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Immunoglobulins Associated with Elevated Riboflavin Binding by Plasma from Cancer Patients[1] (42247)

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Abstract. Plasma from 182 patients with different malignant diseases was tested for riboflavin binding by immunoglobulins, which have been recently identified as major carriers of this micronutrient. A wide range of binding (5.9 to 130 pmole/ml plasma) was observed, and significant elevations were found for patients having breast cancer (21.2 ± 1.9, $P < 0.05$) and melanoma (25.7 ± 1.9, $P < 0.001$) compared to controls (15.5 ± 1.9). The proteins responsible for a majority of the higher binding were identified as immunoglobulins, based on their elution from gel filtration columns and the removal of 57–88% of the non-albumin binding by treating of plasma with Protein A-agarose. The binding was only weakly related to the total concentration of immunoglobulins ($r = 0.11$ by linear regression analysis), however, and is apparently due to a subclass that is elevated in some types of cancer. Elevated levels of these immunoglobulins may contribute to the lower urinary levels and clearance of riboflavin in cancer.

Many cancer patients excrete lower than normal amounts of riboflavin in urine (1). In addition, when compared to control patients, they do not show significant increases in riboflavin excretion after oral or intravenous doses of this vitamin (1, 2). Possible causes of this abnormality include increased binding or metabolism of riboflavin by tumors or host tissues. Greater binding by plasma proteins, for example, could decrease losses by protecting riboflavin from glomerular filtration. This relationship has, in fact, been observed in a patient with multiple myeloma who produced large amounts of riboflavin-binding immunoglobulin G (IgGs) (3, 4).

We have recently shown (5) considerable variation in riboflavin binding by plasma from healthy volunteers and attributed the differences to riboflavin-binding immunoglobulins. Such proteins might contribute to the reduced riboflavin excretion seen in cancer since some neoplastic diseases alter the immune system (6). Other types of proteins might be involved, however, because pregnancy-specific riboflavin-binding proteins have been isolated from bovine (7), rat (8), and human (9) blood, and gestation-distinct proteins are often elaborated in malignant diseases (10).

This study examined patients with cancer to determine if there are differences in riboflavin binding by plasma proteins and, when found, to identify the proteins responsible.

Materials and Methods. *Materials.* [2-^{14}C]Riboflavin (sp act, 60 Ci/mole) was obtained from Amersham (Arlington Heights, Ill.) and purified by paper chromatography. Solutions were handled in dim light to avoid photodecomposition (5). Spectrapor dialysis membranes (12,000–14,000 mol wt cutoff) (Scientific Products, McGaw Park, Ill.) were prepared by soaking in warm dilute Tris–EDTA solution and washing exhaustively with deionized water. All other chemicals and materials were the analytical grade available from commercial suppliers.

Plasma or serum was recovered from blood drawn into Vacutainers (Becton-Dickinson, Rutherford, N.J.) containing EDTA or no anticoagulant, and stored at either 4–8°C for short periods of time or frozen at −20°C. Plasma was used for the majority of the subjects and analyses of plasma and serum samples from several subjects gave identical results; all samples will be referred to as plasma within

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the text. These experiments had been approved by the Human Investigations Committee at Emory.

Measurement of flavin binding. Quantitation of the binding of riboflavin was accomplished by equilibrium dialysis. Samples (1- or 0.5-ml) were dialyzed for at least 8 hr at 4–8°C in an approximately 6- to 10-fold greater volume of 0.01 *M* potassium phosphate (pH 7.4) containing 0.04 μM [2-^{14}C]riboflavin [a concentration within physiological bounds (11, 12)]. Aliquots of the sample and external dialysis buffer were counted in Aquasol-2 (New England Nuclear, Boston, Mass.) using a Beckman LS 7000 scintillation counter with automatic quench correction. Bound riboflavin was determined by subtracting the total cpm in the external buffer from the total cpm inside the dialysis membrane.

Detailed analyses of the reproducibility of the binding method have been conducted with plasma from normal individuals and cancer patients. Replicates of a subject's plasma consistently agree within 3–6% in the same assay, otherwise we repeat the measurement. Comparison of binding values in plasma samples drawn over a period of several weeks from the same patients showed that the measurements agreed within 5–20% (only one patient exhibited greater variation over time and was excluded from the study). The binding value is not affected by freezing or a limited number of freeze–thaw cycles. Plasma collected from a single individual at several different times of the day (fasted and fed) yield identical results; the use of samples particularly turbid due to a high lipid content was avoided, however.

Analysis of IgGs. One milliliter of plasma was applied to a 1.5 × 25-cm column of Sephadex G-100 and eluted with 0.01 *M* potassium phosphate (pH 7.4) at 3 ml/hr. Fractions were assayed for protein by the biuret method (13), for protein composition by polyacrylamide gel electrophoresis (5), and for binding by equilibrium dialysis using [2-^{14}C]riboflavin.

Protein A-agarose (Sigma) was prepared by swelling and washing the beads in 0.15 *M* NaCl–0.1 *M* potassium phosphate buffer (pH 7.4). To 1 ml of a 50% suspension of beads in buffer was added 1 ml of plasma and the mixture was shaken for 2.5 hr at room temperature. Following centrifugation, an aliquot was removed for analysis of binding, and the remainder of the supernatant was added to 2 ml of new beads and mixed for another 2.5 hr. This supernatant was also collected and both were analyzed for binding by equilibrium dialysis. To assess dilution effects and nonspecific binding to agarose, samples were similarly treated using Sepharose CL-2B.

Total IgGs were measured by rate nephelometry using an automated Beckman ICS instrument (Beckman Instruments, Inc., Anaheim, Calif.) and Kallestad reagents (Kallestad Laboratories, Austin, Tex.).

Statistical methods. Data were analyzed by calculating mean ± SEM, by the Students *t* test for unpaired data, and by linear regression analysis.

Results and Discussion. Plasma from 182 patients with a variety of neoplastic diseases exhibited a wider range in riboflavin binding (5.9 to 130 pmole/ml plasma) than was seen with healthy controls (15.5 ± 1.9) (Table I).

The means were considerably higher for

TABLE I. RIBOFLAVIN BINDING BY PLASMA FROM CANCER PATIENTS AND CONTROLS[a]

Type of cancer	*N*	Mean ± SEM, pmole/ml plasma
Controls	34	15.5 ± 1.9
Brain	2	14.6 ± 3.4
Breast	67	21.2 ± 1.9[b]
Colon	19	18.2 ± 2.2
Endometrium	1	22.5
Esophagus	2	21.9 ± 8.0
Ewings sarcoma	1	13.0
Head and Neck	4	13.2 ± 1.9
Hodgkins Disease	4	34.6 ± 15.7
Kidney	1	17.7
Liver	3	18.1 ± 4.3
Lung	14	18.1 ± 1.7
Lymphoma (non-Hodgkins)	13	15.1 ± 3.8
Melanoma	36	25.7 ± 1.9[c]
Mesothelioma	2	81.1 ± 49.3
Myeloma	1	8.7
Ovary	5	16.4 ± 2.0
Prostate	1	12.2
Squamous cell ca.	1	10.9
Stomach	2	13.2 ± 2.5
Testicle	2	15.4 ± 6.3
Uterus	1	8.8

[a] Binding was measured by equilibrium dialysis using 0.04 μM [2-^{14}C]riboflavin.

[b] Significantly different when compared to controls, $P < 0.05$.

[c] Significantly different when compared to controls, $P < 0.001$.

patients with mesothelioma (523% of controls), Hodgkins disease (223%), melanoma (166%), and tumors of the endometrium (145%), esophagus (141%), and breast (137%). The groups for which the differences could be established as statistically significant were breast (21.1 ± 1.9, $P < 0.05$) and melanoma (25.7 ± 1.9, $P < 0.001$). There were no obvious differences within these groups due to age (which ranged from 22 to 87), to sex (64% female, 36% male), or to the type of therapeutic treatment. Approximately half of the patients with melanoma were receiving adjunct therapy with a Newcastle disease virus oncolysate (14), but their binding did not differ from those of the remaining melanoma patients ($P > 0.10$). Comparison of the binding levels of patients receiving antiestrogens (e.g., Tamoxifen) with those who were not did not show any significant difference ($P > 0.20$). The frequencies of different levels of binding for different individuals are shown in Fig. 1. For both melanomas and breast cancer, there was a shift and broadening of the distribution curve compared to healthy controls.

The proteins responsible for riboflavin binding in patients with melanoma and breast cancer were partially identified by fractionation of the plasma with gel filtration chromatography (Fig. 2). Riboflavin binding was predominantly located in the initial fractions, which contained immunoglobulins as seen by electrophoresis (not shown) and as quantitated by nephelometry for control plasma (5). A minor amount of riboflavin was bound to albumin (the major protein peak), which has been shown to account for approximately 13–15% of the riboflavin bound in plasma from controls (5).

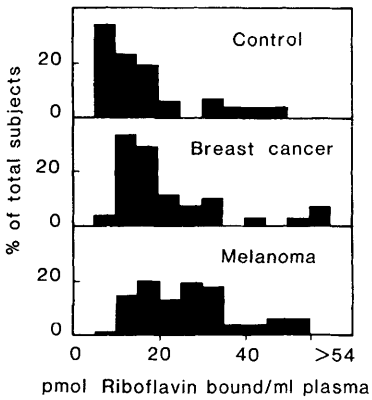

FIG. 1. Individual binding values obtained by equilibrium dialysis for controls, and breast cancer and melanoma patients.

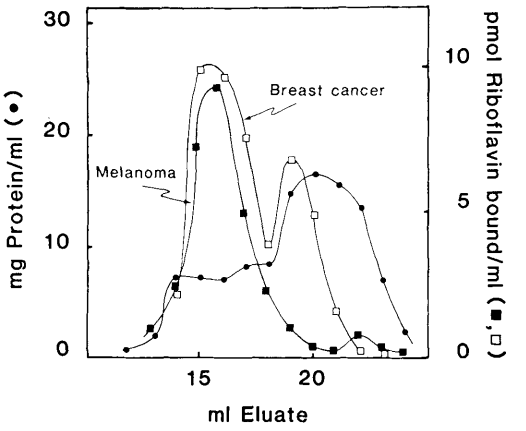

FIG. 2. Chromatographic profiles of plasma from melanoma and breast cancer patients on Sephadex G-100.

The involvement of immunoglobulins was confirmed using Protein A-agarose, which binds IgG (subclasses 1, 2, and 4) and IgA relatively specifically (15, 16). In two consecutive incubations of plasma from individuals with breast cancer or melanoma, or pooled plasma from patients with different types of cancer, 42 to 75% of the riboflavin-binding proteins were removed (Table II). These percentages underestimate the fraction attributable to IgGs because albumin binds approximately 5 pmole of riboflavin/ml plasma (5). With the data in Table II adjusted for only non-albumin bind-

TABLE II. REMOVAL OF BINDING COMPONENTS FROM PLASMA BY PROTEIN A-AGAROSE[a]

Patient	Type of cancer	Initial binding, pmole/ml plasma	% Removed
1	Breast	28.3	47%
2	Breast	33.0	75%
3	Melanoma	19.5	42%
4	Melanoma	36.0	71%
Pooled plasma of cancer patients		30.7	75%

[a] Binding was measured by equilibrium dialysis following two consecutive incubations of the plasma with protein A-agarose. Similar experiments using Sapharose CL-2B served as controls.

ing, 57 to 88% was removed by Protein A-agarose. These results suggest that immunoglobulins, or possibly proteins associated with immunoglobulins, make the greatest contribution to the elevated binding.

Immunoglobulin levels often vary among persons with different carcinomas, although there is no concensus on the generality of this phenomena. Patients with breast cancer have been reported to display increased, decreased, and normal levels of IgG or IgA (17–19); these conflicting studies may be the result of comparison of patients at different stages of disease or treatment. In studies of persons with melanoma, it has been concluded that IgG and IgA may both be elevated (17), but this does not appear to be the rule (14).

To determine if the elevated binding was due to variations in total IgGs, samples from 30 of the 36 individuals with melanoma were analyzed for IgG by rate nephelometry (Fig. 3). There is little or no correlation between these two parameters (correlation coefficient = 0.11). The subjects in our study had normal IgG levels (mean ± SEM = 11.9 ± 3.1). These results indicate that while immunoglobulins may play an important role in causing variations in riboflavin binding in cancer patients, the differences are probably due to a subclass of riboflavin-binding immunoglobulins. A similar conclusion was derived from studies of variations among healthy individuals (5), and from attempts to isolate alternative riboflavin-binding proteins from humans (20). It is not known whether these immunoglobulins bind riboflavin nonspecifically or at an antigenic site, as has been found for the IgGs in one case of multiple myeloma (3).

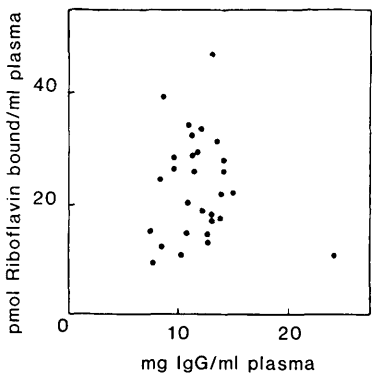

FIG. 3. Comparison of riboflavin binding values and IgG concentrations for plasma samples from patients with melanoma.

Decreased urinary excretion both before and after riboflavin tolerance tests has been reported in several types of cancer (1), and might be a function of retention of the vitamin in circulation due to protein binding. Our measurements indicate that more than 80% of the plasma riboflavin in patients with the highest binding is associated with circulatory proteins. This could decrease glomerular filtration and urinary excretion of the micronutrient. Higher than normal requirements for riboflavin in tumor tissues (21), however, might additionally contribute to reduced urinary excretion. The two may, in fact, be related because elevated binding by immunoglobulins could maintain riboflavin in circulation longer and allow tissues greater opportunity for its uptake, which occurs by passive diffusion followed by metabolic trapping (22). Further investigation of the relation between plasma riboflavin binding levels, the vitamin status of tissues (as indicated by standard indices such as the erythrocyte glutathione reductase activity coefficient), and concomitant urinary excretion levels should be considered.

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