay system is ten times more sensitive than *in vivo* assays for GAF and it can also be used to titrate samples for comparing GAF responses.

This work was supported in part by Grant No. AI-10087 from the National Institute of Allergy and Infectious Diseases.

1. Berry, L. J., *in* "Microbial Toxins" (S. Kadis, G. Weinbaum, and S. J. Ajl, eds.), Vol. V, p. 165. Academic Press, New York (1971).
2. Rippe, D. F., and Berry, L. J., Inf. Immun. **6,** 766 (1972).
3. Moore, R. N., Goodrum, K. J., and Berry, L. J., J. Reticuloendothel. Soc. **19,** 187 (1976).
4. Wicks, W. D., Barnett, C. A., and McKibbin, J. B., Fed. Proceed. **33,** 1105 (1974).
5. Westphal, O., and Jann, K., *in* "Methods in Carbohydrate Chemistry" (R. L. Whistler, J. N. Bemiller, and M. L. Wolfram, eds.), Vol. V, p. 83. Academic Press, New York (1965).
6. Reuber, M. D., J. Nat. Cancer Inst. **26,** 891 (1961).
7. Pitot, H. C., Peraino, C., Morse, P. A., and Potter, V. R., Nat. Cancer Inst. Monogr. **13,** 229 (1964).
8. Ballard, F. J., and Hanson, R. W., J. Biol. Chem. **244,** 5625 (1969).
9. Diamondstone, T. I., Anal. Biochem. **16,** 395 (1966).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
11. Phillips, L. J., and Berry, L. J., Amer. J. Physiol. **218,** 1140 (1970).
12. White, C., Biometrics **8,** 33 (1952).
13. Moore, R. N., Goodrum, K. J., Couch, R. E., Jr., and Berry, L. J., Inf. Immun. **19,** 79 (1978).
14. Benacerraf, B., Thorbecke, G. J., and Jacoby, D., Proc. Soc. Exp. Biol. Med. **100,** 796 (1959).

Received June 23, 1978. P.S.E.B.M. 1978, Vol. 159.