Surface morphology of immunocompetent cells isolated from spleen of *Bufo himalayanus* (Günther)

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Immunocompetent cells were isolated from spleen of *B. himalayanus* and studied surface morphology of the three different cell types - (i) plastic adherent; (ii) nylon wool adherent; and (iii) nylon wool non-adherent cells. As revealed by scanning electron microscopy, they resembled the macrophages, B and T cells, respectively. Presence of such cell types indicated that *Bufo himalayanus* possessed a well-organized immune system. Further work is needed to characterize the functional efficacy of these immunocompetent cells found in *B. himalayanus*.

**Key words:** B cells, *Bufo himalayanus*, Nylon wool column, Scanning electron microscopy, T cells

Amphibians are excellent model to study the evolution of complex immune system in vertebrates. *Bufo himalayanus* (Himalayan toad) is a unique amphibian species, adapted to cold climatic conditions of Eastern Himalayas and shows significant morphological, behavioral and physiological differences from other members of Bufonidae. They are restricted to 1300 m above sea level in the hills of Darjeeling and Sikkim in India and in parts of southern China.

In higher vertebrates, lymphocytes1-3 and monocytes4, 5 are the components of immune system that play an important role in self-defence system. Preliminary observations indicted that *B. himalayanus* has well-organized primary and secondary lymphoid organs. In the present study, blood parameters of the captive and wild toads were investigated and lymphoid cells isolated over percoll gradient6 were characterized on the basis of their adhesiveness to plastic, and nylon wool and then their surface morphology were studied by scanning electron microscopy.

**Material and Method**

*Animal model—*Bufo himalayanus* (Günther), falls under the “least concerned” category of the IUCN red list 2007 (Ref. 7), was undertaken for the present investigation. Adult, healthy individuals were captured from the wild and grouped into two categories. The first group was sacrificed immediately after capturing and serum parameters were studied. The second group was kept in the laboratory under proper hygienic conditions and fed with house flies and chopped chicken and goat liver. The captive toads were sacrificed after seven days for serum biochemical analysis. The animals were sacrificed following the guidelines and approval of the Animal Ethical Committee of the institute.

Serum preparation—Blood (1-2 ml) was collected by cardiac puncture, using 21 gauge needles. Blood samples were transferred into vacutainer tubes and placed on ice until processing in the laboratory, 2-3 hr after collection. Serum was separated by centrifugation at 3000 rpm for 10 min and split in two or more vials.

Estimation of serum SGPT, total protein, albumin and triglyceride—All the reagents for analysis of serum SGPT, total protein, triglyceride and albumin were purchased from Span Diagnostics Ltd. Gujrat, India. The serum levels of SGPT, total protein, albumin and triglyceride, were determined spectrophotometrically (Jasco V-530) as per manufacturer’s instructions (Span Diagnostics Ltd., India).

Isolation of spleenic leukocytes—The spleen was collected aseptically from the toad and cells were dissociated in amphibian phosphate buffered saline (APBS, pH 7.2), with the help of a sterilized stainless steel wiremesh and by passing through a syringe fitted with 27 gauge needle. The cells were washed twice in

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chilled APBS and finally suspended in Leibovitz-15 (L-15, Himedia, India) medium, supplemented with glutamine, $1.25 \times 10^{-5} \text{M}$; HEPES (Sigma, USA) buffer, 200 mg NaHCO$_3$/100 ml, 100 U of penicillin/ml (Sigma, USA); 100 µg/ml of streptomycin; (Sigma, USA), and 50 µg/ml of nystatin (Sigma, USA).

The lymphoid cells were freed from red blood cells by centrifugation over isopycnic percoll density gradient as described earlier. The cell fractions collected from 65% percoll interphase and then washed twice with cold APBS to remove percoll. Viability of the cells was tested by trypan blue exclusion method and then suspended at a concentration of $2 \times 10^6$ cells/ml.

**Adherence property of leukocytes**—Immuno-competent cells suspended in L-15 medium were then layered on a 4 inch sterilized plastic petridish (Laxbro) and incubated in humid environment having CO$_2$ (5%) at 37°C for 45 min. After the incubation period, the non-adherent cells were removed by gentle stirring onto a separate container. The adherent cells were first given a cold shock with chilled medium and then gently scraped with rubber policeman before collection. The plastic non-adherent cells were further fractionated following the method of Julius et al. Briefly, the plastic non-adherent cells were loaded on a nylon wool column and incubated at 37°C in humidified atmosphere having 5% CO$_2$. The non-adherent cells were eluted from the column with warm medium (37°C) after 1 hr of incubation. The cell viability of all the cell types was checked by Trypan blue exclusion method and the cell concentration was adjusted to $2 \times 10^6$ cells/ml.

**Phase Contrast Microscopy**—Purified immuno-competent cells were suspended in L-15 medium at a concentration of $2 \times 10^6$ cells/ml, taken in a glass petridish (Borosil) and observed and photographed under Zeiss Axiosstar Plus microscope (Carl Zeiss Microimaging GmbH, Germany).

**Scanning Electron Microscopy (SEM)**—The cells were fixed with gluteraldehyde (2.5 %) in 0.1M Cacodylate buffer (pH 7.1) for 2 hr. Post fixation was made in osmium tetroxide for 2 hr followed by dehydration in ethyl alcohol gradient. The cells were then subjected to critical point drying using amyl alcohol and liquid CO$_2$ and coated with 150 to 200 Å layer of gold. Surface morphology of the leukocytes was photographed under scanning electron microscope (SEM-Hitachi S530).

**Statistical analysis**—Biochemical variables were expressed as mean, standard deviation (SD) and range. We used analysis of variance (ANOVA) and $t$ test for a comparison between the two groups. Results were considered significant at $P < 0.05$. Statistical analyses were carried out by using STATISTICA version 6.0.

**Results**

**Serum biochemistry**—Results of serum biochemistry analyses have been summarized in Table 1. Almost no difference was observed in the total protein content of serum between newly captured and captive toads which was 2.32 ± 0.16 and 2.33 ± 0.17 g/dL in newly captured toads and captive toads, respectively. Similarly, albumin content in wild group was 0.64 ± 0.06 g/dL and in captive group was 0.62 ± 0.05 g/dL. However, compared to albumin, serum globulin content was much higher in concentration and was 1.68 ± 0.11 and 1.7 ± 0.14 g/dL in wild and captive toads, respectively.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild</th>
<th>Captive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>2.08 - 2.56</td>
<td>2.09 - 2.67</td>
</tr>
<tr>
<td>Albumin (Alb)</td>
<td>0.56 - 0.77</td>
<td>0.56 - 0.72</td>
</tr>
<tr>
<td>Globulin (Glb)</td>
<td>1.52 - 1.87</td>
<td>1.53 - 1.99</td>
</tr>
<tr>
<td>Alb/Glb</td>
<td>0.36 - 0.43</td>
<td>0.33 - 0.44</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>76.21-84.52</td>
<td>78.82-85.62</td>
</tr>
<tr>
<td>SGPT</td>
<td>20.1 - 22.6</td>
<td>20.9 - 22.7</td>
</tr>
</tbody>
</table>

Values for total protein albumin and globulin are represented as g/dL; for triglyceride as mg/dL; and for SGPT as IU/L.

* Differences are non-significant at $P < 0.05$.

5 Differences are non-significant at $P < 0.01$. 


There was a slight increase in triglyceride levels in captive toads. It was $80.5 \pm 2.96$ and $82.3 \pm 2.44$ mg/dL in newly captured toads and in captive toads, respectively. However the difference observed was not statistically significant ($F_{1,18} = 1.96$, at 1% level of significance).

No change was observed in SGPT level of serum of wild and captive toads which was $21.88 \pm 0.69$ and $21.93 \pm 0.53$ IU/L, respectively.

*Isolation and adherence property of leukocytes*—
On the basis of adherence property to plastic, spleen cells were grouped as-(a) plastic adherent cells (PAC); and (b) plastic non-adherent cells (N-PAC). The plastic non-adherent cells were further classified into nylon wool adherent cells (Ny-AC) and nylon wool non-adherent cells (Ny-NAC) based on adherence to nylon wool. Percentage of three categories of cells recovered after each step of separation has been shown in Fig. 1. Number of PACs was found to be extremely low in spleen. Percentage of Ny-ACs was quite significant and was almost three times the number of nylon wool non-adherent cells.

*Phase Contrast Microscopy*—The cells isolated from blood by centrifugation over isopycnic percoll density gradient yielded a pure population of leukocytes as confirmed from the phase-contrast micrograph (Fig. 2B).

*Surface morphology of leukocytes*—Scanning electron microscopy (SEM) of the three category (1-3) of cells showed the characteristics – (1) The plastic adherent cells ranged from 8-10 µm in diameter. Both rough and smooth type cell membrane were observed with some cells having extensive pseudopodia like projections (Fig. 3A). When correlated with the surface morphology of the peripheral blood cells, these large cells appeared to come from monocytes lineage. (2) Nylon wool adherent cells (Ny-AC) had a diameter ranging from 7-9 µm. These cells were round in shape like those of lymphocytes. The surface topography of these cells was relatively rough and showed numerous blunt membranous undulations, minute projections and extensions from cell surface (Fig. 3B). No pseudopodia like extension was visible and this made them distinctly different from the plastic adherent cells and also from the nylon wool non-adherent cells (Ny-NAC). (3) The nylon wool
non-adherent cells (Ny-NAC) also had diameter ranging from 7-9 µm. The cells were round with smooth cell surface morphology. There were absolutely no pseudopodia like projections except that the surface was marked by fissures (Fig. 3C).

Discussion

The blood parameters of the captive animals showed no significant difference from those of the newly captured individuals. There was a slight increase in the triglyceride level, but statistically the difference was not significant. Interestingly, the globulin level was much higher than the albumin in the serum as seen in reptiles and in no way can be compared to humans, but the leukocytes showed features that are present in mammals. The results imply that physiological properties did not change in captivity and the results obtained from the individual could be considered as normal. Further, as there is no previous report about any biochemical parameters of serum of any amphibian species, so the values for those parameters reported in the present study can be taken as reference value for Bufo himalayanus.

Spleen is the most important secondary lymphoid organ in all vertebrates. Bufo himalayanus also has a well organized spleen which is small round pear shaped structure measuring 9-11 mm in diameter, located in the duodenal region of the abdominal cavity. Histological section of spleens showed no special features except that the cells were more loosely arranged than that found in mammals and the red and white pulp areas could not be demarcated clearly. Supporting tissues that form the trabeculae were not very distinct too (Fig. 2A).

Isolation of leukocytes from spleen RBC was a major obstacle that was overcome by adopting a new method of cell fractionation over 65% percoll gradients. This method yielded pure leukocytes as confirmed from the phase contrast photograph (Fig. 2B) of the isolated cells. This single step method of isolation can be utilized to separate leukocyte even from minimum volume of cell suspension. Further, the nontoxic nature of percoll allowed recovery (98%) of viable cells.

The isolated spleen leukocytes were characterized on the basis of their adherence to plastic and nylon wool to understand whether they possess the same differential cell surface characteristics like those found in mammals. B. himalay anus leukocytes showed differential adhesive properties on the basis of which the cells were categorized into three types. The plastic adherent cells in all probabilities appeared to be macrophages as they showed close resemblance with mammalian macrophage cells. Although their number remains low, their presence in the spleen carries great significance as they are likely to process and present antigens to the lymphocytes.

Plastic non-adherent cells were distinctly round with a diameter ranging from 7 to 9 µm and showed resemblance to mammalian lymphocytes. These cells could further be categorized into two types on the basis of their adhesiveness to nylon wool. Elution profile of the plastic non-adherent cells through the nylon wool columns also exhibited characteristics of mammalian lymphocytes i.e., a significant proportion of the plastic non-adherent cells got retained in the nylon wool column (Fig. 3B). Earlier reports suggest that it is B-lymphocytes that get adhered to nylon wool because of presence of microvilli on the cell surface, whereas T lymphocytes get eluted due to relatively smoother cell surface morphology (Fig. 3c).
Interestingly, the nylon wool adherent cells had rough cell surface with various undulating features, small microvilli like projections than the non-adherent cells which were relatively smooth with practically no cell surface projections or undulations. The differences in surface topography along with the differences in surface adhesiveness indicated that the nylon wool adherent and non-adherent cells most likely represented B and T lymphocytes, respectively as observed in mammals. The similarity between amphibian and mammalian lymphocytes was an indication that some of the basic characteristics of lymphocytes probably evolved long back in history. As no specific antibodies are available against the amphibian lymphocytes, it is not possible to confirm the observations, but the findings will definitely help to further characterize the functional efficacy of immunocompetent cells and also throw some light on evolution and origin of immune system.

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References