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Studies on the function of reticulo-endothelial system, I. Effects of the R. E. S. blocking with India ink on the haema-topoiesis and the production of serum antibody*

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Abstract

For the purpose to reveal the role of R.E.S. for hemopoiesis and antibody formation, the R.E.S. of rabbits were severely blocked by the repeated intravenous injection of a vast amount of India ink, reaching 200 to 250 cc in total and the development of anemia and antibody formation by challenging egg albumin were observed while referring to the histologic changes in bone marrow, spleen and lymph nodes. The results were as follows: 1. The repeated intravenous injection of a vast amount of carbon particles induced a severe anemia. The anemia was always normo- or hyperchromic, showing not any disturbance in iron metabolism or hemoglobin formation. The data suggested that anemia is due to the arrest of reproduction of erythroblast or differentiation of the stem cells to erythroblasts, but not due to inhibition of the iron metabolism. 2. R.E.S. had no relation to the proliferation or the differentiation of granulocytes. 3. The functions of R.E.S. related to erythropoiesis and lymphopoiesis are affected by blocking independently of its phagocytic potency. In spite of a severe anemia, the phagocytic potency of R.E.S. could never be lowered and liver and spleen grew much larger in size and weight, showing that the phagocytic ability of R.E.S. is extremely resistant against such a blocking. 4. The serum antibody titer proved to be at the normal level, and no change of the antibody production in spite of heavy blocking of R.E.S. with India ink.

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STUDIES ON THE FUNCTION OF RETICULO-ENDOTHELIAL SYSTEM

I. EFFECTS OF THE R. E. S. BLOCKING WITH INDIA INK ON THE HAEMATOPOIESIS AND THE PRODUCTION OF SERUM ANTIBODY

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ASCHOFF and KIYONO[1], in 1925, were the first to establish the concept of the reticulo-endothelial system (R. E. S.), specific cellular groups having common characteristics of phagocytosis and stainability for vital staining dyes, from the studies of METSCHNIKOFF's macrophage[2] and KIYONO's vital staining[3]. Since then, the morphology of R. E. S. was studied precisely by AKAZAKI[4,5], and in recent years, electron microscopic studies[6,7,8,9,10] have revealed the ultrastructures of reticulo-endothelial (RE) cells, demonstrating phagosomes, vesicles, dense bodies, etc. FRESEN[11] and POLICARD[12] studied the physiopathology of R. E. S. but our present knowledge concerning the functions of the R. E. S. is rather poor in comparison with its morphology. Many investigators have been interested in the responses of this system to the diseased conditions, for examples, the infectious diseases[13,14], malignant tumors[15,16], haematologic diseases, etc[17,18]. Among these the most fruitful results may be the one studied by BESSIS[19]. He observed the reticulum cells in erythroblastic islet and reached the conclusion that erythroblasts take up iron in the form of ferritin from the reticulum cell through rhopheocytotic mechanism which has been clearly demonstrated under electron microscope. It is, however, still uncertain whether the erythroblasts receive iron solely through the way demonstrated by BESSIS or this is only the occasional phenomenon which is not so important for erythropoiesis.

Besides these, reticulo-endothelial cells challenged by antigenic substances take up the antigen by their active phagocytotic mechanism and this is understood as the first step of antibody production, though it is still uncertain whether the lowered activity of the R. E. S. would be nessesarily connected to the lowered antibody formation. The disagreement among the results obtained by several authors[20,21,22,23,24,25] may greatly be due to the difference in the method employed for the blocking of R. E. S. especially the incompleteness in blocking. Taking this into consideration, I have studied first how the functions of R. E. S.

are arrested by intravenous injection of carbon particles by applying carbon clearance method, and then I have tried to elucidate how the normal functioning of R. E. S. supports the normal haematopoiesis and serum antibody production.

MATERIALS AND METHODS

Fifteen adult male rabbits weighing 2.5 to 3.0 kg were used. These were divided into three groups, five animals in each. Each animal belonging to the first group received the intravenous injection of 15 to 25 ml of 20 per cent India ink (Fueki Bokuju) in physiologic saline solution daily, for 50 days and all of them were sacrificed for the histologic observation. Components of Fueki Bokuju are 80 % of water, 6 % of carbon black, 4 % of glucose, 8 % of $CaCl_2$, 0.4 % of camphor and some surface active agents with other related substances in trace. The animals belonging to the second group were challenged with foreign protein. After the termination of India ink injections, the animals received the intravenous injection of egg albumin (1.5 % solution in saline, 2 ml.) 2 times with 2-day interval, and 3 to 20 days after the last injection the precipitin test was carried out and then sacrificed for histologic observation. Those of the third group were of control and received the daily injection of the saline solution only, 15 to 20 ml a day per animal for 50 days and sacrificed.

The grade of the blockade of R. E. S. was estimated by the carbon clearance method, described by SHIBATA[22], a modified method of HALPERN and others[26,27]. At certain intervals, the red blood cell count, reticulocyte count, white blood cell count, hematocrit value and hemoglobin level were observed by the routine method. For the observation of red cell size, one drop of the blood was taken on an object glass, covered with a cover slide, photographed with a scale under microscope, and then the diameter of the red blood cell was measured.

Cytological observations of blood cells were made on blood smears fixed with methanol and stained with May-Grünwald Giemsa by the routine method.

Serum iron was measured wth o-phenanthroline, by a modified method of BOTHWELL and others[28]. Serum precipitin was measured by the routine method.

All the animals were sacrificed by total blood depletion with severance of the carotid artery at the termination of experiment.

Liver, spleen, bone marrow and lymph nodes were checked of their gross changes, liver and spleen were weighed, and then all the tissues were fixed with neutralized formol, dehydrated, embedded in paraffin, sectioned and stained by hematoxylin-eosin by the routine method.

RESULTS

By the repeated intravenous injection of India ink, red cell count and hemoglobin level of the circulating blood decreased gradually developing a severe anemia at the 50th day. The severity of anemia was almost the same in all the animals. During the course of developing anemia the reticulocyte number increased gradually reaching 8 per cent of red blood cells at maximum, around the 20th experimental day, showing a decreasing tendency toward the end of experiment. Color index showed 1.0 at start, the value was kept for a fairly long time unchanged during the progress of anemia, but in later stages it became larger than 1.0 and reached 1.4 at the end stage of experiment (Fig. 1).

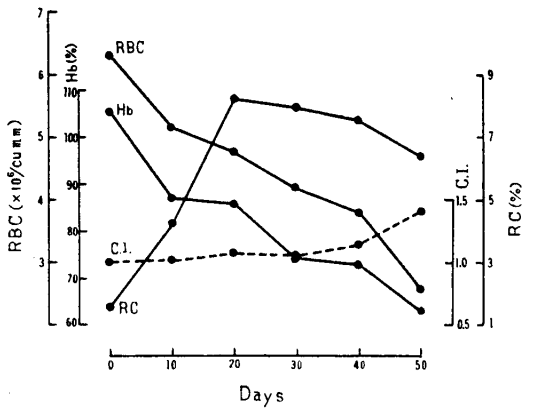


Fig. 1 Changes in red cell number, hemoglobin level, reticulocyte count and color index observed under the treatment of repeated intravenous injections of India ink, 3~5 cc of the ink in about 20 cc of saline solution daily. Each curve shows the mean value of 5 animals and note the development of normo- or hyperchromic anemia with the repeated injections of India ink. RBC: Red blood cell count, Hb: Hemoglobin level (Sahli), RC: Reticulocyte count, C. I.: Color index

Macrocytosis[29] appeared with the advance of anemia and the summit of Price-Jones curves showed a slight right-shift (Fig. 2).

Serum iron markedly decreased around the 20th day, but in the end stage, it recovered nearly to the normal level (Fig. 3).

Granulocyte count of the blood increased in the early stage of experiment but after several days it dropped to the normal level and kept in the same level till the end of experiment (Fig. 4). Monocyte count revealed no specific change. Lymphocyte count showed a gradual decrease but in no cases the number was greater than one half that of control (Fig. 4). Erythroblasts, chiefly orthochromatic ones, often appeared in the circulating blood and sometimes reached 20 per cent

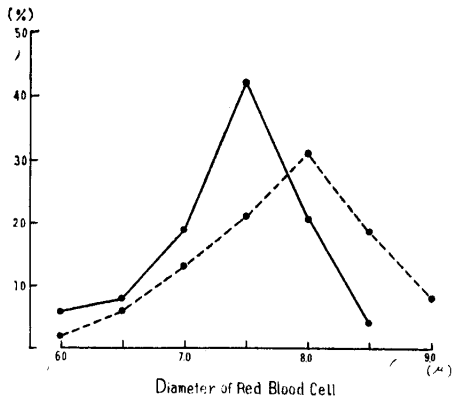


Fig. 2 Right side shift in Price-Jones curves seen in one animal among those introduced in Fig. 1. Observations on the blood taken from the animal after receiving about 50 injections, at the stage of which the animal developed severe anemia, RBC, 3 million per cu mm and Hb level, 65%, Sahli.

Solid line: The curve drawn before the injection of India ink,

Broken line: After 50 injections of India ink

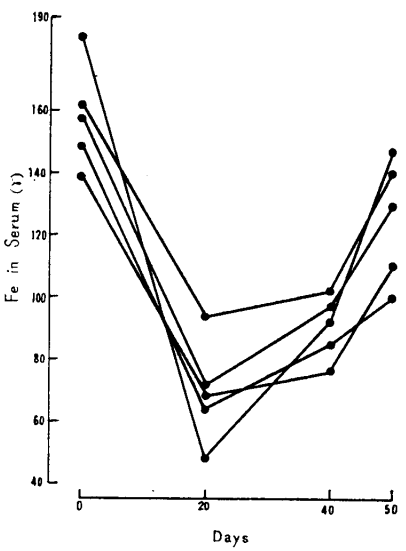


Fig. 3 Changes in serum iron level observed on the same rabbits as in Fig. 1. Each curve shows the value in one animal and a marked drop in Fe level after about 20 injections with some recovery thereafter.

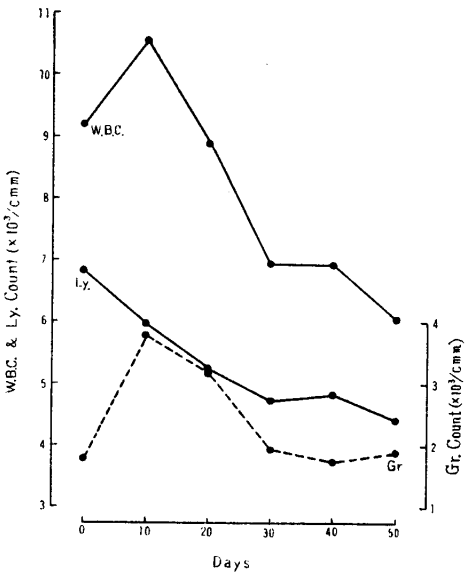


Fig. 4 Changes in white blood cell count Observed on the same animals as in Fig. 1. Each curve shows the mean value of 5 animals. W. B. C.: Total white blood cell count, Ly.: Lymphocyte count, Gr.: Granulocyte count

of the nucleated cells. This phenomenon occurred soon after the injection of India ink, without any relation to the increase in the reticulocyte number.

The carbon clearance was given by reducing the carbon contents of serum obtained at 30 minutes after the injection of India ink (Pelikan black) from that at 5 minutes after the injection. The data indicated that phagocytic potency of

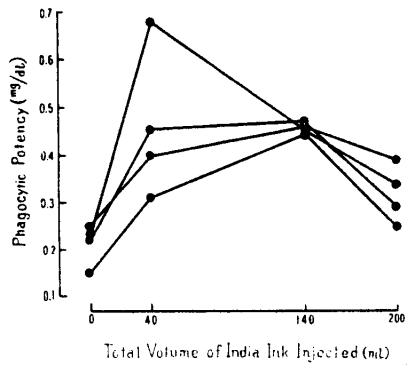


Fig. 5 Changes in the curves of the phagocytic potency estimated on the four animals from those appearing in Fig. 1. Each point was obtained by reducing the concentration of carbon particles in serum obtained at 30 minutes after the ink injection from that at 5 minutes. Each curve shows the value in one animal. Note the well retained phagocytic ability even after the introduction of 200cc of India ink in total for 50 days.

the R. E. S. was markedly accelerated at around the 10th day, when the animal had received 40 ml of India ink in total. This state of stimulated phagocytosis persisted until the 30th day under the continued injection of India ink, by that time the animals received 140 ml of India ink in total.

Thereafter toward the end stage of experiment the phagocytic activity of R. E. S. somewhat decreased, but it was still higher comparing to the original level found after the first injection of Pelikan ink (Fig. 5).

On autopsy liver, spleen and bone marrow of the animals injected 200 to 250 cc of India ink appeared completely black. The weight of liver became about 2.4 times as heavy as that of control, and that of spleen about 12 times of the normal ones (Table 1).

Table 1 The weight ratio of liver and spleen to body weight (×1,000) of rabbits received the repeated injections of ink. 3~5cc of the ink diluted in about 20cc of saline solutien were introduced into vein daily for about 50 days. Control animals received the injections of saline solution in the same volume daily as in those of treated with India ink injection.

Organ \ Animal No.	1	2	3	4	5	mean value
Liver	53.6	47.4	51.7	63.5	63.6	56.0
Spleen	9.84	2.59	7.33	6.00	10.00	7.15

Organ \ Animal No.	6	7	8	9	10	11	12	13	mean value
Liver	20.0	21.2	21.9	22.1	22.7	23.0	27.0	28.0	23.3
Spleen	0.26	0.45	0.50	0.52	0.62	0.59	0.72	0.73	0.55

No. 1 ~ No. 5: The rabbits injected with India ink, No. 6 ~ No. 13: Control group

Microscopically, the reticulo-endothelial cells of liver, spleen and bone marrow appeared as black as a carbon mass being laden heavily with carbon particles. They were markedly swollen and seemed to be increased in number. In liver, the proliferated, swollen and enlarged Kupffer cells made a grouping

and formed large nodules. Among these black nodules, the atrophic hepatic cell cords were observed (Photo. 1). In spleen, a great majority of the sinusoidal spaces were enlarged and occupied by swollen RE cells taking carbon particles similarly as in the case of Kupffer cells in liver. The lymph follicles of spleen were hardly recognizable, being atrophied extremely (Photo. 2).

The erythropoietic foci in bone marrow were completely destroyed by the swollen and proliferated RE cells filled with carbon particles and erythroblasts were rarely encountered (Photo. 3).

Generally, lymph nodes appeared brown in gross appearance and microscopically, a few RE cells containing carbon particles were found (Photo. 4), excepting the periportal lymph nodes which appeared quite black, having the sinuses filled up with RE cells heavily laden with abundant carbon particles (Photo. 5).

On another group of animals, which had received fifty injections of India ink and then challenged with egg albumin, the serum-antibody titer was estimated carefully but against expectation, no suppresed antibody formation was observed, as long as 3 to 20 days after challenging with the antigen (Fig. 6).

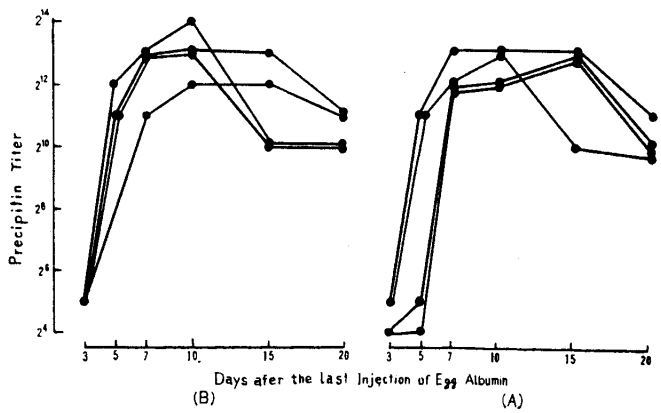


Fig. 6 Curves of the precipitin titer observed on the animals treated similarly as those in Fig. 1. After the completion of India ink treatment the animals were challenged with the intravenous injection of 2cc of 1.5% egg albumin in saline solution, twice at 48 hour interval and the precipitin titers were estimated 3 to 20 days after the 2nd injection of egg albumin. Each curve shows the value obtained on one animal. A: Experimental animals received India ink injection B: Control received saline solution injection

DISCUSSION

Intravenous injection of a vast amount of India ink, nearly the lethal dose, resulted in the complete blocking of the RE cells heavily laden with carbon

particles as far as the microscopic findings are concerned. In spite of this, the phagocytic activity of the R. E. S., as observed by carbon clearance test, was never lowered. The ability of serum-antibody formation which will be triggered by the uptake of antigen by macrophages was proved to be kept normal in spite of the severe blocking of R. E. S. These facts indicate that the adaptability of the R. E. S. to phagocytosis was astonishingly high and it can never be disturbed by the usual blocking method such as carbon particle injection. Sometimes the enhanced phagocytic function may be expected by the so-called blocking, while the haematopoiesis in bone marrow is severely affected resulting in severe anemia. It is clear that RE cells in bone marrow is closely related to haematopoiesis forming erythroblastic islet. Therefore, the retained phagocytic potency of R. E. S. does never assure the well-retained all other functions of R. E. S.

AWAI and SENO[30] reported that the function of R. E. S. concerning iron metabolism is affected independently of its function for phagocytosis. Concerning the anemia induced by blocking R. E. S., KOMIYA[17] and his associates are of the opinion that the anemia is induced by the temporary reservation of the red blood cells in the spleen and also by the disturbance in iron metabolism due to blocking of R. E. S.

RE cells contain hemosiderin, and it has been proved that iron is used for the hemoglobin synthesis through RE cells as revealed by using radioactive iron[30~33].

BESSIS's observations[19] revealed that reticulum cells found in the center of erythroblastic islet supply ferritin molecules directly to the neighboring erythroblasts. However, it is certain that such a pathway of iron transport from reticulum cells to erythroblast will not be so important for the differentiation of erythroid cells or their hemoglobin synthesis, because the anemia induced by blocking R. E. S. with India ink injection is always normochromic or hyperchromic. This fact indicates that in the R. E. S. blocking the erythroid cells are never deficient in iron necessary for hemoglobin synthesis.

In other words, the erythroid cells once orientated toward differentiation can take iron for the hemoglobin synthesis and ripen into red blood cells, quite independently of the injury of RE cells. The development of anemia in R. E. S. blocking is solely due to the arrested erythroid cell multiplication but not to the hemoglobin synthesis. This does not mean the mitotic arrest on the way of erythroid cell differentiation, as there is no sign of serere macrocytosis as in pernicious anemia[51]. The multiplication of the stem cell or its differentiation to erythroid cells should be arrested in R. E. S. blocking. There is no sign of accelerated decomposition of red cell as there is no severe hemosiderin deposition in the tissue.

The histologic picture of bone marrow shows scarcity of the erythropoietic

tissue but there is not any sign of the maturation arrest of erythroblast. Therefore, one of the most important functions of R. E. S. may be supposed to be the supplying of some informations to the stem cells for their multiplication and differentiation to the erythroid cells. These findings are consistent with fact[34,35] that the erythroblasts transferred into a certain culture medium, where the close connection between erythroblasts and RE cells must have been broken, can well differentiate to red blood cells *in vitro* but in any case the reproduction of pro-erythroblasts does never occur.

In the early stage of India black injection, the serum iron level was lowered markedly, probably because of the immobilization[30] of iron from R. E. S. in the normal demand of iron in bone marrow. In the end stage of experiment, the serum iron level[36] rose close to the original, probably because of the decreasing demand in the bone marrow with suppressed erythropoiesis.

Regarding white blood cells, granulocyte counts of the blood never decreased. This is consistent with the observation of KOMIYA[17]. However, the lymphocyte count gradually decreased, probably owing to the atrophy of the lymph follicles in spleen. In lymph nodes, the deposition of carbon particles is relatively slight; at least, histologically, no sign of the inhibition of lymphocyte production could be observed. This is also true in the case of retroperitoneal lymph nodes. The decrease of lymphocyte counts, however, suggests that RE cells play some role in the production or the differentiation of lymphocytes as in the case of erythroblast. It is known that lymphocytes[37–41] and plasma cells[42–47] are responsible for the production of antibody. RE cells take up antigenic substances, digest them, and probably give some information to lymphocytes and plasma cells for antibody formation, but the relationship between RE cells and lymphocytes or plasma cells has not yet been established. Recently, FISHMAN[48] reported that macrophages *in vitro* synthesized m-RNA like substance that gives

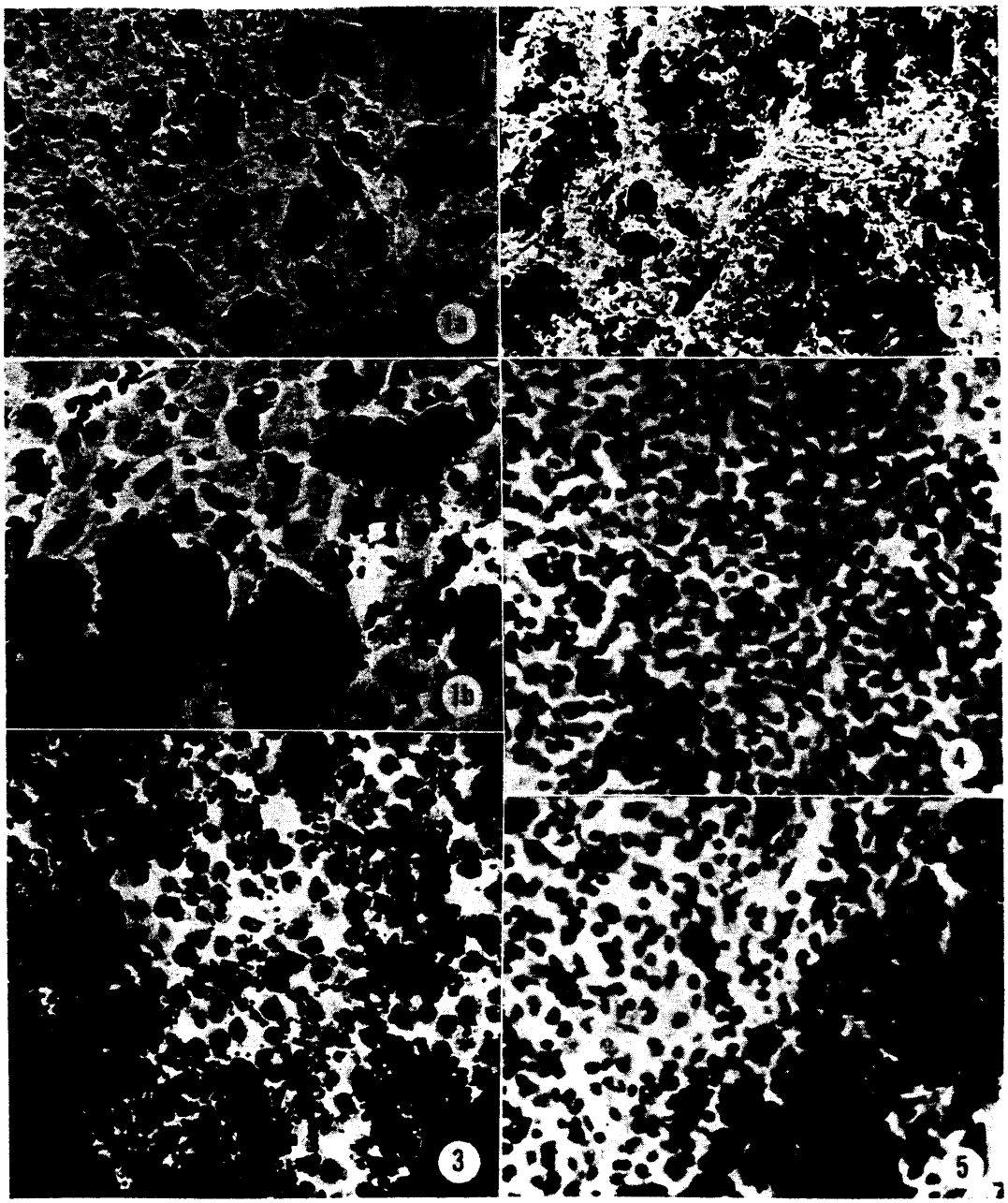
Photos. 1 a & b. Picture of the liver tissue of the rabbit received 50 intravenous india Ink showing the grouping of the swollen and proliferated Kupffer cells taking carbon particles. Hematoxylin-eosin staining, la; ×100, lb; ×400

Photo. 2 Picture of the spleen tissue of the same animal as in Photo. 1 showing a marked proliferation of reticulum cells taking carbon particles and the disappearance of lymph follicles. Hematoxylin-eosin staining, ×100

Photo 3 Picture of the bone marrow of the same animal as in Photo. 1. Erythroid cell proliferation is severely injured with the fairly well retained myeloid cell proliferation. Hematoxylin-eosin staining, ×400

Photo. 4 Picture of the popliteal lymph node of the same animal as in Photo. 1. Lymph node tissue is slightly deposited with India ink but retains nearly normal structure and lymphoid cell proliferation. Hematoxylin-eosin staining, ×400

Photo. 5 Picture of the periportal lymph node of the same animal as in Photo. 1 showing the blockade of sinusoidal reticulum cells. Hematoxylin-eosin staining, ×400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the information for the antibody formation to the cells producing antibody, but it seems not to be acceptable as the general concept for the mechanism of antibody formation. In spite of this, it is obvious that R. E. S. is somehow closely correlated to the antibody formation, as R. E. S. takes up antigen but the lymphocytes or the plasma cells do not, though the latter produce antibody. There are many reports[20, 21, 22, 49, 50] indicating that the R. E. S. blocking results in the decreased antibody formation. However, the intravenous injection of India ink in nearly lethal dose caused not any reduction in serum-antibody production.

This fact suggests that the ability of RE cells to give some information to the antibody producing cells can never be injured by taking up India ink particles. This may be related to the fact that phagocytic potency of R. E. S. has been little affected by the repeated, carbon particle injection, or that the RE cells in lymph nodes are little affected by india ink injection.

SUMMARY

For the purpose to reveal the role of R.E.S. for hemopoiesis and antibody formation, the R. E. S. of rabbits were severely blocked by the repeated intravenous injection of a vast amount of India ink, reaching 200 to 250 cc in total and the development of anemia and antibody formation by challenging egg albumin were observed while referring to the histologic changes in bone marrow, spleen and lymph nodes. The results were as follows:

1. The repeated intravenous injection of a vast amount of carbon particles induced a severe anemia. The anemia was always normo- or hyperchromic, showing not any disturbance in iron metabolism or hemoglobin formation. The data suggested that anemia is due to the arrest of reproduction of erythroblast or differentiation of the stem cells to erythroblasts, but not due to inhibition of the iron metabolism.

2. R. E. S. had no relation to the proliferation or the differentiation of granulocytes.

3. The functions of R. E. S. related to erythropoiesis and lymphopoiesis are affected by blocking independently of its phagocytic potency. In spite of a severe anemia, the phagocytic potency of R. E. S. could never be lowered and liver and spleen grew much larger in size and weight, showing that the phagocytic ability of R. E. S. is extremely resistant against such a blocking.

4. The serum antibody titer proved to be at the normal level, and no change of the antibody production in spite of heavy blocking of R. E. S. with India ink.

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