

**Infection and Malnutrition: Immune Function in Children with Protein—Calorie and Vitamin A Malnutrition**

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**INTRODUCTION:** This is a collaborative project between SMRL, the Faculty of Science, Mahidol University, and the St. Louis Anemia & Malnutrition Research Center, Chiangmai, Thailand.

**BACKGROUND:** The broad purpose of this project is to clarify why malnourished individuals are more susceptible to microbial infections than well nourished persons. In order to elucidate some of the possible mechanisms leading to increased susceptibility, we are evaluating the cellular and humoral immune status of children with protein—calorie malnutrition (PCM). Specifically the humoral parameters being investigated are serum and naso—pharyngeal immunoglobulin levels, serum levels of complement components, including C1q, C1s, C3, C4, C5, C6, C8, C9, C3—proactivator, and total serum hemolytic complement activity (C'H<sub>50</sub>). The cell—mediated immune (CMI) status of the children is being evaluated by measuring the cutaneous response to dinitrofluorobenzene (DNFB) sensitization and to Monilia and Streptokinase—streptodornase (SK—SD) skin test antigens.

We anticipate that better understanding of host defense immune mechanisms against microbial infection in healthy, in addition to malnourished, individuals will result from a study of malnourished children.

**STUDY DESIGN:** This project is designed to conform to the diagnostic and treatment schedule already in effect at the Anemia and Malnutrition Research Center of St. Louis University, Nakorn Chiangmai Hospital (MALAN), Chiangmai, Thailand. The clinical phase of the study outlined below is the responsibility of Dr. Robert M. Suskind, and LTC Robert Edelman, SMRL. The laboratory phase, which consists principally of measuring the levels of immune components in serum, is the responsibility of Dr. Satit Sirising and LTC Edelman.

All patients admitted to the 14 bed research ward of MALAN are treated, studied for 3 months (84—92 days) and then discharged. On admission the patients are clinically evaluated and scored for the presence of marasmus, marasmus—kwashiorkor, or kwashiorkor according to the modified criteria of McLaren and Gomez (see Table 1). Children are admitted to the study if they have primary malnutrition and weigh more than 3.0 kg. and less than 12 kg.

In addition to this protocol each patient is being extensively studied in another and larger research protocol directed by Dr. Suskind for blood coagulation factors, red cell survival, serum proteins (albumin, globulins, ceruloplasmin and copper, TIBC and iron, lipoproteins, glycoproteins, retinol binding protein, haptoglobin, hemopexin, blood & urine amino acid patterns, serum lipids, erythrocyte & leukocyte enzymes, Australian antigen, and electron and light microscopy of liver biopsies.

At the time of admission each patient is placed in one of 4 dietary groups so that approximately equal numbers of marasmic, marasmic—kwashiorkor, and kwashiorkor patients comprise each group. The 4 groups and their diets are listed in Table 2. Details of the diet fed during the day 1—7 stabilization period are

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given in Table 3. The stabilization period may be extended if the patient is not able to tolerate the diet given, but this rarely has been necessary. In addition to the diets listed in Tables 2 & 3, large doses of supplemental vitamins and minerals are given starting on day 2. On admission, all patients are treated vigorously for infection and for fluid and electrolyte imbalance. The large majority of patients have infections on admission.

PROGRESS: A) Cell-mediated immunity

Contact sensitization to 1-nitro, 2, 4-difluorobenzene (DNFB) is a standard method used to test cell-mediated immunity in vivo. Pre-existing sensitization is rare, approximately 95% of normal subjects can be sensitized to DNFB, and circulating antibodies do not develop from contact sensitization.

The children are sensitized with 2 mg of DNFB in acetone applied to the forearm which is then allowed to dry. The site of application was protected for 24 hours with an occlusive dressing. The children are tested for sensitization to DNFB by applying 100 ug DNFB in acetone to the opposite forearm 12-14 days after the sensitizing dose was applied. Two days after the skin test dose, the inflammatory skin response at the test dose site is graded. If positive the children are considered immunized.

A total of 19 patients were exposed to 2 mg DNFB on admission; most were first skin tested with 100 ug DNFB on day 15 while a few patients were first tested on day 29 or 44. Skin tests were repeated at 2 week intervals until positive. The results of the attempt to induce DNFB sensitivity in these patients are given in Table 4, assuming all children were skin test negative on admission. Only 21% of children had converted and were skin test positive when first tested on day 15. An increasing percentage of patients converted to positive after day 15, with 100% of patients' skin test positive by day 56. The low percentage of positive patients on day 15 could be explained by a defective immune response, a faulty inflammatory response, or by defects in both of these mechanisms. The increasing percentage of reactors to the skin test dose after day 15 could be due to improved nutritional status leading in turn to repair of a defective effector (antigen recognition & response) limb of CMI or to repair of a deficient inflammatory response. Further repeated skin test doses of 100 ug DNFB may have contributed to the gradually increasing conversion rate to subsequent skin testing by stimulating the effector (lymphocyte sensitizing) limb of CMI. We therefore designed additional experiments to clarify the mechanism of the defective response to DNFB. These experiments, most of which are in progress, are described below.

DNFB is a potent nonspecific skin irritant in addition to being a contact allergen. Application of a 2 mg dose to the skin of a non-immune results in marked inflammation (erythema and edema) within 12-72 hours which subsides over 3-5 days. We attempted to measure the skin inflammatory response to DNFB. Three groups of non-immune patients were first sensitized with 2 mg DNFB on admission, on day 15, or on day 56. The degree of skin inflammation was graded 2 days after DNFB challenge, and the results obtained to date are shown in Table 5. Only 9 of 25 (36%) of those sensitized on admission had an inflammatory response consisting of induration and/or vesicle or bleb formation, whereas 3 of 3 children sensitized on day 56 had an inflammatory response. Greater than 75% of normal adults can be expected to show an inflammatory response according to a previous report. It therefore appears that in PCM, the inflammatory response may be deficient on admission but improves coincident with nutritional repair. Further studies are planned to study their inflammatory skin responses using the Rebuck skin window technique.

We attempted to determine when the CMI response can be induced by testing 5 patients from each of the 3 treatment groups shown in Table 5. These patients, sensitized for the first time on days 1, 15, or 56, were all skin tested with 100 ug DNFB on day 70. The results shown in Table 6 show that none of 4 patients could be sensitized on day 1, but 3 of 3 patients could be sensitized on day 56. The failure to sensitize on day 1 strongly suggests that a defect in the CMI response exists on day 1. Because the effector portion of the CMI response (Table 7) and the inflammatory response (Table 5) is intact on day 56, the failure to respond implies that a defect existed on day 1 in the ability to be immunologically sensitized (effector limb). Results pending (Table 6) will determine whether this defect is repaired by day 15.

As previously discussed, the 100 ugm skin test doses of DNFB repeated while nutritional repair was occurring may have stimulated the effector limb of a recovering CMI response and may have thereby contributed to the rising conversion rates noted in Table 4. In order to test this possibility the 4 children in Table 6 who were skin test negative on day 70 were challenged again; two were skin tested with 100 ug DNFB on day 84 and two on day 92. One child on each of these 2 retest days converted to skin test positive, indicating that the skin test dose of 100 ugm may indeed immunize some nutritionally repaired children.

Between 50 and 90% of healthy individuals can be expected to have been naturally sensitized to monilia or streptococcal antigens by the age of 6-12 months, and therefore show positive delayed hypersensitivity to intradermal antigenic challenge. Accordingly, in order to test immunological recall in PCM, patients were inoculated intradermally on day 1 with 0.1 ml of monilia (1:100) and streptokinase-streptodornase (SK-SD) (50 units) skin test antigens. The patients served as their own controls in that they were retested when their nutritional status was partially (day 29) and completely (day 70) normal. The results shown in Table 7 indicate that on admission only 7% and 14% of children were skin test positive to monilia and SK-SD, respectively. By day 70, 70% of the children were positive to monilia and 50% were positive to SK-SD. The poor response to antigenic challenge on day 1 can be ascribed either to a deficient inflammatory response (Table 5) or to a defective effector (recall) portion of the CMI response. It is apparent that by day 70, both mechanisms were largely intact. A study is in progress to test the CMI effector limb independently of inflammatory response by measuring lymphocyte transformation *in vitro* in the presence of monilia and SK-SD antigens. In addition, 20 patients are being repeatedly skin tested starting on day 70 in order to determine whether these test antigens can immunize and induce a positive test on rechallenge.

Total peripheral lymphocyte counts were performed on admission. The results, grouped according to clinical diagnosis, show no decrease in lymphocyte counts compared to normal and no differences between 7 marasmic ( $\bar{x} = 6,770$  cumm), 9 marasmic-kwashiorkor ( $\bar{x} = 6,178$  cumm) and 10 kwashiorkor ( $\bar{x} = 5,812$  cumm) patients. Thus the immune defects noted in PCM are the results of factors other than a quantitative deficiency of circulating lymphocytes.

Further correlations were made between clinical diagnosis (m, m-k, k) and the immune and inflammatory response on admission. No clear relationship emerged between the clinical state of the patient and their inflammatory and immune responses on day 1; the 3 groups appear to be equally defective. The levels of serum complement components (C'1q, C'1s, C'3 proactivator, C'3, & C'5) on admission did not correlate with the degree of impairment of inflammation or of CMI. Finally the effect of the 4 protein-calorie diets (Table 2) on DNFB skin test responses on day 15 and 29 was studied; results show no clear differences between the 4 dietary groups; all diet groups showed nearly equal and gradual improvement. It thus appears that the low calorie-low protein diet with vitamin and mineral supplements supplies adequate nutrients for physiological repair of the DNFB skin test. Physiological repair with 1 gm protein/kg diet was not invariable; the low serum complement levels for example, shown in the next section, rose only with a 4 gram protein/kg diet.

#### B. Serum Complement and Immunoglobulin.

**BACKGROUND:** Serum complement plays a major role in mediating inflammation, chemotaxis, immune cytotoxicity and phagocytosis of bacteria. Complement is involved in endotoxin metabolism and perhaps in endotoxin shock. Infection, particularly bacterial with endotoxin shock, is a common event in PCM; the previous section on CMI function provides evidence for defective inflammatory response. Thus a study of complement in PCM may provide additional clues as to the mechanism mediating infections and the many physiological malfunctions noted in malnourished individuals. Our initial studies have dealt with the "profiles" of serum C' component levels and the change in these levels following nutritional repair. Dr. H.J. Muller-Eberhard generously provided the immunodiffusion plates for determining the levels of 9 complement components. Duplicate data for C'3 was also obtained using commercial immunoplates (Hyland Lab).

Serum or heparinized plasma was obtained on days 1, 8, 29, and 84. The serum was stored for weeks or months at  $-20^{\circ}\text{C}$  and then shipped to Bangkok. All specimens were run with complement standards supplied by Dr. Muller-Eberhard or Hyland Labs.

Serum immunoglobulin levels were also determined in these serum specimens by radial immunodiffusion using commercial IgA, IgG, IgM & IgD plates.

**PROGRESS:** The statistical analysis of data from 20 well-nourished control patients and 10 marasmic and 10 Kwashiorkor patients is not completely finished. A narrative summary of the data obtained to date follows. All complement components were depressed on day 1 below the mean control levels; some components, such as C'3 proactivator and C'9, measured more than 2 standard deviations below the control mean, while others, such as C'4 & C'5 were within 1 standard deviation of the mean. The C' levels began to rise by day 8, with recovery of all components by day 29. The levels for several components on day 29 were in fact significantly higher than the mean, but fell to normal levels by day 84, producing a "rebound" effect. In general the levels of C' on day 1 were lower in Kwashiorkor than in marasmic patients. Evidence is accumulating which indicates that the depressed levels of C' on admission are the result of depressed synthesis rather than of increased consumption or loss. For example, comparisons were made of C' levels on days 8 & 29 in children fed 1 gm protein—175 cal/kg diets and those fed 4 gm protein—175 cal/kg diets. The C' levels did not rise between days 8 & 29 in children fed the low protein diet, whereas all C' components rose markedly on the high protein diet. Complement turnover studies are being planned.

The low titers of C' components measured immunochemically does not necessarily reflect impaired complement biological activity. Therefore an attempt was made to elucidate the biological activity of serum C' by titrating total hemolytic complement activity ( $\text{C}'\text{H}_{50}$ ). In a series of preliminary experiments we found that  $\text{C}'\text{H}_{50}$  titers are not lowered by repeated freeze-thawing, storage at room temperature ( $25^{\circ}\text{C}$ ) for 6 hours, or addition of heparin or  $\text{CO}_2$  atmosphere to the storage tubes. However storage prolonged beyond 2—3 weeks, even at  $-90^{\circ}\text{C}$ , seems to result in a gradual loss of  $\text{C}'\text{H}_{50}$  titer over time. Thus the very low  $\text{C}'\text{H}_{50}$  titers we measured in children with PCM may have resulted from prolonged storage of their serum (1—5 months) before testing. We are now measuring  $\text{C}'\text{H}_{50}$  titers in serum stored less than 2—3 weeks in order to eliminate this possible laboratory artifact.

Measurement of serum immunoglobulin (Ig) levels has shown that on admission IgG & IgM are slightly increased in those children clinically infected, while IgD and IgA are markedly increased. In no patient were admission Ig levels below those considered normal for Thai children. The IgA & IgD levels tended to fall to normal levels over several weeks. Plans are underway to test the quality rather than the quantity of serum Ig in PCM by measuring the immune response to specific antigenic challenge.

**SUMMARY:** This data emphasizes the disordered CMI function, inflammatory response, and complement metabolism in PCM. The data thus provides a sound basis for further studies of PCM and infection.

Table 1.

Criteria used to score the clinical nutritional status of each PCM patient on admission

I. Clinical impression

II. McLaren's criteria

<u>Sign</u>	<u>Point Score</u>
1. Edema	3
2. Dermatitis	2
3. Edema + Dermatitis	6
4. Hair change	1
5. Hepatomegaly	1
6. Serum albumin (gm/100 ml)	
Total serum protein (gm/100 ml)	
-1.00	-3.25
1.00-1.49	3.25-3.99
1.50-1.99	4.00-4.74
2.00-2.49	4.75-5.49
2.50-2.99	5.50-6.24
3.00-3.49	6.25-6.99
3.50-3.99	7.00-7.74
≥ 4.00	≥ 7.75

McLaren's score

<u>Clinical diagnosis</u>	<u>Total point score</u>
marasmus	0-3
marasmus-kwashiorkor	4-8
kwashiorkor	9-15

III. Gomez criteria

<u>Clinical criteria</u>	<u>Measured weight/ expected weight for age (Thailand)</u>	<u>Edema</u>
Marasmus	< 60%	No
Marasmus-kwashiorkor	< 60%	Yes
Kwashiorkor	> 60%	Yes
underweight child	> 60%	No

Table 2. Dietary groups

<u>Group</u>	<u>Days following admission</u>			
	<u>1-7</u>	<u>8-29</u>	<u>30-70</u>	<u>70-84</u>
I	Stabilization	1 gm protein/kg 100 calories/kg	4 gm protein/kg 175 calories/kg	solid food ad lib (4 gm. 175 cal.)
II	"	4 gm protein/kg 100 calories/kg	"	"
III	"	1 gm protein/kg 175 calories/kg	"	"
IV	"	4 gm protein/kg 175 calories/kg	"	"

Table 3. Stabilization diet (days 1-7)

<u>Day</u>	<u>Component</u>
1	IV therapy
2 & 3	1 gm protein and 25 cal/kg - if tolerated
4 & 5	1 gm protein and 50 cal/kg - if tolerated
6 & 7	1 gm protein and 100 cal/kg - if tolerated

Table 4.  
Contact Sensitization to DNFB<sup>1</sup> in Protein-Calorie Malnourished Children

<u>Day skin tested<sup>2</sup></u>	<u>Total pts.</u>	<u>Skin response<sup>3</sup></u>		<u>% Converted</u>
		<u>Positive</u>	<u>Negative</u>	
15	19	4	15	21
29	14	7	7	50
44	11	8	3	73
56	8	8	0	100

<sup>1</sup> Sensitizing dose of 2 mgm DNFB applied to skin on day 1.

<sup>2</sup> Day 100 ug skin test dose DNFB applied to skin; skin response graded 2 days later.

<sup>3</sup> Positive = induration and/or vesicle or bleb

Negative = no reaction or erythema only.

Table 5.  
Skin Inflammatory Response to 2 mgm DNFB

<u>Day tested*</u>	<u>Total pts.</u>	<u>pts. completed</u>	<u>Skin response<sup>xx</sup></u>		<u>% Positive</u>
			<u>Positive</u>	<u>Negative</u>	
1	25	25	9	16	36
15	5	0			
56	5	3	3	0	100

\* Skin response read on day 3, 17, and 58 respectively.

xx Negative = no reaction or erythema; positive = induration and/or vesicle or bleb formation.

Table 6.  
Attempt to Induce DNFB Sensitivity on Different Days after Admission

<u>Day Sensitized*</u>	<u>Total pts.</u>	<u>Pts. completed</u>	<u>Skin response<sup>xx</sup></u>	
			<u>Positive</u>	<u>Negative</u>
1	5	4	0	4
15	5	0		
56	5	3	3	0

\* Day sensitizing dose of 2 mgm DNFB applied to skin.  
xx Skin test dose of 100 ug DNFB applied on day 70 and read on day 72; positive = induration and/or vesicle or bleb; negative = no reaction or erythema.

Table 7.  
Skin Test Response to Monilia and Streptococcal Antigens

<u>Antigen</u>	<u>Day* tested</u>	<u>Total No. patients</u>	<u>Skin Response</u>		<u>% Positive</u>
			<u>Positive</u>	<u>Negative</u>	
Monilia <sup>xx</sup> (1:100)	1	13	1	12	7
	29	12	8	4	66
	70	10	7	3	70
SK-SD <sup>***</sup> (50 units)	1	29	4	25	14
	29	27	9	18	33
	70	25	13	12	52

\* Skin test read two days after 0.1 ml antigen injected intradermally.  
xx Monilia positive test = >5mm induration and/or vesicle.  
\*\*\* Streptokinase-streptodornase positive test = erythema and/or induration.