Studies on Antibody-Producing Cells by Hemolytic Plaque Method and Phase Contrast Microscopy

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With the recent development of immunologic chemistry studies of immunoglobulins have made a great progress. Yet the morphologic picture of antibody-producing cells has not been definitely elucidated because of various technical difficulties. Nonspecific reactions and difficulty in cell identification complicate the Coons' fluorescent antibody technic and Nossal's micro-drop method. This results in diversity in the opinions concerning the kind and morphology of the antibody-producing cells. Theoretically, Dameshek expressed the concept of an "immunocyte complex" of cells in 1963, calling the large primitive cells, considered to be the forerunners of the immunocompetent plasmocytes and lymphocytes, "immunoblasts". The functions of these cells in vivo, however, are yet to be studied.

In 1963 Jerne and Nordin presented the hemolytic plaque method which measures quantitatively the antibody-producing cells in vitro. This method requires relatively little technical skill and is an excellent means for identification of individual antibody-producing cells. Harris et al. and Hummeler et al. reported, on the basis of the observations on the fine structure of the plaque-forming cells by electron microscopy, that lymphocytes and plasma cells were present. However, quantitative analysis for these two kinds of cells is impossible with this method.

In the present study, quantitative observations were made as to the number of the plaque-forming cells in the thoracic duct and lymph nodes of the immunized rabbit through a revised hemolytic plaque method using agar on the slide glass at a thickness of 10μ. The type and morphology of the plaque-forming cells were observed with a phase contrast microscope.

Materials and Methods

Animals

Sixty-two adult rabbits weighing 2.5 to 3.0 Kg. were used.
Immunization

The animals were immunized by a single injection in the hind foot-pad with 2 ml. of 20 per cent suspension of sheep erythrocytes washed three times with saline.

Obtaining the Specimen

The cells in the thoracic duct and in the lymph node were examined, each time in four rabbits, everyday from the first to the eighth day and on the eleventh day after the immunization. In addition the cells in the lymph node were examined, each time in four rabbits, on the ninth, tenth, twelfth, thirteenth, fourteenth and seventeenth days after the immunization. Animals once used were never reexamined. A medial incision was made on the neck of the anesthetized rabbit, the left clavicle and cervical muscles resected, and lymph was obtained from the thoracic duct exposed at 5 to 8 mm. distal to the left venous angle. Specimens of the popliteal lymph nodes were simultaneously obtained.

Hemolytic Plaque Method

Four to six ml. of the lymph obtained from the thoracic duct were centrifuged at 2000 rpm for 5 minutes and the sedimented cells were suspended in Medium 199 so as to have its concentration at a level of $3 \times 10^5$ to $3 \times 10^6$ / mm.$^3$. One-tenth to 0.8 ml. of this suspension was mixed with 0.2 ml. of 20 per cent sheep erythrocyte suspension and 2.0 ml. of agar solution and then put into twice diluted Medium 199, thus making a 4.0 ml. final volume. This was incubated at 45 C. for one minute. In order to have an appropriate number of plaques develop on the plate, the amount of the cell suspension was adjusted according to the days elapsed after the immunization. One ml. out of the incubated 4.0 ml. of the suspension was poured into the plate of 6 cm. in diameter and it was incubated at 37 C. for 90 minutes after the agar had hardened. Then with complement added it was again incubated at 37 C. for 30 minutes and the number of the plaques was counted.

As for the lymph node, 150 to 200 mg. of the obtained specimen was chopped with a pair of scissors into small bits within Medium 199. The cell suspension thus produced was filtered with a platina net and centrifuged at 2000 rpm. for 5 minutes. The sedimented cells were processed just as those from the thoracic duct.

For cytological observation of the plaque-forming cells (PFC), a modification of Cunningham's method$^7$ was devised. As seen on Figure 1, a paraffin sheet measuring $15 \times 20$ mm. in inside diameter and 10$\mu$ in thickness cut out with a microtome was set on the slide glass. Within this small chamber, the lymph node cells, sheep erythrocytes, Medium 199, agar solution, and complement were put together. Then it was covered with the cover glass and enclosed with vaseline. This method allows observation of the lymph node cells and erythrocytes arranged in a layer within the small 10$\mu$ thick chamber. This specimen was incubated at 37 C. for 15 minutes, and the cells around the center of the hemolytic plaque were observed by phase contrast microscopy using an oil immersion lens. The PFC were observed, each time in four rabbits, 4, 6, 8, 10, 12, 13, 17, and 18 days after the immunization. Eight specimens were produced of the four rabbits and more than 100 PFC in the eight specimens were subjected to study. On the fourth, sixth, seventeenth

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**Fig. 1.—A hemolytic plaque method using agar on the slide glass at a thickness of 10 $\mu$.**
RESULTS

The objectives of the present experiment were the following four items: 1) changes in the number of the PFC in the thoracic duct, 2) changes in the number of the PFC in the lymph nodes, 3) morphological findings on the PFC in the lymph node by phase contrast microscopy, and 4) chronological changes in each group of PFC.

1) The Number of the PFC in the Thoracic Duct

The average number of the PFC in the thoracic duct for the thirty six rabbits is given in Figure 2. The PFC rose sharply on the fourth day after the immunization reaching the maximum 22.4 per 10⁶. Then it gradually decreased to be 8.2 per 10⁶ on the sixth day and below 1 per 10⁶ after eighth day.

2) The Number of the PFC in the Lymph Node

The average number of the PFC in the lymph node for the sixty two rabbits is given in Figure 3. The PFC, as those in the thoracic duct, rose as high as 242 per 10⁶ on the fourth day after the immunization and remained at such a high level until the seventh day. Then it again increased from the ninth day and the number of the PFC during the ninth to the eleventh days was kept at 570 to 690 per 10⁶, and then tended to decline. That is, the number of the PFC in the lymph node was found to have the first peak on the fourth to seventh days and the second peak on the ninth to thirteenth days after the immunization.
Fig. 3.—Total plaque-forming cells and plaque-forming plasma cells in the lymph node.

3) Morphologic Findings on the PFC in the Lymph Node

It was not easy to have clear-cut cytological findings since the PFC were embedded within 10μ-thick agar. The size and outline of the cell, the amount of RNA in the cytoplasm, the characteristics of the Golgi area, the shape, number, and distribution of the mitochondria, and the shape and size of the nucleolus were observed and these findings enabled differentiation of the cells.

When a single cell is present at the center of the hemolytic plaque, there is no doubt that the cell is a PFC; but when the plaque contains more than one cell, it is difficult to decide which one is a PFC. Therefore, the number of the cells contained within the chamber was adjusted so that whenever possible a hemolytic plaque did not contain more than a single PFC. When a plaque showed more than one cell, they were differentiated by the following point: a PFC has stroma of erythrocytes adhering to it (Fig. 5e), whereas a cell other than a PFC has no such adhesion of the erythrocytic stroma.

From the fourth to the eighteenth days, 56 to 100 and more PFC were observed every day and classified into four groups: 1) lymphogonia, 2) lymphoblast, 3) basophilic mature lymphocyte and 4) plasma cell.

1) Lymphogonia (Amano6) A lymphogonia is the largest of the PFC, its diameter being about 12μ. It appears smaller than the one in blood smear because it is embedded within agar. The rather lucid, large nucleus often has an indentation on one side with an irregular, gigantic nucleolus. It contains little chromatin. The cytoplasm is wide and dark. Rod-like mitochondria are distributed in the centrosphere of the cell or around the nucleus and the narrow Golgi area. No granules or vesicles observed in the cytoplasm (Fig. 4a. and b). Figure 4c shows a lymphogonia in the ordinary wet preparation for comparison. Its diameter ranges 20 to 30μ, and other findings agree with the abovementioned.

2) Lymphoblast A lymphoblast is a round cell and somewhat smaller than
Fig. 4.—(A and B) A lymphogonia seen at the center of a hemolytic plaque. Phase contrast photograph. The scale appearing in the upper part of Figure (4A) shows a graduation by 10μ. All the pictures of the cells were taken in the same magnification. (C) shows a lymphogonia in the ordinary wet preparation for comparison. (D and E) A lymphoblast seen at the center of a hemolytic plaque. (F) shows a lymphoblast in the ordinary wet preparation for comparison.

a lymphogonia. Its round nucleus abounds in chromatin in contrast to the lymphogonia and has two to three rather small nucleoli. The cytoplasm is dark with a few vesicles. The Golgi area is narrow with few granules (Fig. 4 d and e). Figure 4 f shows a lymphoblast in the ordinary wet preparation for comparison. The rod-like or slender mitochondria are distributed in the periphery of nucleus or centrosphere of the cell. Other findings are identical with the foregoing.

3) Basophilic mature lymphocyte. Among the cells classified into this category are included relatively large ones and those as small as a small lymphocyte. But the latter, too, seemed to have somewhat more cytoplasm. The nuclear structure is crude with chromatin clumps. With a few exceptions, a majority of the cells has no nucleolus. The cytoplasm is dark in small and large cells alike, indicating abundant RNA (Fig. 5 a and c). Figure 5 b and d show the basophilic mature lymphocytes in the ordinary wet preparation for comparison. Figure 5 b represents what is considered as the so-called atypical lymphocyte.

4) Plasma cell. A plasma cell is round or oval. Its eccentric nucleus has a thick nuclear membrane. The dark cytoplasm occasionally has Russel bodies and a large Golgi area is noted (Fig. 5 e). Among these plasma cells were seen cells at various maturing stages from those with nucleoli and regarded as plasmoblasts to those regarded as mature plasma cells. But immature cells
Fig. 5.—(A) A large basophilic mature lymphocyte seen at the center of a hemolytic plaque. (B) shows a basophilic mature lymphocyte in the ordinary wet preparation for comparison. The cell called as atypical lymphocyte sometimes. (C) A small basophilic mature lymphocyte seen at the center of a hemolytic plaque. (D) shows a small basophilic mature lymphocyte in the ordinary wet preparation for comparison. (E) The stroma of erythrocytes is adherent to a plasma cell at the center of a hemolytic plaque. (F) shows a plasma cell in the ordinary wet preparation for comparison.

were very rare. Figure 5 f shows a mature plasma cell in the ordinary wet preparation for comparison. The nucleus with a few chromatin clumps is situated eccentrically and the dark cytoplasm with a wide Golgi area has generally scattered round mitochondria.

Thus classified into four groups, the PFC have intermediate forms among them and differentiation was sometimes difficult. Omitted from counting on account of mitosis or degeneration were less than one per cent of cells.

4) Changes in the Number of Each Group of PFC after Immunization

A chronological observation was made as to the ratio among the lymphogonias, lymphoblasts, basophilic mature lymphocytes and plasma cells (Table 1). Lymphogonias are most increased on the fourth day after the immunization, constituting 8.9 per cent of the total PFC on that day, and then decreased. Lymphoblasts, like lymphogonias, attain their peak on the fourth day and then decrease, but the rate of their decrease is less than the former. Basophilic mature lymphocytes are the most frequently encountered of the four kinds of PFC. They maintained on incidence of roughly 60 to 70 per cent and showed no sign of decline until the eighteenth day after the immunization. Plasma cells were very scanty at the early stage after the immuniza-
ANTIBODY-PRODUCING CELLS

Table 1.—Differential Counts of Plaque-Forming Cells (%)

<table>
<thead>
<tr>
<th>Days</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>13</th>
<th>17</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphogonia</td>
<td>8.9</td>
<td>2.4</td>
<td>4.5</td>
<td>4.9</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoblast</td>
<td>16.1</td>
<td>4.9</td>
<td>11.4</td>
<td>16.3</td>
<td>7.8</td>
<td>11.5</td>
<td>7.0</td>
<td>10.7</td>
</tr>
<tr>
<td>Bas. mature lymphocyte</td>
<td>71.8</td>
<td>82.9</td>
<td>71.6</td>
<td>55.8</td>
<td>64.0</td>
<td>65.4</td>
<td>69.8</td>
<td>60.7</td>
</tr>
<tr>
<td>Plasma cell</td>
<td>3.2</td>
<td>9.8</td>
<td>12.9</td>
<td>27.9</td>
<td>23.3</td>
<td>23.1</td>
<td>20.9</td>
<td>28.6</td>
</tr>
</tbody>
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...tion, began to increase on the eighth day, rose as high as 27 per cent on the tenth day and remained above 20 per cent until the eighteenth day.

Of the PFC in the lymph node, the ratio of the plasma cells to the total PFC is given in Figure 3. As is seen from the figure, after the tenth day the change in the number of plaques is roughly parallel to that in the number of plaques with plasma cells.

DISCUSSION

After immunizing rabbits in the hind foot-pad with sheep erythrocytes, the number of the PFC in the thoracic duct and popliteal lymph node was counted daily using the hemolytic plaque method introduced by Jerne and Nordin, and the differential counts and changes in the number of each group of the PFC in the lymph node were observed. The number of the PFC in the thoracic duct attained its maximum on the fourth day after the immunization and then sharply decreased. The number of the PFC in the lymph node showed the first peak on the fourth to sixth days, rose again to reach the second peak on the eighth to thirteenth days and then decreased. The fact that the maximum value of the PFC in the lymph node was observed between ninth and thirteenth days may be attributed to the adjuvant effect of sheep red cells which were injected in the hind foot-pad of the rabbit.

Jerne and Nordin, and Hege and Cole reported that, as the primary response to an intravenous injection, the number of the PFC in the spleen reached its maximum on the fourth day and then decreased. The numbers of the PFC in the thoracic duct and lymph node developed parallel to each other until the fourth day but not afterwards. While the PFC in the thoracic duct rapidly decreased, the PFC in the lymph node turned to rise again on the eighth day or so. The PFC appeared in the thoracic duct in the early stage of immunization; but thereafter they did not appear in the thoracic duct while those in the lymph node increased. This fact may represent a certain immunologic process. It may be explained as follows: The purpose of recirculation of lymphocytes might be to transmit the information of antibody production to the antibody-producing organs through the information-bearing cells. Therefore, the antibody-producing cells should pass through the thoracic duct and appear in the peripheral blood soon after the immunization. Hall et al. reported that the basophilic lymphoid cells in the lymph from an immunized lymph node passed through the lymphatic pathway and induced an initiating immune response in other lymph nodes.

Plasma cells increased after the eighth day of the immunization, and they might specifically remain in situ, not flowing into the thoracic duct.
ham\textsuperscript{11} stated that, from a morphologic observation of the PFC in the lymph and lymph node, plasma cells were noted in the lymph nodes but not in the lymph.

In our present study, the PFC observed within the small chamber were classified into the cells of the lymphocytic series and the plasma cells, the former being further divided into the lymphogonia, lymphoblast, and basophilic mature lymphocyte. Hannoun and Bussard\textsuperscript{12} observed the morphologic picture of the PFC in the lymph node and spleen cells and stated that the PFC were classified into the plasma cell, large reticular cell, macrophage, and lymphocyte. The macrophages were not demonstrated in our experiment.

The lymphogonia (Amano\textsuperscript{8}) is the largest and least differentiated cell of the lymphoid cells in the lymph node. Its nucleus has little chromatin, with a lucid, fine nuclear structure, and large, irregular nucleoli. The term "lymphogonia" has been given by Amano to "the large cell with strongly basophilic cytoplasm, a large nucleus and distinct nucleoli" appearing in the swollen lymph node after immunization with an antigen. This cell is considered to be identical with the ones called the large lymphatic reticulum cell\textsuperscript{13}, transitional cell\textsuperscript{14}, large pyroninophilic lymphocyte\textsuperscript{15}, hemocytoblast\textsuperscript{16}, and immunoblast\textsuperscript{3}.

In the category of the basophilic mature cell are included cells of various sizes, ranging from large to small lymphocytes in size. What is common to these cells is their possession of dark cytoplasm that indicates abundant presence of RNA. Even a small cell appears to have a little wider cytoplasm than a small lymphocyte.

Those that grow dominant after the eighth day of the immunization are mostly mature plasma cells, but rarely were such cells noted to have nucleoli and considered to be plasmoblasts.

As to the morphology of the PFC, Fitch et al.\textsuperscript{17} and Bussard and Binet\textsuperscript{18} made observations by electron microscopy and reported that the cell at the center of the hemolytic plaque is a plasma cell. Afterwards, Harris et al.\textsuperscript{5} and Hummeler et al.\textsuperscript{6} reported that there are two kinds of cells at the center of the hemolytic plaque, a plasma cell and a lymphocyte with endoplasmic reticulum. Amaki et al.\textsuperscript{19} and Irimajiri\textsuperscript{20} observed a closely similar lymphocyte with endoplasmic reticulum in the thoracic duct cells of an immunized rabbit by electron microscopy and speculated that it may be a 19S antibody producing cell. Williams et al.\textsuperscript{21} reported this cell as a plasma cell precursor in an experiment of the kidney transplantation. The cell with endoplasmic reticulum reported both by Harris et al.\textsuperscript{6} and Hummeler et al.\textsuperscript{6} is a mature one and belongs to the basophilic mature lymphocyte group according to our classification. There is no report of immature cells of this kind as for lymphogonias. Most of the PFC are mature lymphocytes. Lymphogonias and lymphoblasts appear in the early stage of immunization, whereas plasma cells increase after the eighth day of the immunization.

Nossal et al.\textsuperscript{2} observed 123 antibody-producing cells and reported, "These comprised 42 19S cells, 64 7S cells, and 17 double producers. All except 4 of the cells in the study could clearly be identified as members of the plasma cell series. No morphologic difference between 19S and 7S cells could be found."
However, it is thought to be rather difficult to differentiate immature lymphocytes and basophilic lymphocytes from immature and mature plasma cells certainly by light microscopy.

The presence of a lymphocyte with endoplasmic reticulum among the cells at the center of the hemolytic plaque undoubtedly indicates that certain lymphocytes possess the ability of antibody production.

The common morphologic features with the PFC revealed by phase contrast microscopy are dark cytoplasm and its basophilia by Wright's stain. But we do not regard all the lymphocytes with basophilic cytoplasm as antibody-producing cells. Their differentiation from the cells with basophilic cytoplasm should be based on the findings of electron microscopy. Those with endoplasmic reticulum are considered to have the ability of antibody production, and those without it are considered otherwise at that particular point.

**SUMMARY**

The numbers of the PFC in the thoracic duct and lymph node of the immunized rabbit were counted by the hemolytic plaque method and the morphologic features of the PFC in the lymph node were simultaneously observed by phase contrast microscopy.

The number of the PFC in the lymph node maintained a relatively high level on the fourth to seventh days, and then rose to attain the maximum on the ninth to thirteenth days, and then decreased.

The number of the PFC in the thoracic duct reached the maximum on the fourth day after the immunization and then dropped rapidly. The mechanism was discussed.

From morphologic observations, the PFC were classified into lymphogonia, lymphoblast, basophilic mature lymphocyte and plasma cell. The lymphogonia and lymphoblast, which belonged to immature lymphocyte series, were frequent in the early stage and the plasma cell was increased in the later stage of the immunization. The basophilic mature lymphocyte constituted more than 55 per cent through the whole process and were most frequent of the plaque forming cells.

**SUMMARIO IN INTERLINGUA**

Le numero del cellulas placa-formatori (CPF) in le ducto thoracic e le nodos lymphatic de conilios immunisate esseva contate per medio del methodo a placa hemolytic, e le characteres morphologic del CPF in le nodos lymphatic esseva observate simultaneemente per microscopia a contrasto (Ic phase.

Le numero del CPF in le nodos lymphatic manteneva un relativamente alte nivello in observationes executate le quarte e le septime dies e montava subsequentemente pro attinger maximos post inter novem e deec-très dies. Postea ille numeros redelinava.

Le numeros del CPF in le ducto thoracic attingeva maximos quatro dies post le immunisation pro declarar postea rapidemente. Le subjacente mechanismo es commentate.

A base de observationes morphologic, le CPF es classificate como lymphogonic, lymphoblastic, matur lymphocytic basophilic, e plasmocytic. Le typos lymphogenic e lymphoblastic, pertinente a immatur series lymphocytic, eseva frequente durante le phases precoco, e le plasmocytos eseva augmentate durante le phases plus tardive del immunisation. Le matur lymphocytes basophilic constitueva plus que 55 pro cento del total durante le integre processo. Illos esseva le typo le plus frequente del cellulas placa-formatori.
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REFERENCES

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