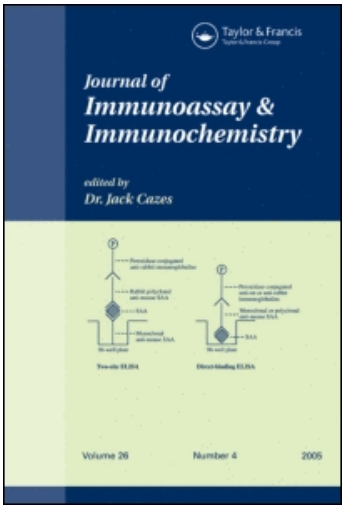

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Radioimmunoassay Measurement of Albumin in Urine Is a Valid Method for Monitoring Analbuminemia in Rats

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RADIOIMMUNOASSAY MEASUREMENT OF ALBUMIN IN URINE IS A VALID METHOD FOR MONITORING ANALBUMINEMIA IN RATS.

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ABSTRACT

Plasma and urinary albumin levels, urinary creatinine and urine volumes were measured at two-week intervals in Nagase analbuminemic rats (NAR) of either sex at ages from 42 to 154 days. Age matched Sprague Dawley (SD) rats were used as controls. Plasma concentrations of immunoreactive albumin (iALB) in NAR ranged from 0.007 $\pm$ 0.002 to 0.023 $\pm$ 0.003 mg/ml, and were lower in younger rats. In SD rats, plasma iALB concentrations ranged from 18.9 $\pm$ 1.0 to 46.2 $\pm$ 6.4 mg/ml. Urinary iALB output in NAR was less than 0.05 μg/mg creatinine measured over a 24 hr period, whereas it was greater than 20 μg/mg creatinine in the SD rats. Measurement of urine iALB concentrations in the NAR colony appears to be a reliable and a non-invasive method for monitoring the persistence of hypoalbuminemia. (Key words: hypoalbuminemia, Nagase, albumin, plasma, urine, radioimmunoassay)

INTRODUCTION

Nagase analbuminemic rats [NAR] are a strain of Sprague Dawley (SD) rat; analbuminemia was found to be an autosomal recessive trait (1). Total

serum protein in NAR was reported to be similiar to that of Sprague Dawley rats, with increased globulins compensating for albumin lack (1-4). Oncotic pressure in NAR was found to average only slightly below normal and only in male NAR, and blood pressure and plasma volume were reported to be normal (5).

The albumin level in NAR at all ages has been described as "less than 0.05 mg/ml" (4). It was shown that the albumin deficiency was not a defect in albumin secretion, but the absence of its synthesis by the liver (6).

Our colony originated from a breeding nucleus of NAR obtained in June 1989. We are indebted to Dr S Nagase for her kind gift of the breeding nucleus. The animals were shipped to Canada from Japan with the help of CLEA Japan, Inc.

The purpose of the current investigation was to develop a method for monitoring the persistence of the hypoalbuminemic trait in the NAR colony.

MATERIALS AND METHODS

Animals. NAR and SD rats were maintained in a controlled environment consisting of fluorescent lighting on a 12 hour cycle, 12 air changes per hour and an air temperature of 21±2°C. The animals (3 per cage) were housed in polycarbonate caging (19"x10.5"x8") on contact bedding (Crown Flushable litter-paper extrusion, Crown Animal Bedding, James River Corp., Camas, WA). Food (Purina Rat Chow, Purina Mills Inc, St Louis, MO) and water were available *ad libitum*, except during urine collection periods when water and food were withdrawn.

Blood sampling. Blood samples were obtained at 42, 56, 70, 84, and 154 day intervals. With animal under manual restraint, the end of the tail was clipped

using a sharp scalpel blade, a 100 μl blood sample was collected into two heparinized hematocrit tubes, centrifuged, separated, and the plasma stored at -20°C. At 154 days (=final sample) the rats were anesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Mississauga, ON; 65 mg/kg) and blood obtained from the inferior vena cava which had been surgically exposed. Blood (approximately 7 ml) was placed into 10 ml tubes containing heparin solution (15 units) and centifuged at 4°C at 2500 g for 5 min. Plasma was stored at -20°C.

Urine sampling. For urine collection, rats were placed in individual metabolic cages for the sampling period (24 h starting at 9 am). Urine collections were performed at 42, 56, 70, 84, and 154 days. Water and food were withheld during this time. Samples were stored at -20°C.

Creatinine measurements. Urinary creatinine was analyzed colorimetrically by a modification of the Jaffe reaction after adsorption on Lloyd's reagent (7).

Albumin radioimmunoassay. The RIA was prepared on ice, and all reagents were refrigerated before use. All standards and unknowns were analyzed in triplicate. The RIA buffer (pH 7.4) contained 81 mM Na_2HPO_4, 19 mM NaH_2PO_4, 50 mM NaCl, 0.01 % (w/v) NaN_3 and 0.1 % Triton-X.

Rabbit anti-rat albumin (Cappel, Organon Teknika Corp, West Chester, PA; 0213-0342 lot 32806) was used at a working dilution of 1:60,000 in RIA buffer. One hundred μl of diluted antiserum was added to each RIA tube (12 x 75 mm, borosilicate glass) with the exception of the non-specific binding (NSB) tubes.

Rat albumin (Sigma, St Louis, MO, 68F9310) was used as the standard. Working standards (0.005 - 10 μg/ml) were prepared on the day of the assay, and 100 μl aliquots of those were pipetted for standard curve determinations.

The standard curve also included non-specific binding (NSB) and triplicate maximum (=zero albumin) binding tubes.

The tracer was prepared by iodination of rat albumin (Sigma A-4538) using a modification of the method of Hunter and Greenwood (8). The reaction was carried out at room temperature. To a tube containing albumin (200 μg/100 μl in 0.1 M phosphate buffer, pH 7.4), 10 μl (approx 1 mCi) of $Na^{125}I$ (Amersham IMS.30, Amersham Canada Ltd, Oakville, ON) was added. This was followed by rapid addition of the following reactants in the sequence indicated: [a] 10 μl chloramine T (Eastman 1022, Eastman Kodak, Rochester, NY) at 1 mg/ml HOH, [b] 10 μl $Na_2S_2O_5$ (MCB SX 630, Mathieson Coleman Bell) at 1 mg/ml HOH, [c] 200 μl of ion exchange resin suspension (Bio-Rad AG1-X10, Biorad, Richmond CA) at 50 mg/200 μl HOH. The reaction tube was gently mixed on a Vortex mixer between each addition. Following the addition of the resin, the mixture was centrifuged for 3 min in a bench centrifuge, and the supernate transferred to a clean tube for column chromatography.

Sephadex G-200 (Pharmacia, Uppsala, Sweden) was equilibrated with 0.1 M phosphate buffer at 4°C in a 10 ml glass pipette (approx 9 x 230 mm) with a 100 mm pressure head. The supernate of the iodination reaction mixture was applied to the column, and 30 x 1 ml fractions were collected at a flow rate of 15 ml/hr. The peak tube from the iodination profile was kept at 4°C, and the tracer diluted for each RIA experiment with the RIA buffer to contain 10,000 - 15,000 cpm/100 μl. New tracer was prepared once a month.

The radioimmunoassay mixture (300 μl) containing 100 μl standard or unknown, 100 μl diluted antiserum, and 100 μl tracer was incubated for 20-24 h at 4°C. Separation of bound from free tracer was achieved by addition of 100

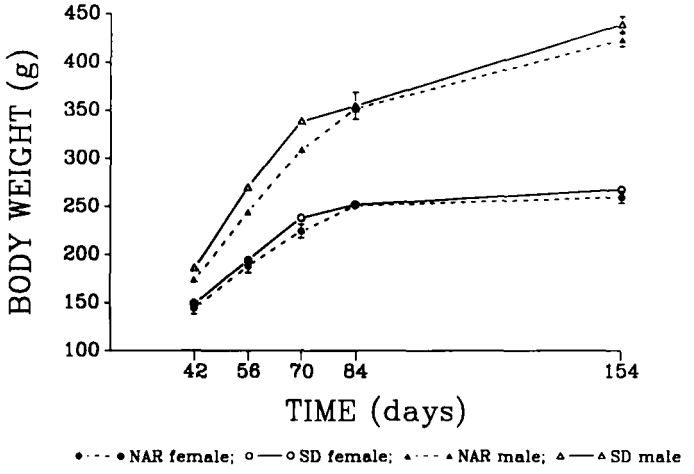

Figure 1. Body weight of male and female NAR and SD rats, ages 42 to 154 days. Six animals in each group.

μl of bovine gamma globulin (Fraction II, Code 82-041-2, ICN ImmunoBiologicals, Lisle, IL), 8 mg/ml in RIA buffer, followed by addition of Fisher Sci Carbowax R PEG 8000), thorough mixing on a Vortex mixer and centrifugation at 4°C, 2600 x g for 1 hr. Both bound and free fractions were counted (LKB 1272 Clinigamma, Stokholm, Sweden), and bound : free ratios calculated using an in-house data reduction program (232-Com, Informatica Information Systems, Vancouver BC, Canada).

All data are reported as mean (M) ± standard error of the mean (SEM).

RESULTS

Body weights obtained for all rats at two week intervals from day 42 to 154 did not differ between NAR and their respective controls (Fig 1).

TABLE 1

CREATININE OUTPUT (mg/24 h/100 g) OF NAR AND SD RATS OF EITHER SEX FROM DAY 42 TO DAY 154 OF AGE

DAY	NAR F n = 6	SD F n = 6	NAR M N = 6	SD M n = 6
42	2.24 ±0.32	2.83 ±0.24	1.88 ±0.10	2.27 ±0.30
56	2.39 ±0.11	2.77 ±0.17	2.54 ±0.11	3.19 ±0.19
70	2.47 ±0.14	2.57 ±0.11	2.85 ±0.24	3.31 ±0.16
84	2.77 ±0.08	3.25 ±0.22	3.16 ±0.16	3.43 ±0.35
154	3.70 ±0.10	3.64 ±0.10	3.66 ±0.07	3.74 ±0.13

All values are M ± SEM

Creatinine outputs did not differ between SD and NAR rats in this study (Table 1).

Albumin radioimmunoassay. The antiserum was found to be stable for at least 6 months when stored at a 1 : 100 dilution at -20°C: there was no difference in the ability to bind ^{125}I-alb of the newly opened freshly diluted vial of antiserum and the frozen aliquots. On the other hand, inferior results (approximately 50% reduction of zero binding of the standard curve, with comparable decrease in

slope) were obtained when the diluted (1 : 60,000) antiserum was stored at 4°C for a period of 2 weeks.

The sensitivity of RIA in terms of 20% and 50% displacement of the tracer was 0.016 $\pm$ 0.003 and 0.038 $\pm$ 0.004 μg/ml (n=16) respectively. The intra- and interassay error (CV%) were 8.3 and 11.0 % respectively.

Crossreactivity of the antiserum with bovine serum albumin (BSA, crystallized, 81-001, ICN ImmunoBiologicals, Costa Mesa, CA) was minimal: 10 mg/ml BSA depressed the binding of the tracer to the antiserum to the same extent as 0.0115 μg/ml rat albumin (Fig 2). Conversely, iodinated BSA did not bind to the antiserum at the dilution of antiserum used in this RIA. Rabbit serum albumin (Sigma, St Louis, MO, A-0764, lot 37F-9306) did not crossreact with the antiserum: 50 mg/ml resulted in a depression of tracer binding equivalent to that of 0.016 μg of rat albumin. Dilutions of SD plasma and urine were parallel to the standard curve (results not shown) as were dilutions of NAR plasma and urine (Fig 2).

The specific radioactivity of the tracer, measured by the self-displacement method (9), was 0.63 - 0.70 μCi/μg. The tracer, ^{125}I-rat albumin, was used for up to one month of storage at 4°C; however, the original tracer dilution was maintained throughout the life of the tracer, thereby maintaining the mass of tracer per assay tube unchanged.

The presence of Triton X in the buffer was found to be necessary to prevent adsorption of the tracer to the test tubes.

The non-specific binding (NSB), obtained by omitting the antibody from the tubes, was found to be indistinguishable among the standards (n = 30), plasma samples (n = 24) and urine samples (n = 24) and was not routinely measured thereafter for the unknown samples.

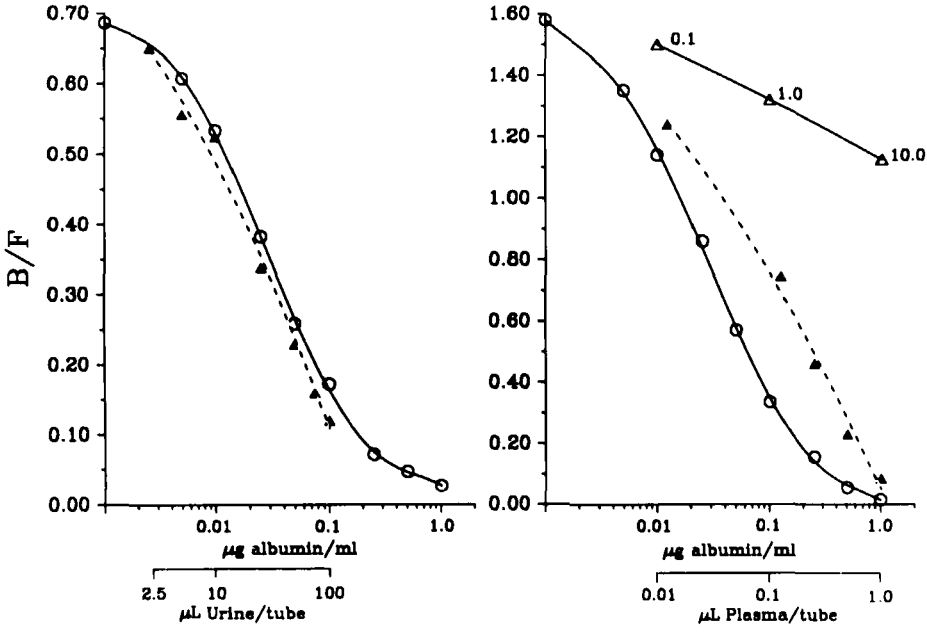

Figure 2. Rat albumin RIA. Left: Comparison of dilutions of NAR urine (------▲------) (μl/tube) with the rat albumin standard (—o—) (μg/ml). Right: Comparison of dilutions of NAR plasma (------▲-----) (μl/tube) and BSA (——Δ——) (mg/tube) with the rat albumin standard (———o———) (ug/ml).

Plasma iALB concentrations. Concentrations of iALB in plasma of NAR and SD rats are shown in Table 2. The concentrations in SD plasma required 1 : 400,000 to 1 : 800,000 dilution of plasma. NAR plasma was diluted 1 : 100 for RIA. Plasma iALB concentrations in NAR were 1,500 - 3,000 times lower than those in SD rats. There was no difference in plasma iALB levels between sexes.

Output of iALB. Output of albumin by NAR and SD rats is shown in Table 3. No differences between the two sexes were observed, and the output remained stable for the duration of the study. NAR urines were not diluted for RIA, while

TABLE 2

PLASMA IMMUNOREACTIVE ALBUMIN CONCENTRATIONS (mg/ml) OF NAR AND SD RATS OF EITHER SEX FROM DAY 42 TO DAY 154 OF AGE

DAY	NAR F n = 6	SD F n = 6	NAR M n = 6	SD M n = 6
42	0.007 ±0.002	-	0.012 ±0.003	-
56	0.013 ±0.002	18.9 ±1.0	0.013 ±0.002	23.1 ±2.3
70	0.014 ±0.003	46.2 ±6.4	0.016 ±0.004	41.7 ±7.0
84	0.016 ±0.001	42.6 ±3.4	0.014 ±0.001	30.0 ±2.1
154	0.021 ±0.001	30.1 ±4.9	0.023 ±0.003	33.8 ±2.4

All values are M ± SEM

SD rat urines were diluted 1 : 100. The output was expressed per mg urinary creatinine. For diagnostic purposes measurement of concentrations of urinary iALB was found to be sufficient, as the concentration difference between the two groups of rats was approximately 1000-fold (Table 4).

DISCUSSION

Our NAR colony has been established for two years. During this time we have not observed increased morbidity, mortality or decrease in reproductive

TABLE 3

ALBUMIN OUTPUT (μg/mg creatinine) IN URINE OF NAR AND SD RATS OF EITHER SEX FROM DAY 42 TO DAY 154 OF AGE

DAY	NAR F n = 6	SD F n = 6	NAR M n = 6	SD M n = 6
42	0.046 ±0.008	30 ±5	0.034 ±0.012	35 ±9
56	0.039 ±0.004	83 ±7	0.048 ±0.007	134 ±34
70	0.041 ±0.010	25 ±4	0.038 ±0.005	25 ±3
84	0.043 ±0.005	35 ±4	0.033 ±0.003	25 ±2
154	0.045 ±0.005	22 ±3	0.042 ±0.002	24 ±4

All values are M ± SEM

ability in NAR as compared to normal rats. According to literature reports, the reproductive capacity of NAR appears unaffected (10). Wound healing in NAR has also been found to be unaffected (11). Mean body weight was reported to be comparable between NAR and controls, although the mean adipose tissue weight was found to be reduced in NAR (12). No differences were seen in the growth rate between SD and NAR in the course of our study (Fig 1).

A mention concerning the control rats must be made. While the NAR strain was derived from SD rats in Japan, it was not possible for us to obtain SD

TABLE 4

ALBUMIN CONCENTRATION (μg/ml) IN URINE OF NAR AND SD RATS OF EITHER SEX FROM DAY 42 TO DAY 154 OF AGE

DAY	NAR F n = 6	SD F n = 6	NAR M n = 6	SD M n = 6
42	0.023 ±0.004	18 ±3	0.020 ±0.008	17 ±5
56	0.027 ±0.002	73 ±5	0.035 ±0.006	143 ±32
70	0.037 ±0.009	33 ±6	0.042 ±0.006	34 ±6
84	0.043 ±0.006	45 ±5	0.033 ±0.003	37 ±3
154	0.040 ±0.008	23 ±5	0.038 ±0.002	30 ±5

All values are M ± SEM

rats from the parental strain. Urinary iALB concentrations have been monitored routinely in our NAR colony, and instances of reversal to normo-albuminemia were not observed. Therefore SD rats of North American origin were used as controls in our study.

Reports of measurements of circulating albumin in analbuminemic rats range from non-detectable to very low levels. Electrophoresis on cellulose acetate confirmed that NAR had no albumin in the serum, while the bromcresol green method led to a massive overestimation of albumin in NAR plasma (13).

Using this method, plasma albumin level was found to be 3.5±0.5 g/dl in SD rats compared to 0.6±0.2 g/dl in NAR (14). When albumin was measured by immunoturbidimetry using rabbit anti-rat albumin (Nordic, Tilburg, The Netherlands) and rat albumin (Fraction V, Sigma, St Louis, MO), albumin was consistently below the detection limit (< 0.2 g/l) in all NAR tested (5).

None of the above studies used methods of comparable sensitivity to that described in the current report. Our experiments suggest that some albumin can be detected in NAR plasma, although the circulating levels are 1,500 - 3,000 times less than in SD control groups. It was reported that a small percentage of hepatocytes in NAR stained positively for albumin (14); thus it is possible that our measurements, due to the inherent sensitivity of RIA methodology, detected the product of still-active hepatocytes. The parallelism of NAR plasma dilutions with the rat albumin standard curve suggests, but does not prove, structural similiarity between the iALB measured in NAR plasma and rat albumin.

Plasma albumin in SD rats was reported to increase with age: from 18.5±0.3 g/l at 8 days to 24.8, 30.0 and 35.3 g/l at 24, 45 and 75 days of age respectively (5). Our results indicate that similiar increase may be occuring in NAR as the plasma albumin concentration in male and female rats at 154 days was significantly greater ($P < 0.05$) than at 42 days. No significant differences were observed among any other time periods.

The RIA as described in this report is too sensitive for plasma albumin measurements both in NAR and in SD rats, as 1 : 400,000 to 1 : 800,000 dilutions of SD plasma and 1 : 100 dilutions of NAR plasma were required. Decrease in incubation time or increase in antibody concentration would be expected to reduce the RIA sensitivity if such a method was to be used only for

plasma measurements. We did not attempt to bring this about, since iALB measurements in NAR urine required the sensitivity of this assay.

In our experiments, mean values for concentrations of iALB in NAR urine at all time periods did not exceed 0.05 μg/ml, a value near the midpoint of our standard curve (0.038 μg/ml). The system was thus well suited for measurement of iALB in NAR urine; SD urine had to be diluted 1 : 100 for RIA measurements. The immunoreactive material in urine of both SD and NAR showed parallelism with the rat albumin standard curve. The absence of increased NSB in NAR urines, which were used undiluted in RIA, and the parallelism with the standard curve over a 40-fold range strongly suggest a structural similiarity with rat albumin.

The relatively high values obtained for urinary albumins in SD rats on day 56 (Table 3) cannot be explained easily. Both NAR and SD urines were measured in the same RIA on this as well as on all other occasions, and NAR urinary iALB concentrations did not exhibit any anomalies. Similarly, quality controls were within the normal range. Urinary volumes were also normal, but the concentrations of iALB in SD urines of both genders (Table 4) were high. Individual results for females were scattered, while the males fell into two groups of three animals each: three with very high urinary iALB values, and three with values within the expected range. We can only speculate that the discrepancies on that day were the result of contamination in the collection vessels.

RIA measurements of urinary albumin excretion have been reported for streptozotocin diabetic rats; control albumin levels in Wistar Kyoto and spontaneously hypertensive rats prior to streptozotocin treatment were in the range of 100 - 500 μg/24 h/animal (15). When corrections for weight and

creatinine are made, these values are comparable to those observed by us in SD rats. Our results also fall in the same range as the urinary albumins reported for control SD rats receiving low protein intake, in which an electrodiffusion assay was used for albumin measurements (16).

It can thus be concluded that measurements of urine iALB concentrations in the NAR colony is an efficient and a non-invasive method for monitoring the presence of hypoalbuminemia. Measurement of urinary albumin concentration, rather than daily albumin output, may provide a sufficient screening method, since the concentrations of iALB in urine of NAR are greatly reduced in comparison to SD rats.

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