Primary Formation of Germinal Centers in the Chick Spleen after Injection with Sheep Red Blood Cells

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Summary. The primary formation of germinal centers was induced in the chick spleen by intravenous injection of sheep red blood cells (SRBC) at 21 days of age. In the spleen, SRBC were trapped in the ellipsoids soon after injection. The number of cells of perielipsoidal lymphoid tissues (PELT) was gradually depleted. Large pyroninophilic cells appeared in the periphery of periarterial lymphatic sheaths (PALS) 4 hrs after injection. Then they increased in number and migrated centralward in the PALS. On the 4th day they were accumulated in the vicinity of the central arteries to make nodular structures. Morphologically mature germinal centers encapsulated by connective tissue were formed for the first time on the 6th day after injection. They were most numerous on the 8th day and then decreased gradually. It was presumed that the primary germinal centers in the chick spleen might have been formed by the cells of the PELT which were originally derived from the bursa of Fabricius. The process of proliferation of germinal centers in the secondary immune response was discussed.

Germinal centers in the lymphoid organs, such as lymph nodes, spleen, and tonsil, are intimately associated with immune response. They are absent at birth and appear postnatally in association with antigenic stimulation (Weiss, 1972; Ravila, 1975). The proliferation of germinal centers following immunization was studied in mature animals by many investigators (Langevoort, 1963; Hanna, 1974; Hanna et al., 1967; Makinodan et al., 1969; Satodate et al., 1977). There are few reports, however, on the primary formation of germinal centers, except that histological studies in germfree animals were done by Kim and Watson (1971) in piglets and by van Ewijk et al. (1977) in mice.

The germinal centers of the chick spleen are oval and delineated clearly by connective tissue investments. They have often been called lymphoid foci (Lucas et al., 1954), secondary nodules (Thorbecke et al., 1957), and colonial nodules (Edwards et al., 1968). Generally, germinal centers appear in the chick spleen for the first time at about 4 to 5 weeks of age (Thorbecke et al., 1957; Cooper et al., 1965; Fukuta et al., 1979). Cells at the centers are regarded as B lymphocytes derived from the bursa of Fabricius (Cooper et al., 1965, 1966; Hoffmann-Fezer et al., 1977). The process of their formation, however, has not been examined in detail.

In the present study, the process of formation of primary germinal centers was
examined histologically in chicks too young to form these centers.

MATERIALS AND METHODS

Young chicks were obtained from eggs of the White Leghorn breed and maintained in the Laboratory of Veterinary Anatomy, University of Tokyo. At 21 days of age, a total of 43 chicks were injected intravenously with $1 \times 10^9$ sheep red blood cells (SRBC) suspended in sterile phosphate-buffered saline (pH 7.2). Two to five birds were killed for examination 10–14 min, 1, 2, 4, 6, 8, 12 and 24 hrs, and 2, 4, 6, 8, 13 and 20 days after injection. The spleen was collected from each chick, fixed in Zenker-formol, Carnoy’s and modified Karnovsky’s fluids, and processed into 3 μm tissue sections by the routine procedure. These sections were stained with hematoxylin-eosin, methyl green-pyronin, Mallory’s Azan and May-Grünwald Giemsa. Microscopic observation was performed mainly on longitudinal sections through the splenic hilus.

The results of observation were compared with those obtained from two other groups. One of these groups consisted of intact chicks 3, 4, 5 and 6 weeks old, and the other of chicks which received the second injection with SRBC 14 days after the first injection.

RESULTS

Normal spleen

The lymphoid tissues of the spleen of the adult chick consist of three parts, i.e., periarterial lymphatic sheaths (PALS) or clusters of small lymphocytes around arteries, perellipsoidal lymphoid tissues (PELT) or concentric lymphoid cell layers surrounding ellipsoids (Schweigger-Seidel sheaths), and germinal centers.

In intact chicks 3 weeks old, the splenic lymphoid tissues were poorly developed and merely consisted of PALS and PELT, with no germinal centers. The PELT were composed of one or two layers of medium-sized lymphoid cells stained faintly with pyronin (Fig. 1). The spleen of the 4-weeks-old intact chick was the same in appearance as that of the 3-week-old one, except that there was a mild increase in cells in the PALS. A few germinal centers appeared in it for the first time at 5 weeks of age. At 6 weeks of age, germinal centers further increased in number, and the other lymphoid structures were also well developed.

Spleen after SRBC injection

In the spleen, SRBC were found in the ellipsoids 10 min after injection. They were readily identified by their intense affinity to eosin and the absence of nuclei in contrast with the nucleated chick erythrocytes (Fig. 3a). Many macrophages containing eosinophilic cell debris were recognized in pulp cords near the ellipsoids and in veins (Fig. 3b, c). These figures suggest the phagocytosis of SRBC by macrophages in the ellipsoids. A few SRBC were still recognized in some ellipsoids and their neighborhood 14 min after injection, but 1 hr later no SRBC were detected histologically.
Fig 1. Spleen of an intact chick 3 weeks of age. Sheathed capillaries (SC) are surrounded by one or two layers of medium-sized lymphoid cells (arrows). These cells compose PELT. May-Grunwald Giemsa. × 600

Fig. 2. Twelve hrs after SRBC injection. Note that no PELT are present around any sheathed capillary (SC). May-Grunwald Giemsa. × 600

Fig. 3. Spleen 10 min after injection. Hematoxylin-eosin.

a. Injected SRBC (arrows) trapped in the ellipsoids of sheathed capillaries (SC). × 600.

b. Macrophage engulfing eosinophilic cell debris in the pulp cord adjoining the ellipsoid (arrow). × 2,250.

c. Macrophage (arrow) in the vein. Nucleated chick erythrocytes (E) and a lymphocyte (L) are also observed. × 2,250
The layers of medium-sized lymphoid cells in the PELT varied in size from area to area even in the same spleen. These cells, however, decreased in number 2 to 8 hrs after injection. They were hardly recognized around the ellipsoids 12 and 24 hrs later, so that the ellipsoids adjoined with one another or with reticular cells in the pulp cords (Fig. 2). The PELT were recovered on the 2nd day and further developed thereafter.

Large pyroninophilic cells appeared in the peripheral areas of the PALS 4 to 8 hrs after injection. They increased in number with the advance in age. They had a large amount of cytoplasm stained strongly with pyronin or a basic dye and large pale nuclei with distinct nucleoli (Fig. 4, inset). They were distributed throughout the PALS by 24 hrs after injection (Fig. 4). On the 2nd day they were accumulated near small arteries, or so-called central arteries (Fig. 5). Finally, they aggregated densely and formed nodular structures in the PALS on the 4th day (Fig. 6).

The nodular structures were formed regularly in the vicinity of the central arteries or their branches. They were encapsulated by connective tissue on the 6th day. Typical germinal centers appeared eventually in the chick spleen. Mature germinal centers were most numerous on the 8th day in the primary response. In them cells proliferated by mitosis. The enlarged centers extruded into the surrounding pulp cords over the PALS (Fig. 7). The cells of the centers, however, were reduced in size and lost affinity to pyronin.

The germinal centers began to decrease in number on the 13th day. On the 20th day, they decreased further to the level of intact chicks of the same age. They showed somewhat irregular contours on the 13th and 20th days. In them most cells

**Fig. 4.** Twenty-four hrs after injection. Large pyroninophilic cells (arrows) are diffusely distributed throughout the PALS. They are much larger than surrounding small lymphocytes. A central artery. Methyl green-pyronin. ×750.

*Inset.* Enlargement of large pyroninophilic cell (*). The cell has a large nucleus with prominent nucleolus. ×1,750
Fig. 5. Spleen on the 2nd day after injection. Large pyroninophilic cells (arrows) increase in number to accumulate near small arteries (A) in the PALS. Methyl green-pyronin. ×600

Fig. 6. Spleen on the 4th day. Large pyroninophilic cells aggregate densely in the vicinity of a small artery (A) in the PALS (surrounded by arrows). Methyl green-pyronin. ×600

Fig. 7. Spleen on the 8th day. An aggregation of large pyroninophilic cells is encapsulated by connective tissue to form a morphologically mature germinal center (GC). Mitotic figures are frequently observed at the center (arrows). Methyl green-pyronin. ×350

Fig. 8. Spleen on the 13th day. Most of the cells in the germinal center are small and fail to show any characteristic of the large pyroninophilic cell. Degenerative cells (arrow) are also recognized. Methyl green-pyronin. ×550
were of the same size as surrounding lymphocytes in the PALS (Fig. 8). Degenerative cells or nuclear debris were recognized in the germinal centers. From the 13th day onward, new cells stained strongly with pyronin also appeared in the affected spleen.

**DISCUSSION**

Antigenic stimulations cause morphological changes in the lymphoid organs. In the treatment of antigens inducing humoral immunity, large pyroninophilic cells appear and germinal centers proliferate in the spleen (LANGEVOORT, 1963; HANNA, 1964; HANNA et al., 1967; MAKINODAN et al., 1969; VAN EWIK et al., 1977). On the other hand, in the case of cell-mediated immunity, small lymphocytes in the PALS are rapidly depleted (ABE and ITO, 1972). Most of the previous investigations were carried out in adult animals, which have the complete lymphoid tissues with germinal centers. In mature animals, it is difficult to examine the process of germinal center formation because of confusion of pre-existing germinal centers with newly formed ones.

In the present study, young chicks having no germinal centers but being immunocompetent received the intravenous injection of SRBC, and the primary formation of germinal centers was induced in their spleens. Large pyroninophilic cells appeared in the periphery of the PALS 4 hrs after injection. They increased in number with the lapse of time. Judging from changes in the distribution of these cells after injection, the cells seemed to have migrated centralward in the PALS and accumulated in the vicinity of small arteries to make nodular structures. On the 6th day, these structures were encapsulated by the connective tissue to form typical germinal centers of the avian spleen for the first time. From the 8th day onward, germinal centers decreased in number and frequently contained degenerative cells, so that they seemed to be in the process of involution.

In the previous papers (LANGEVOORT, 1963; MAKINODAN et al., 1969), large pyroninophilic cells appearing during the period of immune response were regarded as precursors of plasma cells which had no relation to germinal centers. In addition, they migrated from the PALS into venous sinuses via pulp cords in quite a different manner from those observed in this study. Even in the primary immune response of germfree animals (KIM and WATSON, 1971; VAN EWIK et al., 1977), large pyroninophilic cells in the spleen were regarded as the derivatives of small lymphocytes in the PALS. Germinal centers in these animals were considered to be the clusters of small lymphocytes of unknown origin.

In the present study large pyroninophilic cells migrated centralward in the PALS to make germinal centers. This pattern agrees well with the migration of antigen-bearing cells in the chick spleen immunized with human serum albumin (WHITE et al., 1970). Moreover, in the turtle spleen, BORYSENKO (1976) observed that large pyroninophilic cells formed lymphoid foci in the PALS. He did not, however, regard these as germinal centers.

The SRBC injected as antigen were trapped in the ellipsoids soon after injection. Macrophages containing cell debris were frequently recognized in the pulp cords neighboring the ellipsoids and veins. On the other hand, medium-sized lymphoid
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cells in the PELT decreased in number after the SRBC injection. They were regarded as B lymphocytes derived from the bursa of Fabricius, as well as the cells in germinal centers (Mori and Hoshi, 1971; Hoshi, 1972; Hoffmann-Fezer et al., 1977). They were hardly recognized 12 and 24 hrs later.

In the secondary response in this study, large pyroninophilic cells seemed to have migrated from the PALS to the pulp cords, unlike in the primary response. After SRBC were injected for the second time, only a few of them were found in the spleen and seldom detected in the ellipsoids and their neighborhood. Moreover, medium-sized lymphoid cells in the PELT decreased in number, but not so markedly as in the primary response.

In the primary response, SRBC were trapped in the ellipsoids, medium-sized lymphoid cells in the PELT were depleted, and large pyroninophilic cells appeared in the periphery of the PALS in turn. These findings suggest that the primary germinal centers may have been formed directly from B lymphocytes in the PELT by the antigenic information transmitted from the ellipsoids where SRBC were trapped.

Linna et al. (1969) followed labelled bursal cells in the chick spleen for 48 hrs, but recognized none of them entering the germinal center. In the present experiment, it was 6 days after stimulation that a germinal center was formed, so that 48 hrs might not be enough for the bursal cells to enter the germinal center.

Germinal centers were always formed in the cluster of small lymphocytes of the PALS. It is suggested that the formation of germinal centers may require a milieu which is composed of T lymphocytes, and that an interaction between B and T lymphocytes may be important in the immune response, as described by Jankovic et al. (1969).

Changes in cell layers in the PALS could not exactly be evaluated in the present study, because B lymphocytes or large pyroninophilic cells increased in number and formed germinal centers in the PALS. The number of small lymphocytes in the PALS, however, increased more remarkably during the immune response than those in the intact chicks.

ヒツジ赤血球投与による鶏雛の腫臓における胚中心形成

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胚中心のみられない21日齢の白色レグホンにヒツジ赤血球 1×10⁹ 個を静脈内投与し、腫臓に出現する胚中心の形成過程を組織学的に検索した。ヒツジ赤血球は、投与直後、薬毛細血管壁の炎膜により反応した、薬毛細血管壁の薬膜組織（PELT）はビロニンに染染する中型リンパ細胞よりなるが、この細胞はヒツジ赤血球投与後次第に減少し、12ないし24時間後ではほとんど消失した。ビロニン好性大型細胞は、投与4時間後に動脈周囲リンパ組織鞘（PALS）の辺縁部に出現、時間の経過とともに増加し、PALS内を中心に向って移動し、中心動脈に接して集積し、4日後には結節状の細胞集塊を形成した。この細胞集塊は、6日後には結合組織性の被膜によって包まれ、ニワトリの成熟型の胚中心となった。胚中心は投与8日後にその数が最も多く、その後徐々に減少した。抗原刺激によって
REFERENCES


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