A Brief Guide to Current Methods of Assessing Vitamin A Status

A Report of the

International Vitamin A

Consultative Group

(IVACG)

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The mission of the International Vitamin A Consultative Group (IVACG) is to guide international activities aimed at reducing vitamin A deficiency in the world. The group offers consultation and guidance to various operating and donor agencies that are seeking to reduce vitamin A deficiency and its accompanying blindness. As part of this service, IVACG has prepared guidelines and recommendations for

- assessing the regional distribution and magnitude of vitamin A deficiency;
- developing intervention strategies and methodologies to control vitamin A deficiency;
- evaluating the effectiveness of implemented programs on a continuing basis; and
- undertaking research needed to support the assessment, intervention, and evaluation of programs.

Monographs published by the International Vitamin A Consultative Group are

- *Guidelines for the Eradication of Vitamin A Deficiency and Xerophthalmia* (1977) (available in English and French)
- *Recent Advances in the Metabolism and Function of Vitamin A and Their Relationship to Applied Nutrition* (1979)
- *The Safe Use of Vitamin A* (1980) (available in English and French)
- *The Symptoms and Signs of Vitamin A Deficiency and Their Relationship to Applied Nutrition* (1981) (available in Spanish only)
- *Biochemical Methodology for the Assessment of Vitamin A Status* (1982)
- *Reprints of Selected Methods for the Analysis of Vitamin A and Carotenoids in Nutrition Surveys* (1982)
- *Periodic, Large Oral Doses of Vitamin A for the Prevention of Vitamin A Deficiency and Xerophthalmia: A Summary of Experiences* (1984)
- *A Decade of Achievement: The International Vitamin A Consultative Group (IVACG) 1975-1985*
- *The Safe Use of Vitamin A by Women During the Reproductive Years* (1986) (available in English, French, and Spanish)
- *Biochemical Methodology for the Assessment of Carotenes* (1987)
- *Guidelines for the Use of Vitamin A in Emergency and Relief Operations* (1988)
- *Vitamin A Supplements: A Guide to Their Use in the Treatment and Prevention of Vitamin A Deficiency and Xerophthalmia* (published by the World Health Organization in conjunction with IVACG and UNICEF, 1988) (available in English and French)
- *Guidelines for the Development of a Simplified Dietary Assessment to Identify Groups at Risk for Inadequate Intake of Vitamin A* (1989)
- *Methodologies for Monitoring and Evaluating Vitamin A Deficiency Intervention Programs* (1989)
- *Nutrition Communications in Vitamin A Programs: A Resource Book* (1992)

These reports are available free of charge to developing countries and for $3.50 (U.S.) to developed countries. Copies can be ordered from the IVACG Secretariat:

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Preface

Vitamin A is an essential nutrient for humans. Its deficiency causes xerophthalmia, including night blindness and keratomalacia; abnormalities in cellular differentiation, reproduction, and the immune response; and ultimately permanent blindness and death. Because of considerable current interest in the possible pivotal role that an inadequate vitamin A status plays in morbidity and mortality in children, much attention is being given to various new ways of assessing vitamin A status.

In this monograph, experts in various methodologies contribute short descriptions of various current procedures. The physiological basis of the method, a description of the procedure, a discussion of its advantages and limitations, and its application in at least one instance are briefly summarized. This publication is intended as an introduction to various assessment techniques and not as a comprehensive description of the procedure or of its overall use. It will be of help to investigators and program planners in selecting assessment methodologies best suited to their specific situations and available resources. A few key recent references for each procedure are given to aid the reader in gathering more detailed information once a selection is made.

It must be stressed that each author is presenting his or her views about a given method and its interpretation. Thus, differences in suggested cutoff values and feasible public health criteria will appear. These differences are intended to stimulate thought and further study but certainly not to confuse the reader. We hope that you find this brief state-of-the-art introduction to vitamin A assessment to be of value in your own work. We naturally welcome comments and suggestions about the publication from our readers.

Barbara A. Underwood
James Allen Olson
Editors

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Introduction

An unsatisfactory vitamin A status is a major public health problem among preschool children in much of the less-developed world. Clinical manifestations of an unsatisfactory vitamin A status include increased vulnerability to severe infections and xerophthalmia, including night blindness, keratomalacia, and irreversible blindness. Preschool children are at high risk of vitamin A deficiency because of their rapid growth rate, which increases their vitamin A requirements; their typically nutritionally inadequate weaning food diet; and their general aversion to eating green leafy vegetables. As the vitamin A status becomes poorer, total body reserves of vitamin A are first depleted, followed by a reduced concentration of plasma retinol, which in turn leads to abnormalities of tissue function. Because plasma vitamin A is homeostatically controlled and is subject to change as a result of other nutritional deficiencies, infections, and parasitic infestations, it does not serve as a useful indicator of vitamin A status in the individual unless it is very low (<10 μg/dL; <0.35 μmol/L) (1,2).

Because vitamin A normally is well absorbed from the intestine, is efficiently stored in the body, primarily in the liver but also in other tissues, and is well mobilized from storage sites for use in meeting body needs, the total body reserve of vitamin A is a good indicator of vitamin A status. Although the total body reserve can be measured by isotopic methods (3), the procedure is not generally applicable to survey protocols. Other survey procedures have consequently been developed that provide various types of information about vitamin A status. It is nonetheless useful to relate these indicators, whenever possible, to the total body reserves of vitamin A as a general reference point.

Vitamin A status can be considered as a set of five sequential categories: deficient, marginal, satisfactory, excessive, and toxic. A *deficient status* is characterized by clinical signs, primarily by xerophthalmia. Clinical signs, which have been well described in other monographs (4,5), will be considered only briefly here. A very low plasma retinol level (<0.35 μmol/L) is also associated with such signs. A *marginal status* can be detected by impaired vision in dim light, reduction in goblet cell frequency, and epithelial cell abnormalities in many tissues, including the conjunctiva of the eye, as well as by an abnormal proportional increase in plasma vitamin A and plasma retinol-binding protein (its carrier protein) in response to a small oral dose of the vitamin. A marginal vitamin A status is not associated with overt clinical signs. The marginal status is sometimes termed preclinical vitamin A deficiency. A *satisfactory status* means that all physiological functions of vitamin A are being adequately met by existent total body reserves of vitamin A. Furthermore, a satisfactory status, at least as formally defined in documents of the Food and Agriculture Organization of the United Nations and the World Health Organization, also includes a reserve of vitamin A that will provide protection from deficiency for a given period of time (6). An *excessive status* results from the ingestion of relatively large amounts of vitamin A, but without clinical signs, and a *toxic status* is characterized by a large number of clinical signs (7,8). Although excessive intakes of vitamin A are of serious concern, particularly when ingested by women in early pregnancy (9,10), excessive and toxic status categories will not be considered further in this report.

This summary of assessment methodologies places in context the uses, advantages, and constraints of each currently used technique. The booklet provides a conceptual framework

within which to consider alternative approaches to vitamin A assessment, some of which have been extensively employed and others of which have not. Thus far, the only indicators approved by the World Health Organization are clinical signs of xerophthalmia, night blindness, and plasma retinol values <0.35 µmol/L (10 µg/dL). IVACG does not currently recommend without qualification the use of other methods in general surveys. Because each procedure provides different kinds of information, each has somewhat different uses and somewhat different "windows" relative to vitamin A status. Each indicator can be approximately placed on a scale in regard to dietary intake and liver vitamin A concentrations (Figure 1), but this relationship should be considered only a guideline because of both extensive individual variation and the lack of conclusive data on the actual range of utility of each indicator.

This summary should serve primarily as an introduction for scientists to a set of useful indicators; those embarking on an assessment survey or program requiring assessment methodologies clearly should explore in depth both the availability of manuals for specific methods and the published literature. The effects of seasons and differences among geographic regions in a country must also be carefully considered. Other sources also should be consulted for determining the sample size appropriate for a given situation, for example, *Methodologies for Monitoring and Evaluating Vitamin A Deficiency Intervention Programs* (11). Before one or more of these methods are selected, available facilities, personnel, and funds must be matched both with the goals of a given survey and with the requirements and cost of a given method.

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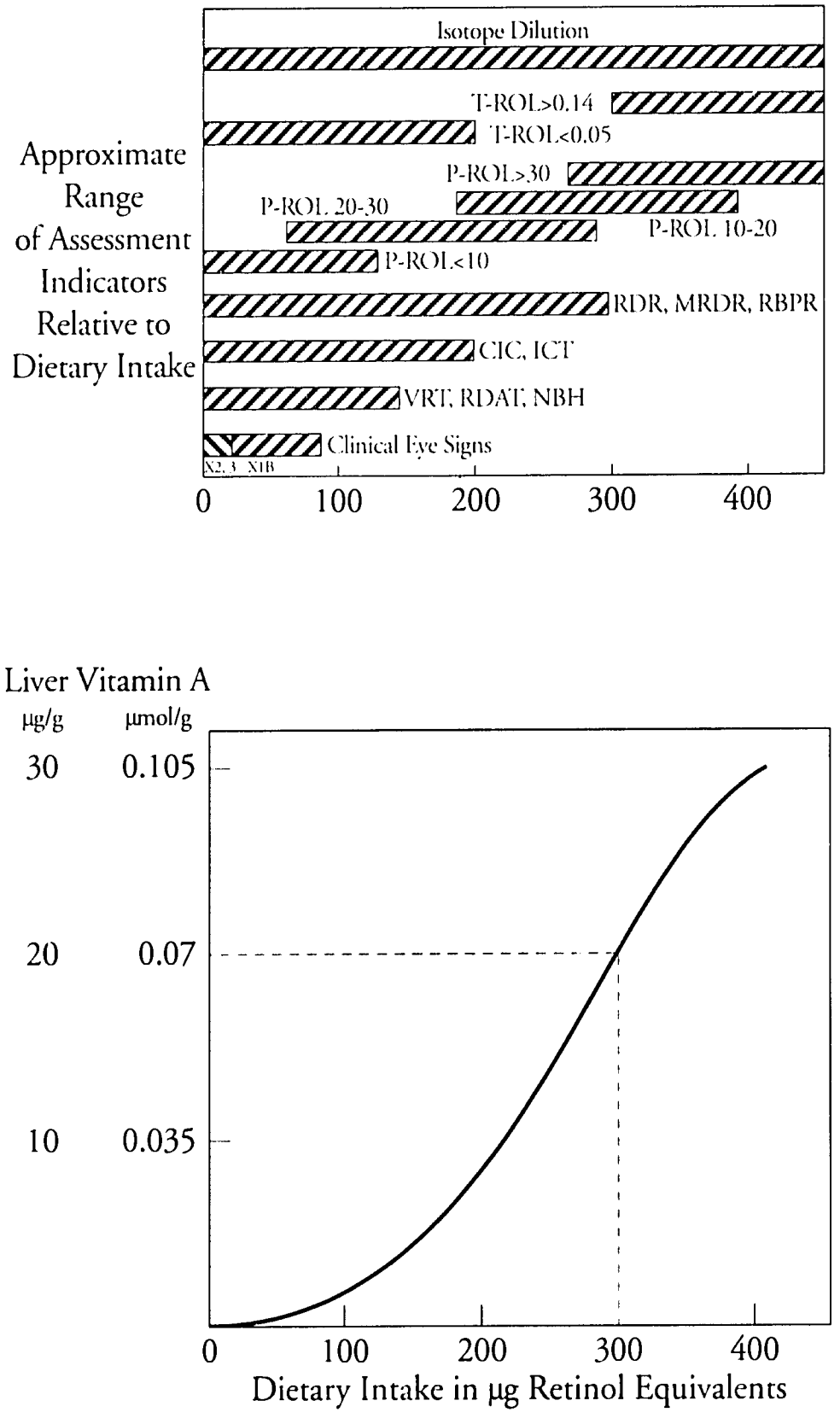

Figure 1. An approximate relationship between the average daily dietary intake of vitamin A and provitamin A carotenoids, expressed as µg retinol equivalents, and the vitamin A concentration in the liver of a preschool child.

Nonhepatic stores of vitamin A are approximately 10% of the liver reserves when total body stores are high (> 30 mg for a 15-kg child) but are proportionally greater (20-50%) when body reserves are low (< 6 mg for a 15-kg child). A satisfactory liver vitamin A concentration is considered to be 0.07 µmol (20 µg)/g wet weight.

The approximate ranges for the responses of various indicators of vitamin A status relative to dietary intake are given in the upper portion of the figure. Thus, clinical signs appear when the average daily intake of vitamin A is very low, which corresponds to negligible liver reserves. Plasma retinol concentrations <10 µg/dL are usually associated with other signs of vitamin A inadequacy, whereas a plasma retinol concentration in the range of 10-20 µg/dL may either be associated with Bitot's spots and other signs of deficiency or be found in a vitamin A-sufficient child, albeit often plagued with infections. Isotope dilution, which involves analysis of the dilution in the plasma of endogenous vitamin A by a dose of deuterated vitamin A, provides information about total body reserves of whatever magnitude.

Abbreviations: X2, corneal xerosis; X3, corneal ulceration and keratomalacia; X1B, Bitot's spots with conjunctival xerosis; VRT, vision restoration time; RDAT, rapid dark-adaptation test time; NBH, night blindness by history; CIC, conjunctival impression cytology test; ICT, impression cytology with transfer test; RDR, relative dose-response assay; MRDR, modified relative dose-response test; RBPR, retinol-binding protein response test; P-ROL, plasma (or serum) retinol concentration; and T-ROL, tear fluid retinol concentration, followed by values expressed in µg retinol/dL. Vitamin A equivalencies are: 1 µmol retinol = 286 µg retinol; 1 µg retinol = 1 µg retinol equivalents = 0.0035 µmol retinol.

PART 1. DIETARY

Semiquantitative Dietary Assessment of Vitamin A Intake

Barbara A. Underwood, Ph.D.

The habitual intake of too little vitamin A, frequently because too few dietary sources high in vitamin A activity (retinol equivalents) are eaten in relation to needs, precedes other manifestations of vitamin A deficiency. This is the underlying cause of vitamin A deficiency as a public health problem. Quantitative dietary assessments of usual food (nutrient) intakes are difficult to conduct even among literate, highly educated populations and, in the case of vitamin A, often are equally difficult to interpret as a measure of vitamin A except when used concurrently with other assessment parameters (1). The problem is lessened when the objective is restricted to classifying individuals into "at risk" categories based on their frequency of intake of vitamin A-containing foods. Due to the social and economic conditions that surround child populations where vitamin A deficiency occurs, it is usual that they consume a limited number of carotene-containing foods that are locally available. Knowledge of the availability of local food sources, their vitamin A content, and how much and how often they are fed to children can help identify populations with inadequate intakes. This information, used with other assessment measures, can identify a population's risk of inadequate vitamin A nutriture and provides a valuable basis for determining appropriate food-based long-term intervention programs to alleviate the problem.

Procedure

The International Vitamin A Consultative Group (IVACG) prepared a guideline for developing a semiquantitative dietary assessment for identifying populations at risk for inadequate dietary intake of vitamin A (2). It does not assess intake of other nutrients.

The first step is to determine the locally available sources of vitamin A activity and their use in the diets of young children by conducting a qualitative survey of a random selection of local markets, gardens, and uncultivated sources and, using food tables, grouping them according to their content of retinol equivalents. The next step is to interview child-care givers to determine local practices in incorporating these foods in children's diets and the usual portion sizes they are fed.

From this information, a locally appropriate food composition table can be constructed to categorize local foods fed to children into high, moderate, and low categories, based on their relative vitamin A content and the smallest portion size fed. A simple dietary questionnaire can then be developed that lists only the local food sources of vitamin A and groups them into categories designating their relative contribution toward meeting the vitamin A requirement if consumed with sufficient frequency. The questionnaire is then administered by local workers by first determining the intake and portion size of the predesignated foods in the last 24 hours and determining the usual frequency of intake daily, weekly, and monthly. "Risk" categories can be assigned on a population basis after calculating a "consumption index" where diets show little variation and/or a "usual pattern of frequency" where variation is more common.

Interpretation

The steps outlined above must be adapted and applied locally. Risk categories using the consumption index (CI) are established based on the recommended dietary intake for preschool children. Risk categories for the usual pattern of food consumption (UPF) are established based on the safe level of vitamin A intake and the basal requirement for preschool children. The validity of these val-

ues and the usefulness of the approach are currently being reviewed in light of numerous applications of the methodology in different countries and cultures (3).

Advantages

The procedure avoids the time-consuming effort to collect strictly quantitative information yet allows identification of high-, moderate-, and low-risk populations. After a locally appropriate dietary questionnaire is developed by an individual knowledgeable in food consumption, it can be administered by locally trained interviewers. The interviewer can inform mothers about what foods rich in vitamin A activity are locally available to feed their children if they are not doing so or if they are not feeding them frequently enough. Because the information is based on the local situation, it provides a relevant baseline from which to develop nutrition education materials and relevant potentially sustainable programs to change children's feeding behaviors and improve their diet. In addition, periodically repeating the assessment is a means of monitoring the success of programs in changing food intake behaviors.

Limitations

The methodology does not provide information on the total adequacy of the children's diet. When complex recipes are usually fed to children, developing an appropriate food composition table can become time-consuming. The validity of the cutoff values to determine risk using the CI and/or UPF needs further testing.

Application

Since the IVACG *Guidelines for the Development of a Simplified Dietary Assessment to Identify Groups at Risk for Inadequate Intake of Vitamin A* were published, they have been applied to varying degrees in at least 20 applications in 14 different countries throughout the world in situations that typify the field conditions for which these guidelines were developed. Each situation presents different challenges, and numerous suggestions have been made for improvements and modifications. An effort is now underway to compile these experiences so that future users will profit from past and current successes and failures, and know the reasons for each.

Others are attempting further simplification of the approach by eliminating the need to establish portion sizes or a local food composition table (D. Rosen, unpublished and undated manuscript from Helen Keller International). The validity of this modification is being field tested.

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PART 2. PHYSIOLOGICAL

Historical Assessment of Night Blindness

Alfred Sommer, M.D.

Night blindness, or inability to see under low levels of illumination such as at early dawn, at late dusk, or in a room lit by weak candle, lantern, or other light, is often the first clinical manifestation of mild vitamin A deficiency. Actually measuring the ability of the eye to adapt to low levels of illumination (dark adaptation) or testing every child for night blindness is difficult and time-consuming.

Instead, an easier approach, simply asking the child's mother or another guardian familiar with the child whether the child is night blind, has proved practical in a number of cultures. This seems most helpful in communities where vitamin A deficiency is sufficiently endemic and chronic that a local term exists for the problem. The existence of this local term is the basis for using a "history of night blindness" as a valid parameter for assessing vitamin A status in that particular population (see Table 1).

Procedure

Determining whether a locally appropriate term exists often requires asking about symptoms rather than asking about "night blindness" literally translated. For example, local terms in Indonesian and Bengali literally mean "chicken eyes" or "chicken blindness," because chickens are night blind. (See Table 1 for some other country-specific local terms.)

The best means of identifying an appropriate local term is to ask the target populations, including pregnant women and schoolchildren. The term often will be specific to the regional language or local dialect. Once a locally appropriate term is identified, it can be validated by specifically inquiring what the population means by the term and what age groups it affects. One can be misled by terms that actually mean "blindness." The distinction is easily made: "night blindness" overwhelmingly involves children and is not present during bright sunlight; "blindness" overwhelmingly involves the elderly and is present most, if not all, of the time.

If in doubt, one can further validate the term by revisiting children with a positive history at dusk or after dark or by placing them in a dimly lit room for 10-20 minutes to dark-adapt. Their ability to find candy, toys, or their parents can be compared with that of children said not to be night blind who have been tested in the same way.

If a mother answers affirmatively to the local term for night blindness, her response can be further confirmed by asking what she means by it. Usually, she will indicate that the child is afraid to move after dusk or cannot find his food or other needs, and that this behavior is different from that of other children in the village. Mothers will sometimes say the child is night blind only after a particularly sunny day or after flying a kite (equivalent to looking up at the bright sky), but not after a cloudy day. This child is definitely suffering from mild night blindness and should be noted as night blind. The exposure to very bright sunlight stresses the ability to dark-adapt and therefore reveals mild vitamin A deficiency.

Interpretation

The World Health Organization's (WHO) recommendations stipulate that vitamin A deficiency is a "serious public health problem when at least 1% of the children are night blind." In rate calculations, only the number of children 2 years and older, if that is the cutoff age, should be included in both the numerator and the denominator.

Table 1. Local Terms for Nighblindness in Selected Countries

Country	Language	Term for Nightblindness	Translation Note
Bangladesh	Bengali	*rat kana*	"night blind"
Brazil	Portuguese	*cegueira noturna*	
Burkina Faso	More	*you zondo*	
	Marka	*souran fie*	
	Samo	*;oro yei*	
Cambodia	Khmer	*quack mourn*	
China	Chinese	*ye mang*	
Chad	Bilala	*mournou* or *mordourou*	
Ethiopia	Amharic	*defint* or *dafint*	
	Eritrean	*ghahmi*	
	Tigrignian	*ghahmi*	
Ghana	Kassim	*titi yibri*	
	Nankani	*yong pelipse*	
Haiti	Haitian creole	*jé poul*	
India	Tamil	*maalaik'kun* or *malai ken*	
	Telegu	*raychekatloo*	"evening eyes"
Indonesia	Indonesian	*sisiak* or *buta ayam*	"chicken blindness" or "eyes"
	Sundanese	*kotokeun*	"chicken blindness"
	Javanese	*kotok ajam*	
Kiribati	I-Kiribati	*mataki nte bong*	
Malawi	Chichewa	*chidwi*	Used in Lower Shire Valley
Mali	Bambara	*suranfiye*	
Nepal	Nepali	*ratandho*	
Niger	Hausa	*dundumi* or *doufoun ido*	
	Djerma	*dundumi*	
Nigeria	Yoruba	*nakoju*	
	Hausa	*dundumi*	
	Boko dialect	*venaguasina*	
Pakistan		*chanjar, andhrata, narata*	
		ratonda, shabkor or *shabkori*	
Papua New Guinea	Pidgin	*ai tutak long nait*	"eye too dark in the night"
Peru	Local Spanish dialect	*nietalopia*	
Philippines	Tagalog	*matang manok*	"chicken eye"
	Bicolano	*barapen* or *barap*	
	Cebu Island language	*mahap sa hapon*	
	Hilagaynon	*kurap*	
	Kinaraya	*barapon*	
Senegal	Wolof	*mbenpéne*	
Sudan	Arabic (proper)	*ashaleli*	
	Arabic (colloquial)	*jahar*	
	Dinka	*juhara*	
	Kordofan Province	*abuginfet*	
	Darfur Province	*amele'li, jhaar, jahman,*	Refers to nightblindness
		toormu, wa'ala ootri, abuginfet	in both cattle and humans
Tanzania	Kiswahili	*kutoona usiku*	
	dialect	*siyakuona ne chilo*	
Zambia	Chibemba	*kafifi*	

Source: Helen Keller International, *Vitamin A News Notes*, Issue 8 (Spring 1992), p. 11. Used with permission.

Advantages

The advantages of this means of assessing the problem are its noninvasiveness, simplicity, and speed. Children need not be examined, samples need not be obtained for laboratory processing, and a physician or ophthalmologist is not required. But the person asking the questions must be highly trained and skillful in collecting and interpreting the mother's response.

Limitations

A "history of night blindness" is probably not a useful index of vitamin A status where locally specific terms do not exist, particularly if the community seems uncertain or bewildered by the clinical description.

A "history of night blindness" is a useful test only in children older than 1 or 2 years of age. Mothers may not notice night blindness in younger children because younger children do not ordinarily walk about the hut or village.

Application

History of night blindness has been utilized as a standard WHO criterion in field surveys in at least a dozen countries. At least three studies (1-3) have validated the sensitivity and specificity of the procedure against other criteria and are consistent with one another. They indicate that it is a reliable test for the diagnosis of mild vitamin A deficiency both in individual children and in the population at large. The prevalence of night blindness tends to approximate or exceed the prevalence of Bitot's spots in younger children, but is sometimes lower in older children, presumably because of the presence of nonresponsive Bitot's spots among older children whose vitamin A status has improved. Because night blindness can also be a problem among children of different ethnic or regional origin in urban slums, the use of local rural terms for the condition may or may not be appropriate.

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PART 3. BIOCHEMICAL

Frequency Distributions of Serum[1] Vitamin A Levels in Cross-sectional Surveys and in Surveys Before and After Vitamin A Supplementation

Hernando Flores, Ph.D.

A. CROSS-SECTIONAL SURVEYS: SERUM/PLASMA DISTRIBUTIONS

Serum levels of vitamin A are not considered a reliable index of subclinical vitamin A status of individuals; a frequency distribution obtained from a suspect population is more useful. The frequency of values below a cutoff, usually taken as 0.70 µmol/L (20 µg/dL) for low and 0.35 µmol/L (10 µg/dL) for deficient, identifies the at risk group (1). These cutoff points have been arbitrarily established based on cross-sectional survey experiences in a variety of countries where clinical vitamin A deficiency did or did not occur, where sampling techniques were not always representative, and where consideration was not given to specific age (particularly preschool children) and sex groups (adolescent girls and fertile women) that may have had cutoff points for functional consequences different from those of their male counterparts (2). Recent work has shown that the frequency distribution curve of serum vitamin A levels in 2–6-year-old children residing in deprived environments who previously received a massive dose of vitamin A has a normal, or close to normal, shape with 95% of the values ≥ 1.05 µmol/L (30 µg/dL) (3).

Procedure

Fasting blood samples are obtained by venipuncture or finger prick from an appropriately sampled population. The specimens must be handled by well-described standard procedures to assure stability until analyzed by standardized analytical procedures that provide for an ongoing program of quality control (4,5). The biochemical values are plotted along the ordinate (x axis) and the frequency of their occurrence along the abscissa (y axis) in intervals of equal size in a continuous figure or as a histogram. For normal or near-normal distributions the data can be summarized using the mean and the standard deviation, but if the distribution is skewed, values are more appropriately presented as median and percentile values. The proportion of the population whose vitamin A status is considered nutritionally adequate or at risk is determined based on reference cutoff points.

Interpretation

Recent data suggest that the cutoff values previously considered adequate, i.e., > 0.70 µmol/L or 20µg/dL should be reconsidered. In a group of 2–6-year-old Brazilian children from a deprived environment whose vitamin A status was known to be satisfactory following supplementation, a cutoff point of 1.05 µmol/L (30 µg/dL) assured that 95% were in adequate vitamin A status. For diagnostic and program evaluation purposes, analysis of a baseline distribution against this reference (i.e., 95% values ≥ 1.05 µmol/L) provides an objective estimate of the proportion of children below the cutoff and thus at risk of vitamin A insufficiency.

Advantages

This approach in a single cross-sectional survey provides a profile of the population's current status. If the sample is randomly selected,

[1]Plasma can be substituted for serum.

the data can be extrapolated to the larger population it represents, and thus provide a baseline against which to measure future changes. It is the most cost-effective biochemical approach to assessing a population's vitamin A status. The parameters of the curve (mean, median, standard deviation, coefficient of variation, percentiles) provide quantitative estimates of the proportion of the population that would likely benefit from an intervention program.

Limitations

The profile obtained reflects a static situation and fails to capture the dynamics that may be occurring because of seasonal or other ecologic, economic, or biologic factors; therefore, it must be repeated to determine whether conditions may be improving or deteriorating. In addition, obtaining blood, although a minimally invasive procedure, is often resisted by parents and children and requires complex logistics to assure safe and proper collection, handling, and analysis of specimens. Sterile collection conditions must be maintained. Obviously, a standardized analytical laboratory is required.

Application

Serum surveys are the most commonly used means for the biochemical assessment of vitamin A status. Unfortunately, many surveys have not used appropriate random sampling, thus making it difficult to extrapolate results from a subsample to the larger population in order to obtain reliable estimates of prevalence. The National Health and Nutrition Examination Surveys (NHANES) conducted at intervals in the United States are notable exceptions (2).

B. DOSE-RESPONSE SERUM SURVEYS (S3ODR)

Blood levels of vitamin A are homeostatically controlled. Several factors in addition to dietary intake, however, can lower the homeostatic set point. The best means of determining whether a population's diet is the responsible limiting factor is to determine its response to vitamin A supplementation, i.e., to conduct a dose-response intervention. The difficulties in the past have been to identify a proper cutoff point that is generally indicative of hypovitaminosis A and to assess whether the hypovitaminosis is primary (low dietary intake or poor absorption) or secondary (due mainly to infections or defective transport mechanisms). If children with low levels of serum vitamin A show an increase of these levels after a massive dose of vitamin A, it must be concluded that hypovitaminosis was caused by an absolute or relative deficit of vitamin A intake (5). Comparison of the distribution curves of cross-sectional surveys conducted on a population-based random sample before and approximately 1 month after high-dose dietary supplementation may indicate whether the population's dietary intake was limiting.

Procedure

A fasting blood sample is obtained as described in section A before and 30-45 days after the administration of a massive oral dose (200,000 IU) of vitamin A in oil. In populations of children who initially have adequate vitamin A status, this procedure is innocuous because the liver's capacity to store vitamin A exceeds the amount of vitamin given by at least two orders of magnitude. In vitamin A-depleted children, of course, the dose also meets a defined nutritional need.

This approach should not be used where the population to receive the dose includes fertile women and women who may be in the early stages of pregnancy. Indeed, massive doses of vitamin A should not be given to fertile women at any time unless they have clinical evidence of severe deficiency (6).

Interpretation

The percentage difference in serum levels of vitamin A a month or so following the dose is calculated as:

$$\frac{T_1 - T_0}{T_1} \times 100,$$

where T_0 = value before supplementation and T_1 = value approximately 30 days after supplementation. The cutoff for the percentage increase in serum vitamin A that reflects replenishment of organic reserves has been set at 20%, for analytical and statistical considerations (7).

Advantages

The S30DR approach virtually eliminates nondietary confounding factors, because the curves are drawn with values from populations living in their own (often deprived) environment before and after treatment. There is less likelihood that significant intervening variables other than the intake of the massive dose of vitamin A during the 1-month interval will alter the distribution. This method permits an estimate of the prevalence of primary vitamin A deficiency, the easily preventable form of hypovitaminosis A that can be corrected by an improved diet or the administration of supplements.

Limitations

Limitations include the need for adequate sample collection, careful handling and storage, and accurate analysis. This includes a well-equipped laboratory for vitamin A analysis, with spectrophotometric or high-performance liquid chromatography (HPLC) analytical facilities. Some consider the need for venipuncture blood samples a constraint. This can be overcome by using finger prick blood, provided that the technicians are properly trained in obtaining community cooperation[2] and that instruments are standardized for micro procedures. Because of the need for accurate quantitative analysis, quality control of the laboratory processing and of the preservation of specimens is essential (1). Because of the widespread prevalence of AIDS infection in many societies, technicians must use appropriate precautions (rubber gloves, etc.) in drawing and handling human blood.

Application

In Recife, Northeast Brazil (3), the postsupplementation frequency distribution curve of serum vitamin A levels yielded the following parameters, in µmol/L (values before supplementation in parentheses): mean, 1.78 (1.31); SD, 0.49 (0.54); median, 1.69 (1.29); mode, 1.42 (1.02); and 95% confidence interval, 1.02-2.90 (0.42-2.63). With the postsupplementation figures as references, 33.2% of the children had deficient or marginal vitamin A status at baseline (i.e., they presented with serum vitamin A levels below the lower 95% confidence interval limit of postsupplementation values). This figure is in good agreement with that obtained using a relative dose-response procedure (40.7%) and with the percentage of deceased children whose livers had vitamin A reserves below 0.07 µmol/g (31.6%) (3).

A survey in Recife, Northeast Brazil (H. Flores, unpublished data), showed that the S30DR technique is accurate for estimating the prevalence of inadequate (deficient plus marginal) vitamin A status. With the relative dose-response as the reference, the S30DR technique yielded the following parameters: sensitivity, 91.7%; specificity, 66.3%; positive predictive value, 75.6%; negative predictive value, 87.5%; and bias, 1.2.

[2]An appropriate approach to the community usually overcomes any objections the mothers may have to this procedure. One strategy is not to ask for blood samples, but to offer a free clinical and anthropometric examination plus a "laboratory check-up." All of the results must be given to the mothers as soon as they are available. A reward to children for donating fasting blood samples is to offer them breakfast and a token gift for their cooperation.

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The Relative Dose-Response Assay

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The relative dose-response (RDR) test, originally developed by Underwood et al. in rats (1), is a good indicator of marginal vitamin A status both in individuals and in populations. The RDR assay has been validated in humans using direct measures of liver vitamin A (2,3). During vitamin A deficiency and marginal status, *apo*-retinol-binding protein (RBP) accumulates in the liver. After a small oral dose of vitamin A is administered, the *holo*-RBP is released into the serum from the liver and transported to target tissues. The response of the individual to this dose of vitamin A is measured in the serum 5 hours after administration of the dose.

Procedure

The RDR involves giving a standard oral dose of 450-1000 µg (1.57-3.48 µmol) of retinol equivalents dissolved in oil (4). The dose has also been given intravenously (1000 µg over a 30-minute interval) as a water-dispersed suspension to children with liver disease (2). Two blood samples are taken, one immediately before and one 5 hours after the dose. Venous or capillary blood samples are taken depending on whether microanalytical procedures are available. After lipid extraction of the serum samples, the serum vitamin A concentration is measured by use of high-performance liquid chromatography (HPLC) or of a suitable spectrophotometric method. The higher oral dosage level is recommended when the analytical procedure is HPLC because this method, when routinely applied, does not measure potential postdosing, residual circulating retinyl esters. The response is measured in the serum as a percentage ($A_5 - A_0 / A_5 \times 100$), where A_5 is the serum retinol concentration 5 hours after the dose and A_0 is the concentration before dosing. Theoretically, the value can be 0-100%. A response of 20% or higher is indicative of inadequate liver reserves (< 0.07 µmol/g liver; < 20 µg/g liver) (2). Negative values often occur among individuals who are in adequate vitamin A status. These values are regarded as a 0% RDR and are interpreted as reflecting an adequate liver store of vitamin A.

Interpretation

If the RDR value is ≥ 20%, the child is almost certainly in a marginal vitamin A status. To determine whether a particular community is at risk, the prevalence of a marginal status can be assessed in a randomly sampled subpopulation. Thus, a relatively small but representative number of children need to be studied to make suitable public health judgments about vitamin A status in a population. After analysis, supplements can be made available to residents of communities who have a high incidence of positive RDR values. Typically, a dose of 50,000-200,000 IU every 4-6 months, depending on age, is enough to lower the morbidity and mortality associated with marginal vitamin A status in preschool children. The same benefits can be achieved by assuring a dietary intake that meets the recommended daily need of this age group. Tentatively, a public health problem might be assumed to exist if > 20% of a population of preschool children show abnormal RDR (> 20%) values.

Advantages

The RDR is a good indicator of the individual's vitamin A status and has application for community assessment as well. The effects of confounding factors, such as infectious disease and moderate protein-calorie malnutrition, on serum retinol concentrations are minimized by

use of this response test. For analysis, investigators have a choice of using either HPLC, which is a fairly widespread technique, or any suitable spectrophotometric or photodegradative assay. The latter methods require much simpler instrumentation. Because the latter methods measure total vitamin A (esters and RBP-bound retinol), however, care must be taken that the dosage given assures hepatic uptake of postdosing esters within the 5-hour wait period.

Limitations

The major drawback of the RDR is that two blood samples are required. In some cultures, it is difficult to draw blood samples, particularly from children. Thus, the need to obtain two samples at a 5-hour interval can pose large logistic and cultural problems. Also, the need to analyze two blood samples to obtain a single human value increases the expense and time of each assay.

Application

The RDR test has been used with success in several clinical and field studies, including France (3), Brazil (4,5), Thailand (6,7), and Belize (8). Flores et al. (4) used the RDR test at four different times over a 6-month interval to determine the duration of effectiveness of supplementing children with a single massive dose (200,000 IU) of the vitamin. The RDR value in an individual reverts to normal soon after supplementation, but then becomes abnormal again when liver stores become depleted. The RDR methodology has also been applied in Brazil to evaluate the effectiveness of a food-based intervention program (9).

The RDR test has been applied to a 20% random subsample in a prevalence survey conducted over two seasons in north and northeast Thailand (7). No clinical signs were encountered in the survey, but 20% of the population were identified as marginally deficient by the RDR, 18% by conjunctival impression cytology, and 9% by a serum retinol < 20 µg/dL (≈40% with < 30 µg/dL).

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The Modified Relative Dose-Response Assay

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The modified relative dose-response (MRDR) assay is similar to the relative dose-response (RDR) assay. An analog of retinol (R), 3,4-didehydroretinol (DR), is used as the indicator in the MRDR test. A single dose of 3,4-didehydroretinyl acetate in oil is given orally. Like R, DR binds to accumulated *apo*-RBP in the liver and is released into the serum as *holo*-RBP. A molar ratio of 3,4-didehydroretinol/retinol (DR/R) in serum is determined 4-6 hours after dosing. MRDR values theoretically can range from 0 to ∞.

Procedure

The MRDR assay involves first giving children a single oral dose (100µg/kg body weight) of 3,4-didehydroretinyl acetate dissolved in an oil and then taking a single blood sample 4-6 hours later. Usually ≥ 0.5 mL of blood is obtained by venipuncture. After the serum or plasma (150-500 µL) is extracted with ethanol/hexane, retinol (R) and dehydroretinol (DR) in an aliquot are measured by high-performance liquid chromatography (HPLC). The monitoring wavelength of the detector is set at 350 nm, which optimizes the measurement of DR. The amount of serum needed is dependent on the limits of detection of the HPLC system. Standards of both R and DR of known concentration are used to calibrate the HPLC system and allow a molar DR/R ratio to be calculated.

Interpretation

Children with DR/R ratios of ≥ 0.060 are judged to be in a marginal vitamin A status. The suggested cutoff value of 0.060 was determined by comparative studies with the RDR and conjunctival impression cytology (CIC) techniques and requires further validation (1,2). A cutoff value of 0.030 was suggested earlier on the basis of ratios found in healthy American adults and children. In the light of subsequent experience, this cutoff value has now been increased. Tentatively, a public health problem might be assumed to exist if > 20% of a population of preschool children show abnormal MRDR ratios (≥ 0.060)

Advantages

The MRDR offers several advantages over other procedures. DR is a naturally occurring form of vitamin A that is found predominantly in freshwater fish, but also to a small extent in mammalian tissues, including human. DR has approximately 40% of the biological activity of retinol. Only one blood sample is required, and the serum samples can be frozen and stored for analysis. The required HPLC instrumentation, although costly and sophisticated, is fairly widespread around the world. The effects of storage on vitamin A stability and of sample extraction efficiency are minimized by employing the ratio of DR/R in a single sample. The MRDR, like the RDR, gives a good indication of an individual's vitamin A status and can be applied also in community assessments. It is easily repeated after a therapeutic dose of vitamin A and can be used for evaluation of the effect of an intervention program. Another advantage is that children can be dosed in the morning at their homes and brought to the clinic or the survey site several hours later for the sampling. Moreover, the MRDR value is reproducible over time in vitamin A-sufficient individuals (2).

Limitations

The MRDR assay has several constraints. Because of the low level of DR in the postdosing circulation, a blood sample sufficient to

yield at least 200 µL of serum or plasma is necessary to obtain the amount of serum needed for the analysis. Free DR is not stable once it is extracted from the serum. Therefore, extreme care must be exercised to protect the extracts from light and to inject them as soon as possible into the HPLC system. Because DR-acetate currently is commercially unavailable, it must be synthesized or isolated from fish liver oils. Also, blood must be taken from the children and the samples need to be analyzed using HPLC, which may pose serious limitations in some cultures and institutions. Because of the widespread prevalence of AIDS infection in many societies, technicians must use appropriate precautions (rubber gloves, etc.) in drawing and handling human blood.

Application

Currently, the MRDR has been validated in rats of varying vitamin A status by directly measuring liver stores of R (3-5). The MRDR has been applied to two populations of Iowa children (6,7). In both studies, the MRDR molar ratio was always less than 0.04. Indonesian children tested to date have not shown any overt signs of clinical vitamin A deficiency but have ratios ranging from 0.001 to 0.83 (1,8). A community assessment of two separate villages in the Bogor, Indonesia, area showed a significant difference in the MRDR values. One village was mildly malnourished while the other was fairly well nourished. The CIC test identified the same population but not necessarily the same individuals, as being at increased risk of vitamin A deficiency. The RDR gave similar results in the second population on an individual level when a 1000-µg dose was given but not in the first group when a 450-µg dose was administered (1).

Conclusions

The MRDR holds considerable promise as a minimally invasive technique by which a marginal vitamin A status can be detected in individual children before clinical manifestations appear. Correlation between the MRDR values of individuals and their liver vitamin A concentration would be useful in defining the best ratio to be used as a cutoff between a marginal and a satisfactory vitamin A status. The procedure also holds promise for application among communities to assess the prevalence of marginal deficiency.

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Plasma Retinol-binding Protein Response Test

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Vitamin A (retinol) is transported in plasma bound to a specific carrier protein, retinol binding protein (RBP) (1). RBP is synthesized in the liver and secreted into plasma as the retinol-RBP complex (*holo*-RBP). In vitamin A-deficient animals, the RBP secretion is blocked, leading to its accumulation in the liver and resulting in high liver and low plasma concentrations of RBP. When these deficient animals are given vitamin A, mobilization of preexisting RBP from the liver occurs as *holo*-RBP, which leads to a rapid decrease in the liver RBP concentration and a concomitant increase in the plasma RBP concentration (1). In vitamin A-sufficient animals, by contrast, RBP does not accumulate in the liver to the same extent, and minimal or no change in the plasma RBP concentration is seen after vitamin A administration. Thus, measurement of changes by simple immunologic diffusion methods of plasma RBP concentrations, which occur in response to a single oral dose of vitamin A, may be employed where appropriate biochemical laboratory facilities are not available.

Procedure

The plasma RBP response test consists of measurement of plasma RBP concentrations in sequentially obtained blood samples just before (baseline) and approximately 5 hours (or at 1, 3, and 6 hours where feasible in institutional settings [2]) after an oral dose of 600-1000 µg of vitamin A, or in institutional settings, an intramuscular injection of a standard dose of water miscible vitamin A (2000 IU retinyl palmitate/kg body weight for very-low birth weight [VLBW] infants [2] or 1000 µg for older children [3]). Each sample consists of a minimum of 80 µL blood drawn by venipuncture or puncture of a warmed finger, heel, or earlobe, into two heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, Pennsylvania). Plasma is separated from each sample by centrifugation (Hemacrit centrifuge, International Equipment, Boston, Massachusetts). Plasma RBP concentration is determined in duplicate by quantitative radial immunodiffusion (LC-Partigen immunodiffusion plate plasma RBP, Behring Diagnostics, Somerville, New Jersey) or by enzyme-linked immunosorbent assay (4). The percentage increase in the plasma RBP concentration (ΔRBP) from its baseline value is calculated by the following equation: ΔRBP (%) = [RBP (maximum) - RBP (baseline)] / RBP (baseline) × 100. The lowest ΔRBP value assigned is zero, even when the plasma RBP concentration decreases from the baseline value.

Interpretation

The plasma RBP response test has been used most extensively among VLBW infants, for whom it should be interpreted in conjunction with other conventional biochemical indices of nutritional and vitamin A status, e.g., energy intake, vitamin A intake, plasma concentrations of vitamin A and RBP, or plasma retinol/RBP molar ratio (2). Among VLBW neonates, a high ΔRBP value (≥ 8%), together with low plasma concentrations of vitamin A (< 20 µg/dL) and RBP (< 2.0 mg/dL) and a low plasma retinol/RBP molar ratio (< 0.6), is compatible with functional vitamin A deficiency (2). A low ΔRBP value (< 8%), together with normal plasma concentrations of vitamin A (20-80 µg/dL) and RBP (≥ 2.0 mg/dL) and normal plasma retinol/RBP molar ratio (≥ 1.0), is compatible with normalized functional vitamin A status (2).

Because this approach has received limited

application among other age groups, appropriate interpretative guidelines need to be developed. Among normal adults in Guatemala, the plasma retinol-binding protein response test (RBP-RDR) gave results comparable to those obtained by the conventional relative dose response (RDR) test (5). In this application, the RBP-RDR was calculated and interpreted using the same timing and interpretative criteria as for the RDR test.

Advantages

The plasma RBP response test is simple to perform and applicable both under field and clinical situations. The dose of vitamin A used in the test is within the range that has been reported to be safe even for VLBW infants (6). The blood volume required for testing is easily within the range obtainable from finger prick and also within the range commonly used for biochemical monitoring. It uses inexpensive equipment and commercially available or easily prepared assay kits, and therefore does not require a sophisticated laboratory. The test provides information that may be used for identification of individuals and screening of populations for the prevalence of functional vitamin A deficiency.

Limitations

The plasma RBP response test has been studied in a limited number of a selected group of VLBW infants (2) and adults (5). Both prematurity and postnatal disease states may potentially affect RBP synthesis in the liver and therefore the plasma RBP response to vitamin A administration. The high ΔRBP values seen even in sick VLBW infants (2), however, suggest that both prematurity and postnatal disease states may not be limiting factors in the elicitation of the plasma RBP response. Nutrient deficiencies, including energy, protein, and zinc, potentially also may affect RBP synthesis in the liver and therefore the plasma RBP response to vitamin A administration. A study in preschool children with protein-energy malnutrition and vitamin A deficiency, however, shows that the plasma RBP response to vitamin A administration is intact in most such children (7).

Application

VLBW neonates are vitamin A deficient because their liver stores of vitamin A are lower than those of term infants (7). The plasma RBP response test (modified in the institutional setting to collect blood samples at baseline, 1, 3, and 6 hours after intramuscular injection of 2000 IU/kg water-miscible retinyl palmitate) was performed in a group of VLBW neonates (birth weight < 1350 g, gestational age < 31 weeks, ventilator-dependent for > 24 hours after birth, n = 24) studied shortly after birth—before the initiation of vitamin A supplementation—and again after a 28-day period of vitamin A supplementation (2). On postnatal day 1, the ΔRBP values in this study were high (mean ± SD = 61 ± 37%, range 24-192%), and the plasma vitamin A and RBP concentrations and the plasma retinol/RBP molar ratios were low, indicative of vitamin A deficiency. Supplemental vitamin A improved the vitamin A status of all infants, as shown by low ΔRBP values (mean ± SD = 8 ± 9%, range 0-33%), by normal plasma vitamin A and RBP concentrations, and by normal plasma retinol/RBP molar ratios on postnatal day 28.

Conclusions

The plasma RBP response test is based on a sound rationale involving RBP synthesis and secretion from the liver in relation to vitamin A status. This test involves calculation of the percentage increase in the plasma RBP concentration (ΔRBP) from its baseline value in response to acute vitamin A administration. A high ΔRBP value is compatible with functional vitamin A deficiency, whereas a low ΔRBP value is indicative of normalized functional vitamin A status. An evaluation of this test in VLBW infants showed its usefulness as a monitoring technique for identification of those infants with persistent functional vitamin A deficiency and possibly with increased risk for bronchopulmonary dysplasia (5). The test is simple to perform and applicable to both field and clinical situations. Further validation of this test, however, is necessary to determine its usefulness as a field-applicable screening test for marginal vitamin A deficiency and as a diagnostic technique for assessment of vitamin

A status in individuals such as older infants and children at risk for diseases associated with vitamin A deficiency.

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PART 4. HISTOLOGICAL

Conjunctival Impression Cytology

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Vitamin A is essential for cell differentiation and maintenance of cell membrane integrity. The presence of vitamin A allows stem cells to transform to mucin-secreting cells (goblet cells) while blocking the transformation from columnar epithelial cells to stratified squamous epithelial cells. The absence of vitamin A causes a loss of goblet cells and an increased squamous metaplasia of epithelial cells.

Conjunctival impression cytology (CIC) is a method that assesses this vitamin A-dependent process in a readily accessible epithelial tissue, the bulbar conjunctiva. Sampled cells are stained and evaluated for histologic changes that are specific to the vitamin A deficient state. Animal models show that in vitamin A deficiency, microscopic changes on the surface of the eye appear 4-6 weeks before clinical signs appear. The pathophysiology of vitamin A deficiency at the target cell level, in this case the bulbar conjunctiva, provides the basis for employing CIC as an indicator of vitamin A status.

Procedure

The CIC technique is well described in Natadisastra et al. (1). Briefly, cellulose acetate filter paper is applied to the inferotemporal bulbar conjunctiva of the eye, removed, and placed in fixative prior to staining. The procedure is repeated on the other eye. There are currently two techniques in use: the strip method and the disk applicator method. Both use cellulose acetate filter paper. The difference is that the strip method employs cut lengths of filter paper of varying sizes and is applied by hand whereas the disk applicator uses a punched-out, 0.5-cm-diameter round disk of paper and is applied to the eye by means of a hand-operated vacuum pump and probe. The disk applicator eliminates contact between the paper and fingers, provides a standard-size area of contact, improves targeting of the sample site, and requires less than 1 second (compared with 3-5 seconds with the strip method) to obtain a sample. Importantly, status derived from disk specimens correlates more closely with serum vitamin A levels than does status using the strip method (2). Both techniques employ the same staining method of periodic acid-Schiff (PAS) to stain the mucin of goblet cells and hematoxylin to stain the nuclei of epithelial cells.

Interpretation

Centers currently using impression cytology have agreed on four diagnostic categories: abnormal, borderline abnormal, borderline normal, and normal—which can be collapsed into: normal and abnormal. (These correspond to the impression cytology with transfer [ICT] categories of deficient, marginal minus, marginal plus, and normal. See chapter on "Impression Cytology with Transfer.") A specimen is normal if at least five goblet cells are present, which occurs in conjunction with sheets of normal epithelial cells. A specimen is abnormal if goblet cells and mucin spots are absent. Mucin spots consist of the contents of goblet cells and often are seen when the paper fails to lift intact goblet cells. A child is considered to be vitamin A sufficient if either of the two temporal specimens is normal, and is considered to be vitamin A deficient if both specimens are abnormal. Because vitamin A deficiency is systemic and both eyes are involved, only one normal eye is sufficient for the child to be considered normal.

Conditions under which CIC and other indicators of vitamin A status are likely to be correlated have yet to clarified (3). It should be remembered that CIC measures a different aspect of vitamin A nutriture than biochemical

indicators, i.e., target tissue cellularity, integrity, and function versus circulating or storage levels of vitamin A. Whereas vitamin A supplementation may rapidly boost serum retinol levels (although not necessarily tissue stores), the resolution of the cellular pathology of the conjunctiva may take several weeks. Hence, the kinetics of response to therapy differ markedly. Several studies employing CIC at baseline and after vitamin A intervention (1,4,5) have noted improvement in the status of all CIC-abnormal subjects from 4 weeks (5) to 6 months (1,4).

Advantages

CIC possesses the following advantages as an indicator of vitamin A status: it is relatively noninvasive and inexpensive, it is readily carried out under field conditions in children > 3 years of age, it does not require cold storage of specimens or reagents, and under ideal conditions it may require only a few days of training (for individuals with clinical acumen) to learn to obtain, stain, and interpret specimens. However, CIC does require more extensive training, particularly practice in obtaining specimens from young children, for nonclinical personnel. CIC appears to be most appropriate for community assessment and for monitoring the impact of vitamin A intervention at the community level. Relative to clinical indicators, a smaller sample size is required, and marginal as well as deficient status is assessed.

Limitations

Disadvantages of the CIC technique include the well-described continuum of cytologic changes that cause difficulty in interpreting specimens. There is clear agreement on what is normal and what is abnormal. It is, however, often difficult to interpret borderline specimens (5-10% of most samples), especially borderline cases between categories. Centers that are currently active in CIC research agree that the four classification categories used at present represent a proper balance between diagnostic resolution and the ability of observers to agree on a classification.

Morbidity owing to causes other than vitamin A also may confound the test. The presence of conjunctivitis increases the number of abnormal specimens by CIC. It is known from biopsy studies (electron microscopy) that vitamin A-deficient conjunctiva has a higher density of bacteria and polymorphonuclear leukocytes than normal tissue. Whether an inadequate vitamin A status predisposes children to conjunctivitis is not known at present. Active trachoma, malaria, and vernal conjunctivitis also are reported to confound interpretation.

Applications

More than a dozen studies employing impression cytology to assess vitamin A status, without or with transfer, have been published. (In general, mean serum vitamin A concentrations were lower in children with abnormal cytology.)

A population-based survey in Guatemala showed comparable results of vitamin A deficiency detected by various indicators. The prevalence of vitamin A deficiency ranged from 8% by relative dose-response (≥ 20%) to 13% by CIC and to 18% by serum retinol levels (< 0.70 µmol/L) (3), although there was poor correlation among the indicators. In a prevalence survey of 468 children in Thailand (4), 39% were abnormal by CIC, 39% by serum retinol levels, and 36% by vision restoration time (VRT). In this study, CIC, VRT, and serum retinol levels covaried with one another in a dose-response fashion.

Conclusions

CIC measures histological changes at the target tissues that may occur as a consequence of inadequate vitamin A status. Additional testing is needed to assure the specificity of this test in all environments. CIC is best suited for assessing communities for vitamin A deficiency and for monitoring interventions, but is less practical for individual assessment. It is relatively inexpensive, can be used in the field, and does not require the cold storage of samples or an extensive waiting period. Concurrent eye infections, however, may confound interpretation. CIC does require that the person obtaining the specimens be well trained and committed to fastidiousness in collecting specimens and interpreting data.

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Impression Cytology with Transfer

Cecile Carlier, Ph.D.

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Currently recognized field-applicable indicators of marginal or deficient vitamin A status include clinical signs and symptoms (xerophthalmia), biochemical markers (serum retinol concentrations and relative dose-response tests), and cytological characteristics (conjunctival impression cytology [CIC]). Each of these indicators has limitations in feasibility and practicality in the field as well as in quantitative and qualitative interpretation. The physiological basis for CIC was presented in the preceding section. This section presents and evaluates a simplifying modification for preparing cells for cytological evaluation.

Experiences from two epidemiological surveys conducted in different rural areas of Senegal were used to check the reliability and validity of impression cytology with transfer (ICT) to standardize the cytological results and to promote its use as a mass screening test.

Procedure

ICT is a modification in the CIC procedure in which the conjunctival epithelial cells are transferred from the filter paper to a glass slide and then stained directly in one step. This is done as follows: a 2.5 x 25-mm strip of cellulose acetate filter paper (Millipore HAWP 304) is placed manually, as far as possible, on the inferoquadrant of the conjunctiva of the eye for several seconds and then carefully peeled away. The cells collected on the paper are immediately transferred from the filter paper to a clean glass slide by applying simple finger pressure. A conjunctival imprint is taken from each eye. The slide to which the two conjunctival imprints are transferred is immersed in a bath containing a mixture of 1:8 (vol/vol) 95% alcohol:staining solution, respectively, for a minimum of 15 minutes. The stain is composed of one volume of carbol fuchsin and two volumes of 0.2% Alcian blue in 5% acetic acid. (The 1:8 mixture of alcohol:staining solution is prepared as required and used up to 2 weeks.) The excess stain is then rinsed from the slide with a gentle stream of running water and the stained cells are observed under a light microscope; epithelial cells stain pink and goblet cells stain blue. In accord with Table 1, samples are classified as normal (N), marginal plus (M+), marginal minus (M-), and deficient (D).

The reproducibility of the four histological interpretations by one reader during two observations (n = 1250 slides) under light microscope was as follows: First reading: D (29), M- (178), M+ (165), and N (878). Second reading: D (25), M- (209), M+ (170), N (846).

Advantages

ICT is cheap, relatively noninvasive, and easy to use under field conditions by a well-trained technician. Minimal equipment is needed, and

Table 1: Characterization and classification of specimens

Goblet cells	Epithelial cells	Classification
Present	Numerous, small, in masses	Normal (N)
Present	Numerous, more small than enlarged, and separated	Marginal normal (M+)
Absent; mucin spots present	Enlarged, more separated than small, in masses	Marginal deficient (M–)
Absent	Enlarged, separated	Deficient (D)

Table 2: Sensitivity and specificity of ICT for three histological classifications.

Classifications	Deficient vs. nondeficient	Positive vs. negative	Abnormal vs. normal
Sensitivity (% ± Confidence interval)			
With or w/out ocular infections	11 ± 6	48 ± 9	74 ± 8
With ocular infections	9 ± 4	58 ± 6	80 ± 9
Without ocular infections	12 ± 5	33 ± 7	65 ± 13
Specificity (% ± Confidence interval)			
With or w/out ocular infections	96 ± 3	70 ± 5	48 ± 6
With ocular infections	96 ± 3	55 ± 7	33 ± 8
Without ocular infections	95 ± 3	85 ± 5	62 ± 8

the reagents are readily available and inexpensive in most countries. The specimen is stained in one simple step and is stable under routine field conditions until read with light microscopy. The procedure also requires a smaller sample size relative to clinical indicators and assesses marginal as well as deficient status. When ICT is used in population-based surveys with other assessment information, it provides another criterion for assessing whether there is a public health problem.

Limitations

In children under 3 years of age, it is sometimes difficult to obtain samples. Patience and care in approaching the child and custodian are important to minimize this resistance and to successfully obtain a specimen. In addition, interpretation of borderline specimens can be difficult, and ocular infections, including trachoma, acute malaria, and vernal conjunctivitis, can confound interpretation.

Application

To evaluate the ICT as a feasible, reliable, and valid indication of vitamin A deficiency, an epidemiological survey (n = 1259) and a case-control study (n = 413) were conducted in children from a rural area in Senegal. These data were used for establishing the reproducibility of ICT classification into normal and abnormal (Table 1), to evaluate sensitivity and specificity of the procedure (n = 413), and then to propose a histological criterion for assessing the public health significance of vitamin A deficiency.

The kappa statistic, which was used to assess the degree of agreement in the reading of the histological findings, was determined for the three possibilities of classifying ICT results. The kappa ± standard error (n = 1259 slides) for the three classifications was: 0.66 ± 0.03 for deficient (D) versus nondeficient (M-, M+, and N); 0.86 ± 0.03 for positive (D, M-) versus negative (M+, N); and 0.91 ± 0.02 for abnormal (D, M-, M+) versus normal (N). The best kappa statistic data correspond to the abnormal versus normal classification (i.e., 0.91).

Sensitivity and specificity were defined with respect to serum retinol levels from the case-control study (n = 413). The mean and the standard error for serum retinol were 0.48 ± 0.02 µmol/L. Thirty percent (n = 123) of the children were classified as deficient (serum retinol level < 0.35 µmol/L), 56% (n = 231) as marginal (serum retinol level ≥ 0.35, < 0.70 µmol/L), and 14% (n = 59) as normal (serum retinol level ≥ 0.70 µmol/L). The sensitivity and the specificity calculated in regard to the three possible classifications of ICT are given in Table 2.

In Table 2, the sensitivity and specificity of ICT with their confidence limit intervals are calculated for the three histological classifications and a retinol threshold fixed at 0.35 µmol/L in 413 preschool Senegalese children with (n = 221) or without (n = 192) ocular infections.

On the basis of the kappa statistic[1] (0.91) and the sensitivity (74%) and specificity (48%)

[1]Cohen's *Kappa statistic* varies from 0.5 (no association) to 1 (complete agreement).

values, the abnormal (M+ + M- + D) versus normal (N) classification is proposed as the standard for interpretation of specimens.

Ocular infections, including trachoma, and vernal conjunctivitis are confounding problems for ICT. They lead to moderate changes in diagnostic values (specificity). The reasons for the histological modifications in these infections are unclear. Nonetheless, vitamin A therapy converted abnormal ICT results into normal ones to the same extent in preschool Senegalese children suffering or not suffering from inflammatory trachoma (relative risk = 1.1) (1).

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PART 5. CLINICAL

Clinical Signs of Xerophthalmia

Alfred Sommer, M.D.

Xerophthalmia ("dry eye") is the most readily recognized clinical manifestation of vitamin A deficiency and has been the most widely employed, definitive criterion for assessing whether vitamin A poses a significant public health problem (1). Xerophthalmia includes all ocular manifestations of vitamin A deficiency, from night blindness through corneal ulceration, scarring, and resultant blindness.

The clinical characteristics of xerophthalmia have been classified (Table 1). A detailed description of diagnostic techniques suitable for field use, including criteria and interpretation, is available (2).

The mildest sign or symptom is night blindness (XN). A simplified screening technique, utilizing the history provided by the parent or guardian, as well as other methods of detection, are described elsewhere in this manual.

Procedure

Keratinization of the surface of the conjunctiva (X1A) results in a dry, "unwettable" surface. Readily recognizable plaques first form adjacent to the temporal limbus and are commonly covered with a foamy or cheeselike material. This easily recognized lesion is known as a Bitot's spot (X1B). The cheesy or foamy material is easily wiped off but will reaccumulate rapidly. With more severe deficiency, the nasal region and ultimately all of the conjunctiva can become involved. Caution: X1A is readily overdiagnosed. Only the presence of the foamy or cheesy material (X1B) is sufficiently specific for assessment purposes, and only when present in children under 6 years of age. Temporal Bitot's spots among older children may represent the remains of prior deficiency and may not respond to vitamin A. Signs among older children are therefore less useful for population assessment.

Keratinization of the cornea causes it to lose its normally glistening appearance. Such corneal xerosis (X2) is almost always accompanied by X1B.

Corneal ulceration (X3) represents loss of corneal stroma. If treated immediately, smaller lesions will heal with localized scars (XS). Larger lesions may involve most or all of the cornea (keratomalacia) and almost always result in large scars, distorted, protruding globes (staphylomata), or small, shrunken, sightless (phthisical) eyeballs. All represent healed, end-stage disease and are collectively classified as XS. Since other conditions like bacterial infections or physical trauma can also result in corneal ulceration and subsequent scarring, the diagnosis of XS (e.g., vitamin A

Table 1: Clinical criteria for assessing the public health significance of xerophthalmia (ref. 1)

Criterion	Minimum prevalence
Night blindness (XN)	> 1.0%
Bitot's spot (X1B)	> 0.5%
Corneal xerosis and/or ulceration (X2, X3A, X3B)	> 0.01%
Xerophthalmia-related corneal scars (XS)	> 0.05%

deficiency-related) depends on the clinical appearance and the historical setting (the existence or history of general protein-energy malnutrition, chronic diarrhea, measles, trauma, etc., at the time of the original ocular disease). Because of the high mortality associated with vitamin A deficiency, associated illnesses, and blindness, X3 and XS are rarely encountered in population-based surveys.

Interpretation

Prevalence criteria for each clinical manifestation of xerophthalmia have been established that are expected to reflect a level of vitamin A deficiency that represents a significant public health problem (Table 1). Note: These criteria are the consensus of expert groups; a government might well wish to select less, or indeed more, stringent criteria. The listed prevalence values are applicable only to a representative survey (of the population of interest) of children 0-5 years of age.

Advantages

Although a simple examination can be quickly conducted, the examiner must be well trained and his or her diagnostic skills must be standardized and reproducible. Interpretation of corneal scarring (XS) almost always requires a well-trained ophthalmologist.

Limitations

Although xerophthalmia surveys have been considered the primary reference standard for the assessment and determination of a public health problem, the relatively low prevalence rates of clinical disease and the clustering of cases (3) require large sample sizes.

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Vision Restoration Time

Emorn Udomkesmalee, Ph.D.

The role of vitamin A in vision is well established (1). In the retina, two photosensitive pigments, rhodopsin of the rod cells and iodopsin of the cone cells, are formed by the binding of vitamin A aldehyde (retinal) to the protein opsin. Rhodopsin is primarily responsible for the ability to see in dim light. In the early phase of the dark adaptation process, however, iodopsin is also regenerated. Exposure to light causes the cleavage of the photopigments into opsin plus retinal, a process called "bleaching." In the dark-adapted eye, rhodopsin is resynthesized. The earliest sign of vitamin A deficiency is a decrease in dark adaptation ability because of a decline in rhodopsin concentration. Classic dark adaptation testing by the Goldmann-Weekers adaptometer is tedious and time-consuming. Furthermore, the instrument itself is expensive and cumbersome, rendering it unsuitable for field conditions (2). In the past several years, attempts to evaluate dark adaptation ability in the field have been made through development of a rapid dark adaptation test (3), the use of the Friedmann Visual Field Analyzer (4), and measurement of the time required to identify a specific object in a dark room (5). These tests, however, have not proven to be reliable under all conditions.

Recently, a portable dark adaptometer was developed at the Institute of Nutrition, Mahidol University, Thailand (INMU). After the eye has been bleached with bright light to dissociate the rhodopsin and iodopsin complexes, the time it takes for the child to identify letters illuminated by a fixed dim light is measured. This time is termed "vision restoration time" or VRT. The rate of rhodopsin and iodopsin regeneration should depend on the amount of vitamin A stored in the retina, at least up to a certain saturation level. Thus, as vitamin A stores of a child are depleted, the rate of rhodopsin synthesis should decrease and dark adaptation measured as VRT should increase.

Procedure

The instrument developed at the INMU consists primarily of a bright light (10,000 candela/m^2), a dim light (0.15 candela/m^2), a screen, and a control switchboard, which modulates the duration of bright light and the intensity of both bright and dim light. (The candela is a measure of the luminous intensity of light.) The entire apparatus is enclosed in a rectangular metal box to prevent entry of external light. As a result of testing in healthy children, the intensity of dim light has been fixed at a certain illumination. A child sits in front of the adaptometer and uses the left eye to look through the eyepiece at a screen inside the instrument. A metal support is used to securely lock the chin and face of the child onto the eyepiece. A black cloth is draped over the face to prevent the entry of any outside light. The eye is first bleached with bright light for 2 minutes. The bright light is then replaced by the dim light, and immediately thereafter a single letter is projected onto the screen. The elapsed time between turning on the dim light and the verbal identification of the letter is recorded as VRT (measured in seconds). The test can be completed in 4-8 minutes.

Interpretation

The 1989 model of the portable dark adaptometer was tested in 973 healthy urban Thai schoolchildren of both sexes who were 6-12 years of age. The average VRT was 122 ± 46 seconds (mean ± SD). By using approximately two standard deviations to calculate the cutoff time for vitamin A-sufficient children, a VRT < 180 seconds is considered normal and a VRT > 180 seconds is considered abnormal.

Advantages

VRT is useful for the detection of subclinical vitamin A deficiency in the community as well as for the evaluation of vitamin A intervention programs. Measurement of VRT is noninvasive, relatively simple, rapid, inexpensive, practical, and applicable for different field conditions.

Limitations

Physiologically, VRT seems to be related to the early phase of dark adaptation, in which cone cells play an important role. Because rod cells are more dependent on vitamin A status, modifications are currently being made to the instrument to measure the rods' function. Owing to the nature of the test, which requires a certain level of comprehension and a verbal response, measurement of VRT is limited to school-age children or adults. Attempts are currently being made to adapt the test to younger children, who are more vulnerable to vitamin A deficiency. In addition, because of the large standard deviation associated with VRT, the test requires a large number of subjects for meaningful results. Moreover, the zinc status of the studied population should also be considered, because inadequate zinc nutriture also results in impaired dark adaptation ability or delayed VRT (6,7). Currently the instrument is available only from INMU in Thailand.

Application

During 1988 and 1989, VRT was employed as one of the indicators of vitamin A status in a project that promoted consumption of vitamin A-rich food in a school lunch program in Nongkhai province in Northeast Thailand (8). The strategies included enhanced motivation, improved communication, and an integrated farming system. VRT measurement seemed to identify the same population of schoolchildren with marginal vitamin A status as that determined by serum retinol concentrations or conjunctival impression cytology. When only VRT was used to monitor the effectiveness of vitamin A intervention in the form of a school lunch program for 1 year (1988-1989), significant improvement to normal values was observed, especially in children whose baseline serum levels were initially classified as low (0.35-0.70 μmol/L) or deficient (< 0.35 μmol/L).

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Rapid Dark Adaptation Time

James Allen Olson, Ph.D.

The process of dark adaptation, in which the rhodopsin in the rod cells of the eye is fully regenerated after the eyes are "bleached" by exposure to strong white light, takes approximately 30-35 minutes. The light perception threshold, measured in candela/m^2, progressively decreases in the dark to reach a plateau of approximately 10^{-5} in a vitamin A-sufficient person. The candela is a measure of the luminous intensity of light, which is related to the total visible light energy emitted from a source per unit time. Because the retina responds as a photosensitive surface, the intensity is expressed per unit of area (m^2). Luminance (candela/m^2) is measured by the electronic response of a photosensitive chemical surface, as in a photographic light meter. The threshold may be 10-fold higher (or more) in a vitamin A-deficient person. When made with a Goldmann-Weekers adaptometer, however, this measurement has not proven useful in surveys of vitamin A status in developing countries, primarily because the major target groups are preschool children, who are fearful of the dark, intolerant of the constraints requisite for such a test, and unaccustomed or unable to provide a valid subjective response to objects seen in very dim light.

During the early phase of dark adaptation, a transient plateau is reached at 1-5 minutes in vitamin A-sufficient subjects at a luminance of approximately 10^{-2} to 10^{-3} candela/m^2, and thereafter the threshold rapidly decreases. The point at which the threshold again starts to decrease is called the "rod-cone break point," i.e., when the full dark adaptation of the cones is followed by the adaptation of the rods. At the rod-cone break point, the sensitivity of the retina shifts toward blue, causing blue to appear brighter than red. In vitamin A-deficient subjects, the rod-cone break point is significantly delayed. A practical, simple method of assessing the rod-cone break point, which involves the sorting of disks of different colors under dim light, was devised by Thornton (1). This procedure was first tested in control and vitamin A-depleted adult subjects by Vinton and Russell (2).

Procedure

Subjects are light-adapted, i.e., their retinas are bleached by having them fix their eyes on an illuminated x-ray view box for 1 minute, immediately after which the lighting is reduced to 0.0068 candela/m^2 and a stopwatch is started. Subjects then sort a randomly mixed pile of five white disks, six blue disks (wavelength maximum of 475 nm), and seven red disks (wavelength maximum of 605 nm) into three appropriate piles. Any incorrectly sorted disks are returned to the initial pile by the monitoring person and then are resorted by the subject until all have been correctly identified. The time required to sort all the colored disks properly in controlled dim light from the time of bleaching is measured in minutes (or seconds). The mean and standard deviation (or standard error) is then calculated.

Because under these lighting conditions colors cannot be distinguished, the different colored disks are perceived by the subject only as being of different brightness, a process of discrimination that is rod-dependent.

Interpretation

If the mean rapid dark adaptation time (RDAT) of an experimental group differs significantly from a vitamin A-sufficient control or treated group, a problem of vitamin A status may well exist. Specific cutoff points for abnormal responses in children have not been set, although a statistical cutoff point based on control values has worked well in adults (2). The sensitivity and specificity of the procedure were 95% and 91%, respectively, when the cut-

off value was set as the mean RDAT of control subjects of the same age plus 1.5 standard deviations. Criteria for a problem of public health concern have not as yet been suggested.

Advantages

The RDAT is noninvasive and rapid, requires little instrumentation, is directly related to a specific vitamin A-dependent physiological function, provides a quantitative value for each subject, and can be repeated very soon after the initial test. The sensitivity and specificity in adults are good, and treatment of vitamin A-depleted subjects with vitamin A restores RDAT to normal. A technically trained individual is required to conduct the test, but not an ophthalmologist.

Limitations

Reliability of RDAT in children is highly dependent on the tester's ability to motivate and sustain motivation throughout the test. A controlled light environment is required, which might either be difficult to provide in rural developing country villages or necessitate studies at night. Although conducted in children as young as 4 years (3,4), RDAT is not usable for very young children, who usually are those at greatest risk of vitamin A deficiency. The procedure also requires a subjective sorting of disks that might be affected by other than nutritional factors. It has been used successfully with children in one survey (3) but not in another (4). Because the variance is fairly large, it is most appropriate for comparing reasonably large groups. Of other nutrients, zinc deficiency also induces night blindness. Congenital night blindness, although relatively rare, also exists.

Application

In vitamin A-sufficient adult subjects, the mean RDAT with various adult groups was 3-5 minutes, with a coefficient of variation of 20-30% (2). Younger adults (20-39 years) sorted the disks faster (3 minutes) than did older (40-60 years) adults (4.4 minutes) (2). The mean RDAT in vitamin A-depleted patients, who were suffering primarily from alcoholic liver cirrhosis, pancreatic insufficiency, or small-bowel resection, was 7.6 minutes, with a coefficient of variation of 23% (2). The RDAT correlated well with the mean rod-cone break time and the final dark adaptation threshold, as measured by a Goldmann-Weekers adaptometer. After vitamin A-depleted subjects were treated with repeated doses of vitamin A, the RDAT as well as other related measurements fell in the normal range (2).

The RDAT procedure has been used as an assessment tool in American (5), Guatemalan (3), and Brazilian children (4). In Guatemala, the RDAT was normal in all children with plasma retinol values > 26.9 µg/dL and was inversely related to vitamin A intake (3). In Brazilian preschool children, however, the RDAT was not related to estimated vitamin A intake (< 300 vs. > 300 µg retinol equivalents), to plasma retinol concentrations (correlation coefficient of zero), or to a positive response in serum retinol levels after the administration of a 200,000-IU oral dose of vitamin A (4). In the latter study, the mean RDATs were 2.7-3.3 minutes, with coefficients of variation between 16% and 40%; individual values ranged from 1.5 to 4.7 minutes (4).

Conclusions

Although RDAT is a noninvasive, rapid, readily repeatable method that is directly related to a primary physiological function of vitamin A, successful use in children is directly related to the tester's ability to motivate and sustain their interest. The test is likely to be most useful on a population basis as an objective means of evaluating dark-adaptation. However, the procedure requires further attention and reliability testing.

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Isotope Dilution Analysis

Harold C. Furr, Ph.D.

Newly absorbed vitamin A, labeled with either radioactive or stable-isotope tracer, will mix with body pools of vitamin A (1). If there is no subsequent dilution of tracer by dietary unlabeled vitamin A, after a period for equilibration the total body vitamin A pool can be calculated by a simple dilution equation:

$$\text{Stores} = \text{Dose} \times \frac{\text{Specific activity of dose}}{\text{Specific activity of plasma retinol}}.$$

Procedure

A known dose of labeled retinyl acetate of known isotopic content (expressed as specific activity for radioactive tracer, or as isotope ratio for nonradioactive tracer) is administered orally to the subject. For stable-isotope studies in humans, a dose of 1 µmol $^2H_4$-retinyl acetate/kg body weight (in corn oil solution) is adequate. After a period for equilibration, preferably 3-7 days, a blood sample is taken; plasma retinol is extracted and is purified by high-performance liquid chromatography (HPLC) and the amount of label (specific activity, or isotope ratio) in plasma retinol is determined.

For radioactive tracer, the amount of label is determined by an appropriate counting technique (usually liquid scintillation counting for ^3H- or ^{14}C-labeled vitamin A) and the specific activity is calculated (radioactivity per mass, where radioactivity typically is expressed as disintegrations per minute). For stable-isotope tracers (^2H or ^{13}C), mass spectrometry is required to determine isotope ratios (2).

Calculation of total body stores of vitamin A is made according to the above equation. In practice, a correction (usually taken as 50%) is made for absorption inefficiency and for catabolism of tracer before it mixes with body stores:

$$\text{Stores} = \text{Dose} \times \text{Correction factor} \times \frac{\text{Specific activity of dose}}{\text{Specific activity of plasma retinol}}.$$

Advantages

In this method's simplest application, where the subject ingests no unlabeled vitamin A after administration of tracer dose until the blood sample is taken, isotope dilution analysis is conceptually simple and offers an unambiguous estimate of total body vitamin A stores.

Limitations

This approach currently has technical and theoretical limitations. Technically, the appropriate labeled (radioactive or stable isotope) vitamin A must be available or readily synthesized. The label must be in a metabolically inert position; ^2H or ^3H labeling on the C_{15} position is not appropriate because hydrogens are readily removed and replaced in the body. For stable-isotope labeling, the tracer should have mass at least 3 amu (atomic mass units) higher than that of unlabeled retinol to avoid complications owing to naturally occurring ^{13}C in vitamin A (natural-abundance ^{13}C and ^2H contribute 0.22% to the "m + 3" peak of retinol and thus limit the lower limit of detection of tracer). The labeled vitamin A dose must of course be of high purity. HPLC must be available for purification of the dose, if necessary, and for plasma retinol quantitation and purification for analysis of label. For analysis of radioactive vitamin A (dose and plasma retinol), appropriate counting equipment (typically a liquid scintillation counter) and appropriate laboratory facilities must be available. The use of stable-isotope vitamin A for human studies requires access to a suitable

mass spectrometer: a basic quadrupole gas chromatograph-mass spectrometer is sufficient. The isotope-ratio response of the mass spectrometer must be calibrated by use of appropriate mixtures of labeled and unlabeled standard retinol (2).

The other restriction on the use of isotope dilution analysis results from our limited knowledge of vitamin A metabolism. When a subject continues to consume unlabeled vitamin A after a dose of labeled vitamin A tracer, dilution and loss of tracer to catabolism will occur. This loss of tracer, if not appreciated, will be interpreted as dilution of label and will lead to overestimation of body vitamin A pools. The extent of catabolism (fraction of dose lost) depends on the vitamin A status of the subject: in the rat, a greater fraction of the labeled dose is lost as vitamin A status deteriorates (3). Recently, the relationship between the fraction of dose lost per day and the liver vitamin A stores was used in a rat study to correct the isotope dilution equation for loss of label (4). The nature of this relationship between fraction of dose lost per day and liver vitamin A status is now known for the rat but is not yet known for the human, thus complicating application of isotope dilution to the assessment of human vitamin A status. The problem does, however, contain the seeds of its own solution: application of stable-isotope technology to study vitamin A metabolism in humans should allow estimation of vitamin A disposal rates as a function of vitamin A status, using the kinetics approach of Green et al. (3).

Although a period of time is required for mixing the tracer dose of vitamin A with body pools before sampling for isotope dilution determination, consideration of the rate of catabolism of vitamin A (loss of tracer) suggests that sampling should not be delayed too long: our calculations suggest that 3-5 days after dosing is the most appropriate time for sampling for isotope dilution for the rat and that beyond 7 days is too long. At longer equilibration times, the low amount of remaining tracer owing to the more rapid loss of tracer label in animals with poor vitamin A status cannot be distinguished from dilution of tracer label in animals with large vitamin A stores.

Early isotope dilution studies found that the fraction of tracer dose recovered in the liver was lower in animals with poorer vitamin A status. It has been suggested that this reflects a decrease in absorption efficiency or in liver storage efficiency with decreasing vitamin A status. Consideration of vitamin A disposal rates, however, explains this phenomenon: animals with poorer vitamin A status lose a greater *fraction* of their total vitamin A per day (albeit a smaller absolute amount) than do well-nourished animals.

Application

^{3}H-labeled vitamin A has been used for isotope dilution analysis of vitamin A status in rats (1,4-7), in sheep, and in a human subject (6). Stable-isotope-labeled vitamin A has been used in studies in rats (6,8), sheep and cattle (6), and humans (6,9). In one human study, the problem of dilution of tracer label due to catabolism and ingestion of unlabeled vitamin A was approached by using an estimate of vitamin A half-life (9). Studies that did not allow ingestion of unlabeled vitamin A between dosing with tracer and blood sampling (1,6) or that corrected for dilution of label by catabolism and by ingestion of unlabeled vitamin A (4,9) gave estimates of vitamin A status that correlate well with direct analysis of liver vitamin A.

Conclusions

Isotope dilution analysis has not yet lived up to its promise as a minimally invasive, accurate method for determining vitamin A status. However, correction for dilution of tracer label by dietary unlabeled vitamin A and for the loss of tracer label by catabolism can provide accurate estimation of vitamin A status. Estimation of the rate of vitamin A catabolism as a function of vitamin A status in humans is an important next step for isotope dilution analysis as well as for our general understanding of human vitamin A requirements.

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Tear Fluid Retinol Analysis

Eric J. van Agtmaal, Ph.D.

Tear fluid, which covers the corneal and conjunctival epithelia, serves to protect the eye from exogenous antigenic substances and to maintain the structural integrity of the corneoconjunctival mucosa. In tear fluid a variety of proteins, enzymes, lipids, vitamins, and electrolytes, which are mainly secreted by lacrimal glands, can be measured (1). Because tear fluid is also the exclusive transport medium for metabolites to the corneal epithelium, analysis of the fluid may provide information about the (patho)physiological condition of the mucosal surface of the eye. Retinol was initially found to be present in human tear fluid by Ubels and McRae (2). As part of a vitamin A trial on preschool children in northeastern Thailand (3), tear fluid was collected and analyzed for retinol (4).

Procedure

For tear fluid collection in field surveys, a simple technique using cellulose sponges provides sufficient quantities after rapid tear stimulation. Current collection techniques using glass capillary or Schirmer strip are not suitable under field survey conditions. Cellulose sponges were found to provide sufficient volumes of tear fluid (range 50-400 µL) within a period of minutes, to be easy to handle, to have a low risk for eye damage, to be covered entirely by the lower eyelid (thereby minimizing evaporation), and to provide undiluted tear fluid after centrifugation (4-6).

Materials and equipment needed to obtain tear fluid specimens are:

- sterile cellulose sponges (Sugi [Kennenbach, Germany]), 5 x 5 x 1.5 mm
- 400-µL polyethylene tubes and storage box
- forceps
- Petri dish
- scissors
- table-top clinical centrifuge
- cool box (in the field)
- freezer (in the laboratory)

Collection of tear fluid requires the cooperation of three persons (4). Two persons sit knee to knee, facing each other. The child is placed flat on its back on the two laps, while its head is fixed by the hands of (preferably) a physician, who controls the child's eyelids with the thumbs. The child can freely move its legs, but its hands are fixed by the other sitting person, usually the mother of the child. As one of the lower eyelids is pulled downward, a third person, using forceps, places a cellulose sponge into the lower conjunctival sac at the nasal side. During collection, the sponge is entirely covered by the lower eyelid to minimize evaporation. After reflex stimulation of the tear fluid, the filled sponge (containing about 50 µL tear fluid) is taken out, temporarily kept in a Petri dish, and replaced by a new one; three or four sponges are filled for each eye. Immediately after the collection period, all of the filled sponges for each individual are transferred into a single 400-µL polyethylene tube, which is then sealed and stored at 4°C (cool box).

Undiluted tear fluid is recovered from the sponges by centrifugation (Figure 1). After 1 day of storage of the filled sponges at 4°C in a sealed tube, the recovery of retinol was 98% (5), whereas the recovery of protein was at least 92% (6). The recovery of total protein, amylase, and immunoglobulin A progressively decreased during storage for up to 7 days. Therefore, wet sponges containing tear fluid should be stored at most for 1 day at 4°C, followed by centrifugation and subsequent storage of the tear fluid at -20°C. Retinol and protein concentrations were not changed during storage at -20°C for at least 7 days.

Retinol in tear fluid is quantified by a fast

and sensitive high-performance liquid chromatography (HPLC) technique (7) that is a modification of one used for microdetermination of retinol in plasma (8). After deproteinization of a 50-µL aliquot of undiluted tear fluid with ethanol and extraction with n-hexane, retinol is separated from the extract on a straight-phase HPLC column and detected fluorometrically (excitation/emission wavelength pair: 333/470 nm). The HPLC run takes 4 minutes per sample, with a detection limit of 0.4 ng/mL. The within-assay and between-assay coefficients of variation were 3.1% and 4.2%, respectively. The between-assay analytical recovery of retinol added to tear fluid was 97.4 + 6% (mean ± SD).

Interpretation

Until now, tear fluid retinol analysis (TRA) was used in only one vitamin A intervention study (5). Therefore, follow-up studies with larger sample sizes are needed to investigate its reliability as a new vitamin A assessment method. TRA currently can be used only as a direct indicator of vitamin A levels at the surface of the eye. Further studies are needed to elucidate a possible relationship between tear fluid retinol level and other indicators, such as clinical signs of xerophthalmia, serum retinol, serum retinol-binding protein (RBP), and conjunctival goblet cell density. Furthermore, the effects of various storage conditions on tear fluid retinol need to be investigated.

Advantages

TRA is minimally invasive and does not require blood. It provides a direct measurement of vitamin A levels at the corneoconjunctival surface of the eye. The procedure is useful for field surveys because it requires minimal equipment and supplies to collect tear fluid. TRA can be applied by any laboratory with HPLC facilities for serum vitamin A determination.

Limitations

Storage, handling, and analysis can be problematic and expensive. Collection of tear fluid can be stressful for the donor, and sudden movements of the head may happen at any moment during collection, especially with young children. Therefore, it is essential to inform the child and mother beforehand about the procedure and to work with them strictly on a voluntary basis. During our study (5), cellulose sponges caused no clinically detectable eye damage. Our procedure for tear fluid collection, it should be stressed, was well accepted by parents and members of the community. Correlation with other parameters for vitamin A status is as yet unknown, and its usefulness for individual and community vitamin A assessment is therefore uncertain.

Application

The lacrimal gland is the major source of retinol at the surface of the eye (9). Routine collection of tear fluid from adults and from preschool and school children can be performed in hospitals and under field survey circumstances by well-trained personnel. All donors should be selected only after clinical eye examination to exclude subjects with serious corneoconjunctival pathology.

The median retinol concentration in the tear fluid of a group of control rural Thai children (5) was 0.7 ng/mL (range 0-2.7 ng/mL) at the beginning of a study and was 0.4 ng/mL (range 0-3.3 ng/mL) two months later. In contrast, the median retinol concentration in the tear fluid of a similar group of rural Thai children was 0.5 ng/mL (range 0-1.3 ng/mL) initially but increased to 1.7 ng/mL (range 0-8.7 ng/mL) two months after supplementation with 200,000 IU of retinyl palmitate. Thus, supplementation with vitamin A increased tear fluid retinol concentrations in these children by two to threefold. For comparison, tear fluid retinol concentrations in well-nourished Dutch children and adults were 1.8 ng/mL (range 1.4-2.7 ng/mL) and 4.3 ng/mL (range 0.4-10.6 ng/mL), respectively (7). Median tear fluid retinol values in supplemented Thai children and in well-nourished Dutch children were similar, although the ranges of values in both cases were very broad.

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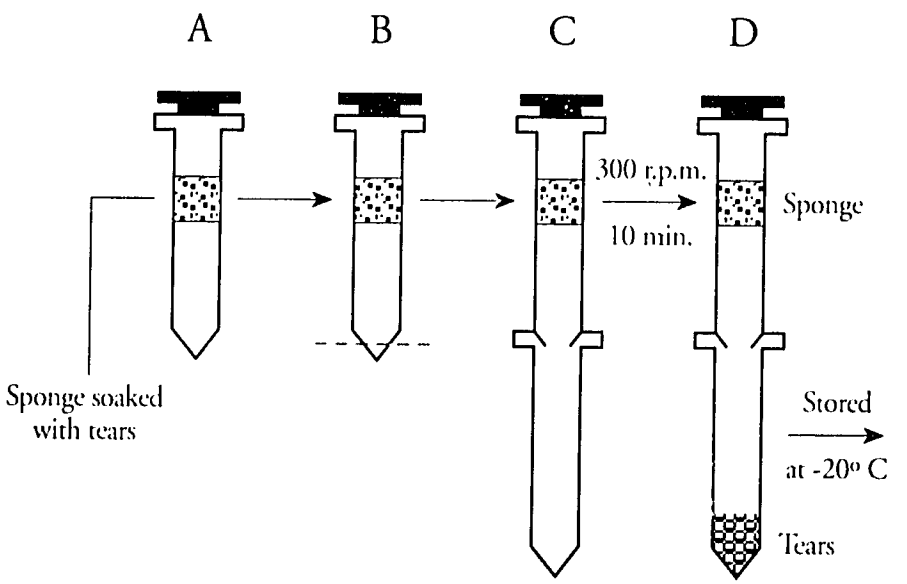
Figure 1. Schematic representation of tear fluid sample treatment after collection. The bottom of the closed polyethylene tube, containing the filled sponge (A), is cut off (B). The tube is placed on another, identical tube (C) and centrifuged. Undiluted tear fluid moves to the lower tube while the sponges remain in the upper tube (D). The lower tube is closed and stored at -20°C for further analysis.



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