Correlation between Concentration of Serum Free Fatty Acids and Ketone Bodies in Alloxan Diabetes (33530)

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Söling *et al.* (1) reported the ketone body formation by the isolated perfused rat liver to be dependent on the quantity of free fatty acids (FFA) provided. On the other hand Heimberg *et al.* (2) found the ketone body production by the perfused rat liver was not proportional to the concentration of perfusate FFA. Similarly, it is not clearly established, whether the development of ketosis in the whole animal is due solely to increased FFA levels in blood or also to concomitant metabolis alterations. The relationship between FFA and ketone bodies in the blood seems to be limited. Blackard and Omori (3) as well as Willms *et al.* (4) observed different blood ketone body responses to norepinephrine-induced FFA elevation in various groups of diabetic patients. During investigations on alloxan diabetic rats in this laboratory, we made the following observations: 2 days after injection of alloxan the mean serum FFA level increased to 1800 μeq/liter and the blood ketone body concentration averaged 8000 μmoles/liter. In untreated chronically diabetic rats (from the fifth week after alloxan) with only moderately increased serum FFA levels and normal blood ketone concentrations, oral fat loading produced a rise in the FFA up to 4000 μeq/liter, but the ketone concentration did not exceed 4000 μmoles/l (5). Thus it appears that the development of severe ketosis occuring within the first few days after alloxan administration can not be accounted for on the basis of elevated serum FFA alone, since very high FFA levels led to a relatively moderate ketonemia in chronically diabetic rats. It must be noted, however, that although in the acutely and chronically diabetic rats serum FFA elevation was of similar order of magnitude in our experiments, the source of the FFA was somewhat different. In the acute state, serum FFA originated in the adipose tissue and were mainly derived from fat mobilization, whereas in the chronic state the rise of FFA was produced by the hydrolysis of the dietary triglycerides.

In the present study, therefore, we investigated the correlation between serum FFA level and blood ketone body concentration both in acutely and chronically diabetic rats after oral fat loading. Thus the major source of FFA was the same in both groups.

Materials and Methods. Male Sprague-Dawley rats, weighing approximately 250 g, were used. Diabetes was produced by subcutaneous injection of 135 mg of alloxan monohydrate (freshly prepared 9.0% solution in 0.9% NaCl) per kg of body weight.

Since an intense ketoacidosis develops in the rats during the first days after alloxan, the fat loading of acutely diabetic animals was carried out 6 days after alloxanization. At this time the initial ketonemia and FFA elevation is reduced almost to the characteristic values of untreated chronic alloxan diabetes. Based on previous work (6) we assume these rats are on the borderline between the two stages of diabetes and develop ketosis more easily than those with long standing diabetes. Only rats which exhibited a 4+ urine test for glucose as indicated by Tes-Tape (Lilly) and showed ketonuria judged with Acetest tablets (Ames) were used. Fat loading of chronically diabetic rats was carried out 6 weeks after alloxanization. Selection of diabetic rats was based on the following criteria: (a) retardation in growth; (b) daily output of more than 80 ml of urine containing more than 2% glucose; and (c) an average daily food intake of more than 20 g. The rats thus selected have fed blood sugar levels above 500 mg/ 100 ml in the acute and chronic stage, as determined with a glucose oxidase technique (7).

In order to study more closely the effect of

TABLE I. Serum FFA and Blood Ketone Body Concentrations in Acutely and Chronically Alloxan Diabetic Rats after Oral Fat Loading.[a]

Time after alloxan administration	No lard feeding			2 hr after first lard feeding		
	Serum FFA (μeq/liter)	Blood acetone (μmoles/liter)	Ratio FFA/acetone	Serum FFA (μeq/liter)	Blood acetone (μmoles/liter)	Ratio FFA/acetone
6 days	638 ± 62	2154 ± 254	0.34 ± 0.1	2150 ± 147	3835 ± 284	0.59 ± 0.07
6 weeks	491 ± 44	534 ± 96[b]	1.35 ± 0.2[b]	2855 ± 321	1890 ± 201[b]	1.58 ± 0.18[b]

[a] Values are means ± SE of means; $n = 12$.

[b] Differences between acutely and chronically diabetic rats were statistically significant ($p < 0.01$).

the duration of insulin deficiency a second experiment was performed. In Expt. 2, subcutaneous injections of 4 units of Zinc-Protamin-Insulin Novo (a gift from Novo Industry A/S Copenhagen) were given daily from the second to the twentieth day after alloxan. The fat loading was carried out 6 days and 6 weeks after the last insulin injection. The selection of the diabetic rats was based on the same criteria as described above.

Without prior fasting, lard was fed twice by stomach tube, 2.5 ml/100 g of body weight 60 min apart, to acutely and chronically diabetic rats. Twelve rats from each group were killed by bleeding from the jugular vein at 2 hr after the first lard feeding. The blood samples were collected in chilled tubes. Free fatty acids were determined in serum by the procedure of Dole (8) modified by Trout *et al.* (9) with Nile blue as indicator. Total ketone bodies were measured in whole blood by the method of Bessman and Anderson (10). The ratio FFA/acetone was calculated separately for each animal.

Results. Expt. 1 As shown in Table I the serum FFA level as well as the blood ketone concentration were higher at 6 days than at 6 weeks after alloxan. Fat loading resulted in an increase of FFA and ketone levels in both groups. In the acutely diabetic rats, however, the blood ketone concentration was significantly higher but the serum FFA level was lower than in the chronically diabetic rats. Thus the ratio FFA to ketone bodies in the acute diabetic state is low in comparison to that calculated for chronic diabetes.

Expt. 2 The results of this experiment are recorded in Table II. In the alloxan diabetic rats maintained on insulin for 18 days the FFA and ketone body concentrations were about the same both at 6 days and at 6 weeks after insulin with drawal. After fat feeding, blood ketone levels were greater than the corresponding values obtained in Expt.

TABLE II. Serum FFA and Blood Ketone Body Concentrations in Alloxan Diabetic Rats after Oral Fat Loading (Expt. 2).[a]

Time after insulin withdrawal	No lard feeding			2 hr after first lard feeding		
	Serum FFA (μeq/liter)	Blood acetone (μmoles/liter)	Ratio FFA/acetone	Serum FFA (μeq/liter)	Blood acetone (μmoles/liter)	Ratio FFA/acetone
6 days	538 ± 56	1649 ± 201	0.40 ± 0.07	1768 ± 166	6422 ± 1097	0.33 ± 0.04
6 weeks	416 ± 35	1789 ± 215	0.28 ± 0.05	2938 ± 251[c]	3107 ± 598[b]	1.29 ± 0.24[c]

[a] Rats were injected with 4 units of PZI daily for 18 days. Values are means ± SE of means; $n = 12$.

[b] Difference between acutely and chronically insulin deficient rats was statistically significant ($p < 0.02$).

[c] Differences between acutely and chronically insulin deficient rats were statistically significant ($p < 0.01$).

1. There were higher ketone body concentrations and lower FFA levels in the rats 6 days after insulin withdrawal than in the rats at 6 weeks of insulin deprivation. The difference in the FFA/ketone ratio after fat loading is highly significant.

Discussion. As generally accepted, there is a marked mobilization of fat from adipose tissue immediately after alloxan, which results in elevated blood FFA levels and severe ketoacidosis. In the chronic state the fat stores are exhausted and subsequently fat mobilization ceases, followed by a decrease of the FFA and ketone concentration in blood. Our study, however, demonstrated that also after fat feeding, when the FFA levels were almost the same in the two groups, the ketonemia was greater in the acutely diabetic rats than in those with long standing diabetes. When the fat loading was carried out in diabetic rats that had been insulin treated the FFA/ketone ratio was significantly higher at 6 weeks than at 6 days after insulin withdrawal. These results confirm the opinion that the degree of hyperketonemia is only partially dependent on the serum FFA levels. Furthermore they indicate that elevated FFA levels lead to a greater ketonemia in the acute than in the chronic state of insulin deficiency.

Two hr after feeding a rather large amount of fat we expected the results to indicate mainly the relation of exogenous fat to the ketonemia. In Expt. 1 without fat feeding, the FFA/ketone ratio was higher at 6 weeks than at 6 days after alloxan, suggesting that even at low FFA levels the ratio depends on the duration of the diabetic state.

It is of interest that the degree of ketonemia in insulin treated rats (Expt. 2) was different from that in untreated rats (Expt. 1). Six weeks after insulin withdrawal the ketone concentration was still somewhat elevated while the FFA levels were low, resulting in a low FFA/ketone ratio. In addition, after fat feeding we observed higher ketone concentrations than in Expt. 1 at each time interval. These findings are in agreement with the results reported by Steiner *et al.* (11) in respect to an increased susceptibility to ketonemia of insulin-treated alloxanized rats after insulin withdrawal. In our experiments, however, this is not related to fat mobilization, since the ketone concentrations changed independently of the changes in the FFA levels.

More recently Foster (12) demonstrated the degree of ketonemia in starved rats was unrelated to changes in the mobilization and oxidation of fatty acids. He suggested that the control of ketonemia may be in part mediated by insulin itself. The finding that after insulin treatment the FFA/ketone ratio was altered at low FFA levels and was related to the duration of insulin deprivation at high FFA levels seems to support this hypothesis.

On the other hand there are numerous biochemical and endocrine factors regulating ketoacidosis in alloxan diabetes and even more factors may act on the ratio FFA/ketone. The mechanism, by which these factors are causally related, has not yet been established.

The finding that after fat feeding serum FFA levels are lower at 6 days than at 6 weeks of insulin deficiency may be due to impaired absorption from the gastrointestinal tract. In the first few days following insulin withdrawal or pancreatectomy a marked gastric dilation was observed (11, 13).

Summary. Lard was given orally to rats 6 days and 6 weeks after alloxan administration and to insulin-treated alloxanized rats 6 days and 6 weeks after insulin withdrawal. Two hr after the fat feeding serum FFA and blood ketone concentrations were determined. The blood ketone concentrations were higher but serum FFA levels were lower in the acutely than in the chronically insulin deficient rats. The results indicate that in alloxan diabetic rats the degree of ketonemia is not proportional to the level of serum FFA but seems partly to depend on the duration of insulin deprivation.

We wish to thank Dr. S. Chernick for many helpful suggestions in the course of this work.

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Received July 11, 1968. P.S.E.B.M., 1969, Vol. 130.

A Rapid *in Vivo* Assay for SV40 Tumor Immunity in Hamsters* (33531)

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The appearance of several viral or viral stimulated neoantigens in SV40 transformed hamster cells is well documented. These include the virion or V antigen(s), a class of intracellular tumor or T antigens reactive in both the complement fixation and fluorescent antibody test, a cell surface reacting S antigen detected by fluorescent microscopy and transplantation antigen detected by transplant rejection tests *in vivo*. The detection of the presence of transplantation antigen in a tumor cell preparation capable of stimulating an immune reaction and of suppressing the development of SV40 induced tumors in hamsters is a prolonged procedure requiring several hundred days for completion (1).

In previous studies, radiation inactivated, 5-iododeoxyuridine-treated or disrupted SV40 tumor cell preparations were evaluated for immunizing efficacy against SV40-induced tumors in hamsters or against challenge with homologous tumor cells (2, 3). The observation was made that both systems of assay provided similar results with equal specificity. The tumor cell challenge assay required some 40–60 days after completion of immunization to evaluate the efficacy of a preparation.

The present report describes a 5-day *in vivo* method for detecting transplantation immunity in a homologous hamster system employing immunodiffusion chambers. The role of a diffusable immunity factor in tumor transplant rejection is discussed.

Materials and Methods. Hamsters. Four to 7-week-old random bred Syrian golden hamsters were used in this study (Lakeview Hamster Colony, Newfield, New Jersey). Males and females in equal proportion were used, however, males were used in recent work to eliminate problems associated with abdominal surgery on female hamsters.

SV40 hamster tumor cells. The virus-free F5-1 line (4–6) of SV40 hamster tumor was used. The cells were cultivated in medium 199 containing 5% heat-inactivated calf serum plus antibiotics. Cell cultures from passage 80 to 125 *in vitro* were removed from bottle culture by brief trypsinization, washed once with Hank's balanced salt solution (HBSS), resuspended to the desired concentration and employed.

* Supported by Grants from the National Cancer Institute (No. CA 10429) and the Atomic Energy Commission [AT(40-1)-3646-02]. This research reported, in part, at the Symposium on "Recent Progress in Tumor Immunology" at the University of Tennessee, Knoxville, Tennessee, May 31, 1968.