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Retinol binding protein as a surrogate measure for serum retinol: studies in vitamin A–deficient children from the Republic of the Marshall Islands[1–3]

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ABSTRACT

Background: Serum retinol is transported by retinol binding protein (RBP), which has one high-affinity binding site for retinol; consequently, the molar ratio of retinol to RBP in the circulation is ≈1 to 1. In vitamin A deficiency (VAD), both serum retinol and RBP decline. However, the retinol-RBP relation has not been well studied in populations with a high incidence of severe VAD.

Objective: The purpose of this study was to determine whether RBP is a good surrogate for serum retinol at the very low retinol concentrations encountered in VAD.

Design: The stoichiometric relation between retinol and RBP was studied in 239 Marshallese children: 65 with severe VAD (≤0.35 μmol retinol/L), 94 with moderate VAD (0.36–0.70 μmol retinol/L), and 80 with vitamin A sufficiency (>0.70 μmol retinol/L).

Results: Excellent correlation between retinol and RBP (r = 0.94) was observed across all retinol concentrations. Severe VAD was predicted with 96% sensitivity and 91% specificity on the basis of an RBP cutoff of ≤0.48 μmol/L, whereas moderate VAD was predicted with 87% sensitivity and 98% specificity on the basis of an RBP cutoff of ≤0.70 μmol/L.

Conclusions: The use of RBP results in the classification of essentially the same children with VAD as does retinol, and RBP is an excellent surrogate for serum retinol. Considering the relative ease of measuring RBP with immunodiagnostic kits compared with that of serum retinol by HPLC, the use of RBP concentrations to assess VAD may be particularly advantageous in field settings. Consequently, measuring RBP concentrations may be a practical alternative to measuring serum retinol in population surveys assessing the prevalence of VAD. *Am J Clin Nutr* 2001;73:594–601.

KEY WORDS Vitamin A, vitamin A deficiency, micronutrient deficiency, retinol, retinol binding protein, RBP, nutritional status, nutritional assessment, Republic of the Marshall Islands, children

INTRODUCTION

Vitamin A deficiency (VAD) persists as a problem of significant public health concern in many regions of the world (1) and is associated with serious increases in morbidity and mortality (2–7). The World Health Organization estimates that ≈250 million preschool-age children have moderate-to-severe VAD (1). Many studies are undertaken each year to assess the vitamin A nutritional status of populations; consequently, there is a demand for a simple, economical method for assessing vitamin A status to facilitate the identification of populations at risk of VAD and to study the effect of vitamin A intervention programs.

A true gold standard for assessment of the body's vitamin A reserves is not readily available outside of a research laboratory setting. Nevertheless, a general understanding of vitamin A metabolism allows us to propose indicators for estimating vitamin A nutritional status on a population level. All vitamin A in the body originates from the diet and is absorbed in the small intestine, where it is packaged along with other dietary fats into chylomicrons, which are secreted into the lymphatic system (8, 9). After entering the general circulation, chylomicrons undergo lipolysis and are eventually taken up by the liver, where the bulk of the body's vitamin A is stored as retinyl esters (8). To meet constant tissue needs despite day-to-day variability in dietary vitamin A intake, a steady concentration of circulating retinol is maintained by drawing on hepatic reserves. This is accomplished through cosecretion from the liver of retinol bound to its specific carrier protein, retinol binding protein (RBP) (10, 11). Circulating retinol is taken up by target tissues, where it is converted to its active forms, retinoic acid [the ligand for nuclear retinoid receptors (12–14)] or 11-*cis*-retinal [the chromophore of the visual pigment rhodopsin (15, 16)]. If the liver becomes depleted of retinol stores, as occurs in the late stages of VAD, apo-RBP is retained in the endoplasmic reticulum of the hepatocyte and is not secreted. Studies using hepatocytes from rats with VAD indicate

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that this block in apo-RBP secretion is nearly absolute (11, 17, 18); however, this has not been established in humans.

Many techniques for assessing vitamin A status have been developed and each has its own strengths and limitations. Clinical indexes of VAD are inherently limited because increases in morbidity and mortality are observed well before the clinical symptoms of VAD appear (1). The most commonly used assessment technique is measurement of serum retinol concentrations, but this requires relatively expensive and sophisticated HPLC equipment. Considering the magnitude of the worldwide public health problem of VAD, development and validation of a method for vitamin A assessment that is inexpensive, practical for use in the field, and accurate is well warranted. In this report we describe the use of RBP as a surrogate measure for serum retinol in a population with a high prevalence of severe VAD.

SUBJECTS AND METHODS

Study population and design

Serum samples were collected for the Republic of the Marshall Islands (RMI) Vitamin A Deficiency Study, a community-based survey designed to assess the prevalence of VAD in the RMI. Ethical approval was obtained from The Ministry of Health of the RMI, the Institutional Research Board of the Pacific Health Research Institute of Hawaii, and the collaborating institutions.

The RMI includes 29 atolls, each composed of many small islands, located in the region of the South Pacific known as Micronesia. The RMI is composed of 1225 islands, with only 181 square km (70 square miles) of total land mass scattered over 1 940 000 square km (750 000 square miles) of open ocean. The maximal width of the islands is 0.8 km (0.5 miles), leaving only a small amount of arable land. Historically, the Marshallese (population: ≈60 000) were a subsistence culture, surviving primarily on fish, breadfruit, coconut, pandanus, bananas, and papaya. The traditional staple foods of the Marshallese have been replaced largely with imported foods, mainly white rice and canned meat. A national nutrition survey conducted in 1991 showed that the Marshallese have a high prevalence of protein-energy malnutrition, VAD (among children), anemia, type 2 diabetes, heart disease, gout, and stroke (19). At that time, the minimum prevalence of VAD among children was estimated to be 4% on the basis of ophthalmologic evaluations by nonphysicians.

Details of the community-based survey to assess the prevalence of VAD in Marhsallese children were described elsewhere (20, 21). All children between the ages of 1 and 5 were eligible for the study. A cross-sectional sample of 919 children representing ≈20% of the total population of 1–5-y-olds living in the RMI was obtained. The sample included ≈200 children from each of 4 strata: 2 urban centers, outer islands, and radiation-affected atolls. With a minimum prevalence of VAD of 4%, a sample size of 200 per stratum was determined to yield a prevalence estimation with an accuracy of ±3%. Of the 919 children in the study, serum samples from 239 children were included in the present study for analysis of RBP. These samples represented a broad range of retinol concentrations, but purposely encompassed a larger percentage of children with ≤0.35 μmol retinol/L because it was important to test the ability of RBP to predict low serum retinol concentrations. According to World Health Organization criteria, a retinol concentration ≤0.35 μmol/L classifies a child as having severe VAD, a concentration of 0.35–0.70 μmol/L as having moderate VAD, and a concentration >0.70 μmol/L as being vitamin A sufficient (1). Thus, 65 (57%) of the 114 children with severe VAD, 94 (21%) of the 444 children with moderate VAD, and 80 (22%) of the 361 children with vitamin A sufficiency were selected for measurement of RBP. Within each serum retinol category, samples were selected randomly for inclusion in the present study.

Sample collection and handling

Study teams—consisting of at least one Marshallese-speaking health care worker, a phlebotomist, and a medical doctor—visited every household selected for the study and obtained serum samples from all eligible children residing within the household. The sampling strategy was based on the 1988 census of the RMI, which provided information on the average number of children of the target age group within each household, determined by dividing the number of children in a locality by the number of households in the locality. This number was then divided into the number of children to be sampled to obtain the number of households to be visited. The visited households were chosen at random. When available, the date of birth was ascertained from the children's health care cards.

From each participating child, a blood sample was collected in a red-top serum separator tube wrapped in aluminum foil, immediately stored at 4 °C, and continuously shielded from light. On the same day, cells were separated from serum in a desktop centrifuge (200 × *g,* 10 min, room temperature) in a local laboratory and aliquots of serum were placed in liquid nitrogen. Serum samples were shipped from the RMI to New York City in liquid nitrogen by overnight air express and were not thawed until retinol analysis. Particular care was taken to ensure that none of the serum samples were exposed to sunlight or to room light from the time of collection until after completion of the retinol analyses.

Radioimmunoassay of serum RBP concentrations

RBP concentrations were measured in 10 μL serum by using specific and sensitive radioimmunoassay (RIA) procedures described in detail elsewhere (22). Briefly, rabbit anti-human RBP generated against purified human serum RBP was used in the analyses. The RBP used to generate this antiserum and the standards used in the RIAs were purified to homogeneity as assessed by overloaded Coomassie blue–stained sodium dodecyl sulfate polyacrylamide gel electrophoresis; specificity was confirmed by Western blot analysis. The lower limit of detection of this RIA for human RBP was 0.5 nmol/L. The within-assay CV for the RBP RIA was 5% and the between-assay CV was 7%. Similar CVs were reported for measuring RBP with commercial radioimmunodiffusion kits (23, 24) and nephelometry (23, 25–27); furthermore, these methods were all reported to correlate well with one another (23, 25–29).

Serum retinol determinations

Serum retinol was measured in 500 μL serum by using reversed-phase HPLC (30). This method uses an internal standard consisting of a known mass of retinyl acetate added in 0.5 mL ethanol to 0.5 mL serum. The serum samples were denatured in ethanol (1:1, by vol), and retinol was extracted into 2 mL HPLC-grade hexane. After one backwash of the hexane extract with 0.5 mL deionized water, the hexane was evaporated to dryness under a gentle stream of nitrogen and the extracted retinol

was redissolved in 40 μL benzene and analyzed by reversed-phase HPLC (31). The amount of retinol in each extracted serum sample was quantitated by comparing the integrated areas under the HPLC peaks with a standard curve relating integrated peak areas with known masses of retinol and an internal standard of retinyl acetate prepared by using published extinction coefficients for retinol and retinyl acetate (31). Retinyl acetate was purchased from Eastman Kodak, Inc (Rochester, NY). All retinol analyses were performed by the same individual. Approximately one-half of the serum samples were assayed in duplicate. The within-assay and between-assay CVs for this assay were 3% and 8%, respectively. The lower limit of detection was 0.02 μmol/L.

To ensure the quality of our serum retinol measurements, we evaluated retinol concentrations of quality-control reference standards (Standard Reference Material 968C; National Institute of Standards and Technology, Gaithersburg, MD) and a control serum sample obtained from a volunteer in our laboratory. Aliquots of this control serum were stored at –70 °C and measured at regular intervals throughout the duration of the study. At no point did the retinol concentrations determined for this control serum sample differ by >16% (twice the between-assay CV for our retinol assays; *see* above) from the mean.

Reversed-phase HPLC procedures

Retinol was separated by reversed-phase HPLC on a 250 × 4.6-mm Ultrasphere C_{18} (5 μm) column (Beckman Instruments, Inc, Fullerton, CA) by using a mobile phase consisting of acetonitrile:methanol:methylene chloride (70:15:15, by vol) at a flow rate of 1.8 mL/min. The running column was preceded by a C_{18} guard column. Retinol was detected at 325 nm by using a 996 Photodiode Array ultraviolet absorbance monitor (Waters Inc, Milford, MA).

Acute phase protein determinations

C-reactive protein (CRP) and α_1-acid glycoprotein (AGP, or orosomucoid) were measured by standard turbidimetric procedures with a Beckman Array (Beckman, LaBrea, CA) in serum samples from a subset of 40 children. These positive acute phase reactants were selected for their differential time courses after infection: CRP rises and falls rapidly whereas AGP has a slower time course. Reference limits used to indicate a positive acute phase response were those suggested by the manufacturer, ie, >0.67 μmol/L (>8 mg/dL) for CRP and >22 μmol/L (>100 mg/dL) for AGP. The within-assay CV for CRP was 1% and the between-assay CV was 4%. For AGP, the within- and between-assay CVs were 1% and 4%, respectively.

Stability of RBP measures

To characterize factors that might influence the immunologic detection of RBP, the effects that variability in serum transthyretin or triacylglycerol concentrations or harsh handling of serum specimens might have on measurements of RBP by RIA were assessed. For these studies, serum samples from children with severe and moderate VAD and from vitamin A–sufficient children were used. Aliquots of serum were subjected to extreme handling conditions by incubating them for 23–25 h at 30, 40, and 45 °C in a room receiving bright sunlight, ie, conditions known to result in significantly diminished retinol concentrations.

Statistical methods

The retinol-RBP relation was analyzed by simple linear regression. Chi-square analyses were performed to compare RBP saturation in different retinol strata. A histogram of RBP saturation with retinol was analyzed to study the distribution of RBP saturation across the population. Some samples appear to have >100% saturation with retinol. With measurement errors of 8% for retinol and 7% for RBP, the CV for the percent saturation of RBP with retinol is ≈11% (ie, the square root of $8^2 + 7^2$), which means that roughly 16% of samples with fully saturated RBP can be expected to appear to have a saturation of >111%.

To investigate whether the predictive value of RBP for retinol is the same at all concentrations, the subjects were divided into 3 groups: those with low, moderate, or normal RBP concentrations. The root mean squared error of the residuals of retinol around the best fit line was calculated for each group and compared. The percent saturation of RBP with retinol was compared with 100% with a one-sample *t* test. Serum retinol concentrations between children undergoing an acute phase response or not undergoing an acute phase response were compared by using Student's two-tailed *t* test.

To determine a test's utility as an indicator for another measure, the receiver operating characteristic (ROC) curve plots a test's true-positive rate (sensitivity) against its false-positive rate (1 − specificity) at every possible cutoff. A high cutoff results in 100% sensitivity, but a specificity of 0. If the test is good, sensitivity decreases little as the specificity increases. Ideally, there is a place on the curve where sensitivity and specificity are both very close to 100%. The higher the sensitivity and specificity for each cutoff, the closer the area under the curve will be to 1, with 1 being a perfect indicator. ROC curves were generated by calculating sensitivity and specificity for every RBP concentration encountered in the study population and plotted as sensitivity versus 1 − specificity (32).

For the purposes of the present study, children with serum retinol concentrations ≤0.35 μmol/L were oversampled for measurement of RBP. Thus, the proportions of children in the subgroups of vitamin A status (severe VAD, moderate VAD, and vitamin A sufficient) were not the same it the 239 children with RBP measurements as in the random sample of 919 children. For this reason, the specificity and sensitivity of RBP as a surrogate measure for severe and moderate VAD, respectively, were calculated by weighting the results in each stratum by the sampling factor for that stratum (114/65 = 1.75 for severe VAD, 444/94 = 4.72 for moderate VAD, and 361/80 = 4.51 for vitamin A sufficiency). The effect of sample handling on RBP measurements was analyzed by repeated-measures analysis of variance. All results are reported as means ± SDs. Statistical calculations were made by using EXCEL (version 9.0; Microsoft, Redmond, WA) and, when necessary, SAS (version 8.0; SAS Institute Inc, Cary, NC).

RESULTS

The RMI Vitamin A Deficiency Study assessed the prevalence of VAD on the basis of serum retinol for a total of 919 children (retinol: 0.66 ± 0.29 μmol/L). The results are summarized in **Table 1**. The relation between RBP and retinol in serum from a subset of 239 of the 919 children is illustrated in **Figure 1**. The dotted line represents the best fit whereas the upper line is the line of identity, ie, the line predicted if RBP and retinol are at a molar ratio of 1 to 1. The correlation between RBP and retinol was good (r = 0.94), with most of the points being very close to the correlation line. When the subjects were divided into groups of low, moderate, and normal RBP, the root mean squared

TABLE 1
Prevalence of vitamin A deficiency (VAD) in the study population from the Republic of the Marshall Islands

VAD classification according to serum retinol	Population
	n [%]
Severe VAD: ≤0.35 μmol/L	114 [12.4]
Moderate VAD: 0.36–0.70 μmol/L	444 [48.3]
Mild VAD to sufficient vitamin A: 0.71–1.05 μmol/L	281 [30.6]
Sufficient vitamin A: >1.05 μmol/L	80 [8.7]
Total	919

error was similar in all groups, as shown in the legend to Figure 1, indicating that the linear relation between retinol and RBP was at least as good at the low end as at the high end. The points generally fell below the line of identity, indicating that the percent saturation of RBP with retinol tended to be <100%. The average percent saturation of RBP with retinol was 89 ± 19%, which is significantly different from 100% ($P < 0.0001$).

The frequency distribution of the percent saturation of RBP with retinol is presented as a histogram in **Figure 2** and shows a wide range of values in this population. The values were approximately normally distributed, with most of the subjects having a saturation between 80% and 110%. In **Table 2**, the percent saturation of RBP is categorized as <80%, 80–100%, or >100% according to serum retinol concentrations. Children with retinol concentrations ≤0.35 μmol/L had a greater tendency to have a low percent saturation of RBP with retinol (<80%) than did children with retinol concentrations of 0.36–0.70 or >0.70 μmol/L (51%, 14%, and 6%, respectively). This observation was also reflected in the negative intercept of the relation between retinol and RBP (Figure 1). Percent saturation of RBP with retinol values were 79 ± 25%, 90 ± 14%, and 96 ± 13% for children with retinol concentrations ≤0.35, 0.36–0.70, and >0.70 μmol/L, respectively.

As can be seen in **Table 3**, on the basis of a cutoff of 0.48 μmol RBP/L (10 mg/L), 63 of 65 children with a serum retinol concentration ≤0.35 μmol/L would have been correctly identified as having severe VAD, whereas 2 children would have been incorrectly identified as having moderate VAD. Because a random sampling of the total Marshallese population was done to determine the prevalence of VAD according to serum retinol, whereas children with severe VAD were oversampled for measurement of RBP, it was necessary to weight each retinol stratum so that sensitivity and specificity calculations for RBP would not be biased by the sampling strategy, as described in the Methods section. As calculated in the footnote to Table 3, RBP concentrations predicted severe VAD with 4% false-negative values, or 96% sensitivity. Of 174 children with retinol concentrations >0.35 μmol/L, 158 children would have been correctly identified as not having severe VAD, whereas 16 children would have been incorrectly identified as having severe VAD. RBP concentrations predicted severe VAD with 9% false-positive values, or 91% specificity. Alternative RBP cutoff values, eg, as calculated from the regression equation or by the method of Leibel et al (33), gave similarly high sensitivity and specificity (data not shown).

On the basis of a previously established cutoff of 0.70 μmol RBP/L indicating moderate VAD (34), 144 of the 159 children with retinol concentrations ≤0.70 μmol/L would have been correctly identified as having moderate VAD, whereas 15 children would have been incorrectly identified as being vitamin A sufficient, representing 87% sensitivity. Of 80 children with retinol concentrations >0.70 μmol/L, 78 children would have been correctly identified as not having moderate VAD, whereas 2 children would have been incorrectly identified as having moderate VAD, representing 2% false-positive values, or 98% specificity. These data are summarized in **Table 4**. The cutoff to predict moderate VAD calculated by using the method of Leibel et al (33) was also 0.70 μmol RBP/L. The cutoff calculated from the regression equation that corresponds to 0.70 μmol retinol/L was 0.77 μmol RBP/L, and resulted in 96% sensitivity and 88% specificity.

ROC curves illustrate graphically the effect of using alternative cutoffs and were generated to further characterize the utility of RBP as a surrogate measure for serum retinol (32). The ROC curve for severe VAD is shown in **Figure 3**. (The ROC curve for moderate VAD was nearly identical and thus is not shown.) The integrated area under the curve was 0.98 for both severe and moderate VAD, convincingly showing a high degree of agreement between RBP and serum retinol as predictors of VAD.

Because retinol and RBP concentrations are known to be influenced by infection, retinol and RBP were compared in children experiencing (acute phase positive) or not experiencing (acute phase negative) infection. CRP was elevated (>0.67 μmol/L) in 3 of the 40 children and AGP was elevated (>22 μmol/L) in 18 of the 40 children. Mean retinol concentrations were not significantly different between the acute phase positive and acute phase negative children (0.64 ± 0.28 compared with 0.77 ± 0.31 μmol/L, respectively); the results were similar for RBP (0.74 ± 0.3 compared with 0.83 ± 0.26 μmol/L, respectively).

As evident in **Table 5**, RBP concentrations were not influenced significantly by harsh handling conditions. Likewise, measures of serum RBP were not influenced significantly by exogenously added transthyretin (up to 10 μmol/L serum) or triacylglycerol

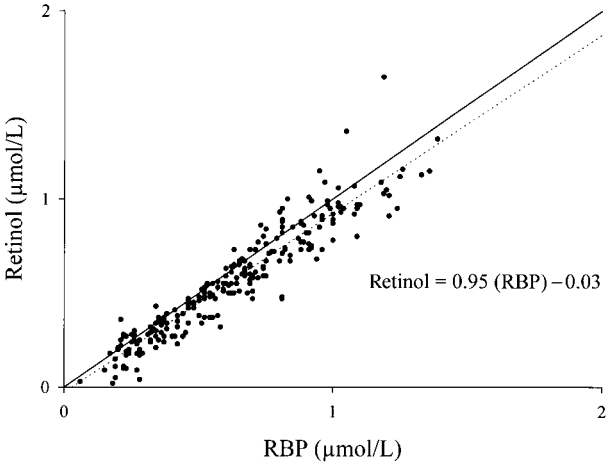

FIGURE 1. Regression analysis of serum retinol versus serum retinol biding protein (RBP); $n = 239$. The solid line represents the line of identity and is equivalent to 100% saturation of RBP with retinol. The dotted line represents the line of best fit of the data. The root mean squared error of the residuals of retinol around the best fit line was 0.097 μmol/L overall. When the subjects were divided into groups according to low, moderate, or high RBP concentrations, the root mean squared error was 0.068 μmol/L in subjects with RBP < 0.48 μmol/L, 0.070 μmol/L in subjects with RBP of 0.49–0.70 μmol/L, and 0.128 μmol/L in subjects with RBP > 0.70 μmol/L.

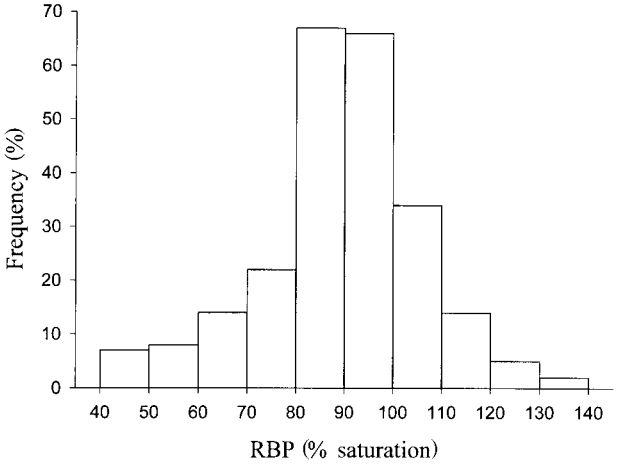

FIGURE 2. Frequency distribution of the percent saturation of retinol binding protein (RBP) with retinol; $n = 239$. The data were categorized into 10% increments of saturation with retinol (40–50%, 50–60%, etc). There was >100% saturation of RBP with retinol in 23% of the samples and >110% in 9% of the samples. As described in the text, this result is to be expected even with small errors in the measurement of retinol and RBP.

(up to 6.25 mmol/L serum) (data not shown). Thus, quantitative immunologic measures of serum RBP under these assay conditions appear to be relatively stable in response to extreme handling conditions (ie, exposure to high temperatures and sunlight) and to variations in transthyretin and triacylglycerol concentrations that may be encountered physiologically.

DISCUSSION

The high prevalence of severe VAD in the RMI provided us with a large number of serum samples in which to assess the ability of RBP to predict retinol across a wide range of serum retinol concentrations, including significant numbers of samples with retinol concentrations ≤0.35 μmol/L. The potential for RBP to serve as a surrogate for retinol follows from the physiology of vitamin A transport and metabolism. Retinol is carried in the circulation by RBP and each molecule of RBP has only one binding site for retinol (35). Under normal conditions, RBP and retinol exist in the circulation at approximately equimolar concentrations (36). In rats with VAD, however, serum RBP concentrations seem to drop and then level off as serum retinol drops below detection limits, resulting in a reduction in the percent saturation of RBP with retinol (17, 18). On the basis of these animal studies, it was suggested that RBP concentrations may not accurately reflect serum retinol concentrations in humans with severe VAD. However, this possibility has not been addressed in human populations with a high incidence of severe VAD (37, 38). The few known human studies that focused on retinol-RBP relations showed that correlations ranged from 0.62 to 0.93 (39–41). An important limitation of some of these earlier studies was the measurement of retinol by use of fluorometric methods, which are less precise and specific than are current HPLC methods. The present study was undertaken to determine whether serum RBP concentrations can correctly classify children as having severe or moderate VAD, compared with retinol concentrations used for the same purpose. Particular care was taken to use very sensitive techniques in the assessment of both indexes and to use sufficient numbers of serum samples with very low retinol concentrations.

TABLE 2
Distribution of the percent saturation of retinol binding protein (RBP) with retinol according to serum retinol concentration

	RBP (% saturation)			
Serum retinol	<80%	80–100%	>100%	Total
	n [%]			
≤0.35 μmol/L	33 [51]	18 [28]	14 [21]	65 [100]
0.36–0.70 μmol/L[1]	13 [14]	64 [68]	17 [18]	94 [100]
>0.70 μmol/L[2]	5 [6]	51 [64]	24 [30]	80 [100]
Total	51 [21]	133 [56]	55 [23]	239

[1] Significantly different from children with serum retinol ≤0.35 μmol/L, $P < 0.0001$ (chi-square test).
[2] Not significantly different from children with serum retinol of 0.36–0.70 μmol/L, $P = 0.08$ (chi-square test).

The reported tendency in rats with VAD for RBP concentrations to drop and then stabilize at low concentrations but for retinol concentrations to continue declining (17, 18) is perhaps the strongest argument against the use of RBP as a surrogate measure for retinol. The rationale is that an excess of apo-RBP might cause misclassification of an individual as less vitamin A deficient than will serum retinol. However, even at very low retinol concentrations, where RBP may not exist at a molar ratio of 1 to 1 with retinol, RBP correlates strongly with retinol and predicts retinol with very high sensitivity and specificity. In agreement with previous studies (37, 38), children with severe VAD in the present study had low percent saturation of RBP with retinol. However, by using a cutoff for RBP of 0.48 μmol/L (rather than 0.35 μmol/L, ie, equimolar to retinol), it is possible to compensate for the decrease in the percent saturation of RBP with retinol seen in severe VAD.

There are no published RBP concentrations that indicate severe VAD (retinol ≤ 0.35 μmol/L). The RBP cutoff of 0.48 μmol/L (10 mg/L) was reported previously to demarcate the 0.025 fractile for children in the 1–5 y age range in a study conducted in healthy children of mixed ethnicities in Vancouver, Canada (24). This cutoff served well for the Marshallese population, as did alternative cutoffs calculated either from the regression equation or by the method of Leibel et al (33). Ultimately, a final choice of a cutoff should depend on the purpose of the survey because the optimal cutoffs for assessing the nutritional status of populations and for monitoring the effect of an intervention may

TABLE 3
Use of retinol binding protein (RBP) to predict severe vitamin A deficiency (VAD) as determined by retinol (96% sensitivity, 91% specificity)[1]

RBP	Retinol ≤ 0.35 μmol/L	Retinol > 0.35 μmol/L	Total
		n	
≤0.48 μmol/L	63	16	79
>0.48 μmol/L	2	158	160
Total	65	174	239

[1] Because the group with serum retinol >0.35 μmol/L is made up of 2 strata with slightly different sampling fractions (444/94 = 4.72 for moderate VAD and 361/80 = 4.51 for sufficient vitamin A), specificity was calculated by weighting the results (16/94 for moderate VAD and 0/80 for sufficient vitamin A) by their respective sampling fractions: $100 - (4.72 \times 16 + 4.51 \times 0)/(4.72 \times 94 + 4.51 \times 80)$.

TABLE 4
Use of retinol binding protein (RBP) to predict moderate vitamin A deficiency (VAD) as determined by retinol (87% sensitivity, 98% specificity)[1]

RBP	Retinol ≤ 0.70 μmol/L	Retinol > 0.70 μmol/L	Total
		n	
≤0.70 μmol/L	144	2	146
>0.70 μmol/L	15	78	93
Total	159	80	239

[1] Because the group with serum retinol ≤0.70 μmol/L is made up of 2 strata with different sampling fractions (114/65 = 1.75 for severe VAD and 444/94 = 4.72 for moderate VAD), sensitivity was calculated by weighting the results (0/65 for severe VAD and 15/94 for moderate VAD) by their respective sampling fractions: $100 - (1.75 \times 0 + 4.72 \times 15)/(1.75 \times 65 + 4.72 \times 94)$.

differ (32). Furthermore, any chosen cutoff requires validation in more than one population.

In contrast, there is a published cutoff for RBP of 0.7 μmol/L (34), which corresponds to the equimolar cutoff for retinol of 0.7 μmol/L established by the WHO as indicating moderate VAD (1). Using this RBP cutoff, we predicted moderate VAD with a sensitivity and specificity equivalent to that associated with the RBP cutoff we chose for severe VAD, providing added confidence in the ability to predict retinol with RBP on the basis of a previously established criterion.

The advantages of measuring RBP rather than retinol to assess the vitamin A status of a population merit discussion. First, the HPLC equipment and solvents required for measurement of retinol are expensive and technically complicated, whereas much simpler immunologic methods can be used to measure RBP. Second, although retinol is stable when handled and stored properly, it is photosensitive; therefore, samples must be immediately and continuously shaded from light (42), whereas RBP is not photosensitive (Table 4). RBP is also less temperature sensitive than is retinol. These factors are not trivial, considering the tropical climate and limited access to adequate on-site refrigeration and laboratory facilities in areas in which many surveys of VAD prevalence are conducted. In fact, one could argue that retinol is inferior to RBP as an indicator of vitamin A status because of its poor stability and high false-positive rate attributable to sample-handling difficulties. In studies that used serum samples stored for several years, we observed that RBP was considerably more stable than was retinol, even under optimal handling conditions (43); therefore, the use of RBP may be preferable to that of retinol in retrospective studies. Measurement of RBP is further facilitated by its immunoreactivity and the high sensitivity with which antibodies are able to detect it, thus enabling RBP to be accurately measured in volumes of serum that can be readily obtained by a finger prick. Although measurement of RBP by RIA is not routinely done outside of a research laboratory, other more readily available methods—including radioimmunodiffusion and nephelometry—yield results for RBP that agree well with each other and with RIA (23, 25–29). Finally, considering the presently available technologies, there is definite potential for developing an immunodiagnostic kit to measure RBP in the field.

Although many conditions affect circulating retinol and RBP concentrations (44, 45), an acute phase response is one that is particularly relevant to populations likely to have a high incidence of VAD because they also have a high prevalence of infectious disease. An acute phase response is characterized by significant overexpression of some proteins (positive acute phase response proteins, eg, CRP and AGP) and depressed expression of others (negative acute phase response proteins). RBP is a negative-acute phase response protein (46); thus, RBP and retinol are depressed during an acute phase response, irrespective of hepatic retinol stores (45, 47–57). Retinol and RBP have been reported to be positively correlated during measles (47), a condition with a pronounced acute phase response.

In the present study, RBP predicted retinol well, although a significant fraction of the children were undergoing an acute phase response. Thus, the use of RBP as a surrogate measure for retinol does not seem to be significantly compromised by an acute phase response. However, the relation between retinol and RBP may be transiently altered in some circumstances, for example, as was described in pregnant women immediately before delivery (58) or possibly in other acutely stressful situations. However, under these circumstances, it is unclear which (if either) metabolite (RBP or retinol) more closely reflects vitamin A nutritional status. Thus, further studies are needed to establish the validity of RBP as a surrogate for serum retinol under stress-related conditions.

Functional tests, namely the relative-dose-response (RDR) (59–61) and the modified RDR (MRDR) tests (62), assess the adequacy of liver retinol reserves, taking advantage of the physiologic retention of RBP in the endoplasmic reticulum of hepatocytes under conditions of VAD and its rapid release when vitamin A becomes available. However, the RDR requires 2 blood samples and both the RDR and MRDR require HPLC technology and administration of retinol or a retinol analogue with a 5-h blood sample. It follows that RBP should function in a dose-response test as well as does retinol.

In conclusion, serum RBP successfully identified Marshallese children as having moderate or severe VAD with 87–98% sensitivity and specificity. Because RBP is more stable than retinol and because the measurement of RBP is considerably simpler and cheaper than the measurement of retinol, the use of RBP to

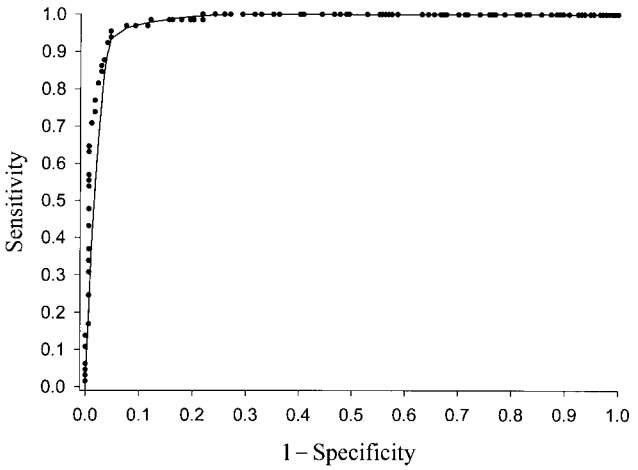

FIGURE 3. Receiver operating characteristic curve for severe vitamin A deficiency (VAD). Sensitivity and specificity were calculated for every concentration of retinol binding protein encountered in the study population for predicting severe VAD (ie, ≤0.35 μmol retinol/L). The higher the sensitivity and specificity of each cutoff, the closer the area under the curve will be to 1, with 1 being a perfect indicator (*see* text for details). The area under the curve is 0.98.

TABLE 5
Effect of sample-handling conditions on the retinol binding protein (RBP) radioimmunoassay[1]

Sample	RBP concentration			
	No exposure	30 °C for 1 d	40 °C for 1 d	45 °C for 1 d
		μmol/L		
1	0.15	0.16	0.12	0.11
2	0.30	0.31	0.25	0.33
3	0.42	0.42	0.41	0.41
4	0.44	0.41	0.45	0.42
5	0.46	0.49	0.51	0.55
6	0.56	0.50	0.55	0.52
7	0.56	0.52	0.51	0.58
8	0.63	0.61	0.62	0.62
9	1.25	1.21	1.10	1.11
10	1.25	1.22	1.19	1.11
11	1.26	1.25	1.32	1.23
12	1.71	1.61	1.66	1.62
Change[2]	—	0.02 ± 0.04	0.03 ± 0.06	0.03 ± 0.07

[1] Aliquots of serum were exposed to bright room light at 30, 40, and 45 °C for 1 d (23–25 h).

[2] $\bar{x}$ ± SD for the changes in RBP concentration between exposure and no exposure. None of the exposures produced a significant change in the RBP values when assessed by repeated-measures analysis of variance. The changes are very small, showing tight correspondence between RBP concentrations determined with or without exposure to temperatures of 30–45 °C for 1 d.

assess the vitamin A status of populations to identify those at risk of VAD should be given serious consideration. Additional studies are needed to validate the use of RBP as a surrogate for retinol in populations with a significant incidence of protein-energy malnutrition or concurrent infections.

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