MATERIAL AND METHODS
MATERIAL AND METHODS

The present study was conducted in the department of Paediatrics, M.L.B. Medical College, Jhansi, between November 1988 to December 1989.

Selection of cases: Children suffering from protein energy malnutrition between the age of 6 months to 5 years, attending Paediatrics Out Patient Department and those admitted in Paediatrics ward were selected for the study.

The diagnosis of Protein Energy Malnutrition was based on present weight (weight below 70% of the 50th centile of NCHS standard for age) history and clinical examination. Suspected cases of childhood tuberculosis, those taking steroids or suffering from any allergic disorder were excluded from the study. Children on immuno-suppressive drugs and those suffering from immune deficiency disorder were not considered for the study.

1. Healthy normal control:

Healthy children (6 months - 5 years) whose weight-for-age was more than 80% of 50th percentile of NCHS standard were considered for inclusion in the present study.
2. *Malnourished children*:

Malnourished cases were further grouped on the basis of McLaren Classification (1967). Besides name, age, sex, address and socio-economic status, following facts were recorded in each case.

i) **Present, past and family history** :- From parents or other family members detailed history was obtained regarding illness, in a chronological order. Emphasis was given to elicit the history of chronic diarrhoea, worm infestation, failure to thrive, fever, cough and vomiting. An enquiry was made about the definite history of tuberculosis in parents, sibling, near relatives and neighbours.

ii) **Physical examination** :- A thorough clinical examination was done to observe psychomotor change, pallor, oedema, skin and hair changes, status of subcutaneous tissue and muscle mass. Eyes were examined for any evidence of xerosis. Skin lesion like hypo-pigmentation/hyper-pigmentation, hyper-keratosis or any other dermatosis was noted. Examination of lips, gums and tongue was carried in each case to detect the presence, if any, of angular stomatitis, cheilosis, sponginess and glossitis respectively. Skeletal system and thyroid gland was examined for abnormalities.
iii) **Dietary history** :- Dietary history was recorded with special emphasis on the following points:

(a) Age upto which breast milk was given.

(b) Age at which artificial milk was started, type of artificial milk and quantum of dilution used in the milk formula.

(c) Age at which semi-solid and solids were started.

(d) Present calorie and protein consumption of the child and also his past calorie and protein consumption were assessed.

iv) **Immunization status** :- History of immunization was recorded from the parents or family members. Also, left upper arm of the child was examined for any scar mark pertaining to BCG vaccination, given in the past.

v) **Antenatal, natal and postnatal history** :- History relating to intake of drugs in the mother and also the type and mode of delivery, condition of baby after birth was taken, to rule out the secondary factors which give rise to malnutrition.

vi) **Developmental history** :- History of developmental mile-stones achieved in the fields of gross motor, fine motor, social and speech was recorded to rule out the presence of mental retardation and cerebral palsy, as the causative factors of malnutrition.
vii) Anthropometric examination:

- **Weight:** It was recorded nearest to 0.05 kg by infant weighing machine for infants weighing less than 10 kg, while adult type weighing machine with an accuracy of 0.5 kg was used for cases weighing more than 10 kg.

- **Crown-heel length:** It was recorded nearest to 0.1 cm. by an infantometer, for all patients up to the age of 5 years.

viii) Laboratory techniques:

Material used -

- Heparin (Preservative free)
- Minimum essential medium (MMS) - Eagle
- Alsever's solution
- Phosphate buffer saline (PBS)
- Pooled normal human serum
- Anti-sheep haemolysin (Amboceptor)
- Methylene blue 0.2%.

**Alsever's solution**:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>24.6 gm</td>
</tr>
<tr>
<td>Trisodium citrate (dehydrate)</td>
<td>9.6 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>50.04 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1200 ml</td>
</tr>
</tbody>
</table>

pH of Alsever's solution was adjusted to 6.1 with 10% citric acid. Solution was sterilized by low.
Phosphate buffer saline (PBS)

A) 0.15 M - $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (23.4 gm/litre)

B) 0.15 M - Na$_2$PO$_4$ 21.3 gm/litre

C) Normal saline - 9.0 gm NaCl/litre.

For phosphate buffer saline (pH 7.4) solution A (18 ml) was mixed with solution B (82 ml) and then solution C (100 ml) was added. The solution was then sterilized by low pressure autoclaving and stored in a refrigerator.

Pooled normal human serum: - Venous blood was drawn aseptically into clean and dry test tubes 15 ml each, from 4 persons. Test tubes were incubated in water bath at 37°C for 30 minutes and then at 40°C for 120 minutes. The clot from each tube was removed gently with a glass rod and the tubes were centrifuged. The clear serum from each of the tubes was collected and mixed with each other. Pooled serum was stored at -20°C. Small aliquotes were used only once after thawing.

Collection of blood sample: - 10 ml heparinized peripheral blood sample (25 unit of heparin/ml of blood) was collected in the sterile tube from each patient for T and B cell counts. Also the venous blood was simultaneously collected in double oxalate vial from the patient, for total and differential leukocyte counts.
Total leukocyte count (TLC) :- One in 20 dilution of blood was made by adding 0.12 ml blood to 0.38 ml of WBC diluting fluid (lurk's fluid) in 7.5 x 10 mm test tube. The suspension was mixed by gentle tilting and rotating by hand for 2 minutes. The Neubaur's counting chamber was charged with suspension and viewed with 5 mm objective under a microscope. The number of leukocyte were counted and calculated as below:

\[ TLC = N \times 200 / \text{cu mm.} \]

'N' is number of leukocyte counted in each mm square area. Total four squares was counted, in which each large square contain 5 small square (total 80 small square will count).

Differential leukocyte count (DLC) :- A thin and uniformly prepared peripheral blood smear was stained for 8 to 10 minutes with Leishman stain washed with buffered solution (PH 6.8) containing \( \text{K}_2 \text{Fe} \text{C}_4 \), 9.19 m/l & \( \text{Na}_2 \text{HPO}_4 \), 9.5 m/l (mixed together in the ratio of 1.03 : 1). The slide was then dried in air. Leukocytes were counted using oil emersion lens and the percent distribution of different leukocytes was calculated after counting 200 cells.

Absolute lymphocyte count (ALC) :- Absolute count was calculated in every case from the total and differential leukocyte count using the following formmulla

\[ ALC = \frac{\text{TLC} \times \% \text{lymphocytes}}{100} \]
Evaluation of T and B lymphocyte:

1. Preparation of lymphocyte rich plasma:

   The lymphocytes were separated from the heparinised peripheral blood by gravity sedimentation method. 10 ml of heparinised blood (25 unit/ml blood) collected in a sterilized test tube was kept up-right at room temperature for one hour. The leukocyte-rich plasma was collected and centrifuged at 1000 rpm for 15 minutes. The clear plasma was separated and the cell button was suspended in phosphate buffer saline (PB/MEM) minimal essential medium. The concentration of lymphocyte suspension was adjusted to $2 - 3 \times 10^6$ per ml in PB/MEM.

2. Preparation of sheep RBC solution:

   Sheep blood collected in equal volume of Alsever's solution was stored in a refrigerator for 3 - 5 days and thereafter used up to 14 days. Sheep blood was washed thrice with buffer saline. One volume of packed cells was suspended in 18 volumes of buffer saline to give a slightly greater concentration than 5% suspension. One ml of this suspension was lysed with exactly 14 ml of distilled water and optical density (OD) was measured at 540 ohm with distilled water as blank. A lysate with optical density of 0.7 represented 5% concentration or $1 \times 10^9$ cell/ml. From the O.D. of sample tested and volume of the suspension (v_i), the final volume (v_f) was calculated according to the relationship:
\[ v_f = \frac{v_i \times O.D.}{0.7} \]

Finally suspension was adjusted to make a standard solution of sheep RBC.

**Demonstration of T cell by sheep RBC Rosette (E Rosette):**

Sheep RBC's were washed thrice with PBS and 0.5% suspension was made in M.E.M. solution. Lymphocyte count was adjusted to 2-3 \( \times 10^5 \) \( \mu \) ml in PBS. To 0.5 ml of sheep RBC suspension was added 0.5 ml of lymphocyte suspension in PBS and mixture was incubated for 15 minutes at 37°C in water bath. After centrifugation for 5 minutes at 500 rpm, mixture was incubated at 4°C over night. Supernatant was removed and pellet was resuspended in M&E (2-3 drops). Finally wet preparation was made and stained with methylene blue (0.2%) and 200 rosette forming cells were counted under a microscope to calculate the percentage of rosette forming cells.

When three or more SRBC's were seen adhering to a lymphocyte, it was considered as a rosette forming cell. The absolute T cell count was calculated as follows -

\[ \text{Absolute T cell count} = \frac{\text{ALC} \times \% \text{T cells}}{100} \]

**Demonstration of B cells by forming EAC rosette (Fleisher et.al., 1975 and Shevach et.al., 1972):**

B lymphocytes in normal peripheral blood can be identified by the presence of at least three surface marker,
receptors for modified components of complement, surface immunoglobulins and receptors for aggregated IgG.
Complement receptor bearing lymphocytes can be detected by the binding of antigen, erythrocyte (E), antibody (A) and complement (C) to form EAC rosettes.

To 0.5 ml of 5% of SRBC suspension, 0.5 ml of anti-sheep haemolysin in appropriate dilution (1 : 400 assessed earlier) was added and incubated for 15 minutes at 37°C. After washing three times with phosphate buffer saline and resuspending in PBS and thereafter adding 0.5 ml of 1 : 10 diluted complement (Human serum), tube was incubated for 45 minutes at 37°C. The cells were washed with phosphate buffer saline and then resuspend to make a concentration of 0.5% of EAC in phosphate buffer saline.

To 0.5 ml suspension of lymphocyte (2-5 x 10⁶ ml), 0.5 ml of EAC in PBS was added and incubated at 37°C for 30 minutes. The solution was resuspend and wet preparation was prepared and stained with 0.2% Methylene blue. Finally 200 cells were counted under the microscope to calculate the percentage of EAC rosette forming lymphocytes.

A group of three or more SRBC’s adherent to a lymphocyte was considered as EAC rosette. Absolute B cell count was calculated as follows:

\[
\text{Absolute B cell count} = \frac{\text{ALC x } \% \text{ EAC rosettes}}{100}
\]
2.4 2,4-dinitrochlorobenzene (DNMB) contact sensitization test:

Stock solution of DNMB in acetone of 1000 µg/0.1 ml and 50 µg/0.1 ml concentration was made and stored in an amber coloured bottle at room temperature. This solution was changed after every 3 months. Stainless steel ring of 2 cm diameter was placed at the site of application of DNMB so that fixed area was obtained. Sensitizing dose of 1000 µg/0.1 ml was applied on the right upper arm on volar surface slightly towards the medial side. Simultaneously a challenge dose of DNMB (50 µg/0.1 ml) was applied on the medial aspect of the flexor side of right forearm.

After the application of DNMB these sites were covered and the subject's parents were instructed not to wash the site for 24 hours. Site was examined after 48 hours for a spontaneous flare, indicated by the appearance of erythema, induration and vesiculation.

Reaction was graded according to the criteria proposed by Aisenberg (1962):

4+ unequivocal spontaneous flare occurring at both sensitizing and the challenge dose site.

3+ A spontaneous flare occurring at only the sensitizing dose site.
2+ in the absence of a spontaneous flare, reapplication of a challenge dose elicited an unequivocal reaction.

If no spontaneous flare occurred even after the reapplication of challenge dose, the reaction was considered as negative.