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INFLAMMATORY REACTIONS IN THE SKIN OF NORMAL AND IMMUNE CANARIES AND MONKEYS AFTER THE LOCAL INJECTION OF MALARIAL BLOOD

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The primary object of the present experiments was to demonstrate whether or not the macrophages of the skin, which are not normally involved in defense against the erythrocytic stages of the malarial parasite, respond in the same manner to these stages as do the macrophages of the spleen, liver and bone marrow, which are normally involved in defense against these stages. This study required the injection of blood into normal as well as into immune skin for comparative purposes. A secondary object was to ascertain the nature and speed of the inflammatory process in the skin of birds and monkeys. This study necessitated the introduction of 1% trypan blue, as well as of blood, into normal and immune animals in order to compare the inflammatory reactions in our animals with those in rats which have been studied in detail by W. and M. v. Möllendorff (1926) and Maximow (1929) and their students.

The closely spaced serial observations of Cannon and Taliaferro (1931) have demonstrated the important role of macrophages of the spleen, liver and bone marrow in the recovery from initial infection and the prevention of superinfection with *Plasmodium cathemerium* in the canary. In 1936 this work was extended to *P. brasilianum* in Central American monkeys by Taliaferro and Cannon, and in 1937 to *P. knowlesi* and *P. cynomolgi* in rhesus monkeys by Taliaferro and Mulligan.

The present paper deals with the inflammatory reactions in the skin and subcutaneous tissue under the following circumstances:

A. In normal and immune canaries after injecting red cells parasitized by *P. cathemerium* or trypan blue subcutaneously;

B. In normal and immune monkeys (*Cebus* and *Ateles*) after injecting red cells parasitized by *P. brasilianum*, normal red cells and/or trypan blue intracutaneously; and

C. In normal and immune monkeys (*Macaca mulatta*) after injecting red cells parasitized by *P. knowlesi* intracutaneously.

We have not found any reports on inflammatory reactions in the skin and subcutaneous tissue to malarial blood, although Huff and Coulston (1944) have reported on the introduction of sporozoites of *P. gallinaceum* into the skin of the chicken. They ascertained that sporozoites, although they entered neutrophils, only grew and segmented in cells of the lymphoid macrophage system, and that succeeding generations of exoerythrocytic stages developed in endothelial cells as well as in cells of the lymphoid macrophage system.

MATERIALS AND METHODS

The following host species were used: canaries, *Serinus canarius;* cebus monkeys, *Cebus capuci-*

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...; red spider monkey, *Ateles geoffroyi*; black ... monkeys, *A. fusciceps robustus* (=*A. ... ensis* of previous papers, see Kellogg and ...elman, 1944); and rhesus monkeys, *Macaca ...ulatta* (=*Silenus rhesus*).

The animals designated immune had acquired an immunity to superinfection which was associated with a low grade or subpatent infection with the homologous parasite. Canaries and Central American monkeys were immune to their respective malarias from one to several months after having undergone a typical acute infection, following the intravenous injection of parasitized blood. Rhesus monkeys were treated with quinine to control their otherwise fatal acute infections (2 of them were subsequently superinfected) until acquired immunity developed.

Normal animals, i.e., not injected with malaria until the time of the test, were similar in every ... to the immune animals as regards ... acclimatization to the laboratory, etc.

... red blood cells were drawn from ... ly infected with the proper plasmo... by cardiac puncture into a syringe contain... in physiological salt solution (1 ml ... ml blood) and were washed once in ... excess of 0.85% salt solution. They were then ... centrifuged at the lowest speed and for the short... time compatible with sedimenting the red ... blood cells. The supernatant fluid was discarded, ... a mixture of half 0.85% salt solution and ... red blood cells was used as the inoculum in ... *P. vivax*, *P. cathemerium* and *P. brasilianum* work. In ... *P. lophurae* work, the blood was washed ... times with physiological salt solution before being ... The inoculum was usually injected ... hour after being drawn from the donor ... animal, but sometimes several hours elapsed. During these intervals, the blood was kept in a refrigerator at about 6 C. Normal red blood cells were treated similarly. The same strain of parasite was used for the test inoculum as had been used to produce the initial infection.

A 1% solution of trypan blue in 0.85% salt solution was injected into some animals to produce control aseptic inflammatory lesions. To this solution India ink was occasionally added (2 drops of India ink to 1 ml of the trypan blue solution) to facilitate finding the site of the injection when the tissues were examined microscopically. ... the feet of canaries were injected, but the ... ... and abdomen and back of monkeys were ... In the latter animals, injections were never ... nearer together than 3 cm and were usually ... closer than 4 cm.

The site to be injected in the canary was simply wiped with 70% ... but the skin of the monkey had to be shaved and then wiped with 70% alcohol. The shaving was done carefully to avoid breaking the skin.

The injection was made with a 27 gauge needle into an area of the skin that contained no surface abrasions or other gross evidences of inflammation.

The inoculum was contained in 0.05 ml for the canary and in 0.1 ml for the monkey. It was injected subcutaneously in the canary and intracutaneously in the monkey. In the latter animal it caused a bleb of about 1 cm in diameter.

The point of the injection was marked by a ring of India ink—about 1 cm in diameter for the canary and about 3 cm in diameter for the monkey. This ring had to be examined every day in case the tissue was not excised. It was reinforced with more ink when it was ... effaced.

At appropriate intervals, the animals were ... cised and fixed as follows: In the canary ligatures were tied above and below the injection site and the whole thigh dropped into Zenker-formol. In the Helly-Maximow ... for 4 to 8 hours the monkey, a circle of skin, including the subcutaneous connective tissue about 3 to 4 cm in diameter was cut out, while the animal was anesthetized, with the point of the infection at the center of the circle. This skin was stretched to normal size on a cork frame, pinned in situ with thorns and immersed in the same fixative for the same length of time. After thorough washing and changes of 50% alcohol, 70% alcohol, 70% alcohol and 95% alcohol, it was removed from the cork frame. These methods precluded the formation of formalin precipitate which is easily confused with malarial pigment.

Tissues were embedded in nitrocellulose and sectioned serially, in a plane parallel with the surface of the skin, through the epidermis and as much of the dermis as contained inoculum. As the canary tissues were not satisfactorily embedded by ordinary paraffin or "double embedding" celloidin methods, they were kept in dioxan for 24 hours before being placed in absolute alcohol and alcohol-ether. They were then embedded by the routine method in nitrocellulose. Serial sections were stained with hematoxylin-eosin-azure II, which is the method of election for the study of inflammation and also of malarial parasites in sections. The technic used is described in Buchbinder and Loosli (1936).

THE ORIGIN AND FATE OF ... IN INFLAMMATION

Before describing the ... reaction in the skin ... br... ... ion ... connective tissue ... will be

given to avoid repetition in describing our experimental results. These findings agree with the findings of Maximow (1902 and 1927) and Marchand (1913) that cells in inflamed areas are derived from both local connective tissue (the histogenous cells) and circulating blood (the hematogenous cells). It may be noted that these authorities differed merely in their idea of the relative importance of the sources from which inflammatory exudate cells originated (see Bloom, 1938).

As is well-known, the inflammatory reaction begins with a dilation of the blood vessels in the irritated area which, in turn, is shortly followed by an exudation from the vessels into the connective tissue of fluid and of granular and nongranular leucocytes. One result of this migration of cells, together with the mobilization of tissue macrophages, is the local accumulation of enormous numbers of macrophages from several sources in an area usually sparsely supplied with them. The exudation of fluid permits specific and nonspecific substances to enter into a tissue from the blood. The 3 stages of the inflammatory reaction, involving neutralization of the noxious agent, removal of the injured or killed tissue, and repair of the damage, show degrees of completeness and efficacy which vary with the tissues involved, the nature of the injurious agent and the resistance of the host.

Histogenous cells

The cells found in normal connective tissue are chiefly fibroblasts, macrophages, adventitial cells (potential macrophages) in the wall of blood vessels, and endothelial cells lining the blood vessels, together with small numbers of connective tissue mast cells, eosinophils, lymphocytes and monocytes.

The macrophage (also known as histogenous macrophage, histiocyte, resting-wandering cell, clasmatocyte, rhagiocrine cell, phagocyte or tissue polyblast) of the local connective tissue is extremely active in inflammation. It begins to phagocytose foreign material soon after such material is introduced and continues to be active throughout inflammation. Although mitoses can be found among the local macrophages, they are few in number and are seldom found before about 3 days. Pre-existing macrophages cannot, therefore, account for the enormous increase of nongranular phagocytes which are seen after 24 hours in locally inflamed areas. Our studies have convinced us that at least 90%—probably more—of the macrophages involved in intense local inflammation arise by the transformation of emigrated, hematogenous, nongranular leucocytes rather than by the proliferation of local histogenous macrophages. This idea disposes of the dilemma so frequently encountered in the literature of inflammation, viz., that local macrophages are not often seen actively proliferating in spite of the fact that they are supposed to be the source from which the greatly increased number of macrophages arises.

The fibroblast (also known as fibrocyte) in mammals remains essentially unchanged in appearance for several days after the inflammatory stimulus is introduced in spite of the active processes going on around it. In later stages of inflammation, it proliferates by mitosis and assumes an active role. When dividing mitotically, it becomes basophilic and its nucleus has somewhat larger masses of chromatin and a distinct nucleolus. It is also augmented in number by the transformation of many macrophages into fibroblasts. The fibroblast apparently has a dual function. When the inflammatory stimulus cannot be satisfactorily removed, it walls off the

material, or, when extensive damage has been inflicted on the tissue, it is the predominant cell involved in scarring and in the repair of connective tissue. In addition, when the stimulus is intense enough, "activated fibroblasts" may develop into macrophages or possibly even into other cells of the connective tissue as happens in tissue culture according to some investigators (see review in Bloom, 1931). This frequently occurs in the bird, but not in the mammal. Thus, we (1938) have found that cells, occurring in the spleen of canaries during inflammation and repair and having the morphological characteristics of fibroblasts, apparently become true reticular cells, i.e., have full mesenchymal potencies of development into other cells of the blood and connective tissue.

The connective tissue mast cell is one of the first tissue elements to undergo observable cytological change during inflammation. Within the first hour, it shows such striking degenerative changes that its disintegration is one of the most delicate indicators of the presence of an inflammatory irritant. During reparative stages, it reappears and probably arises for the most part from adventitial cells. As its function in local inflammation is unknown, it will not be considered further.

The endothelial cell lining the smaller blood vessels becomes active in local inflammation only when new blood vessels are forming. During this process, new capillaries may sprout from preexisting vessels and, as part of this process, the endothelial cells proliferates by mitosis. In this work, we have never observed the slightest indication of an endothelial cell being phagocytic in situ or desquamating to form the so-called endothelial leucocyte. Our work corroborates in detail Maximow's contention that the developmental potencies of this cell appear limited to the formation of other endothelial cells or fibroblasts.

The adventitial cell (also known as pericyte or perivascular undifferentiated mesenchymal cell) occurs in the adventitia of the blood vessels. It is similar in morphology to the fibroblast, but, according to Marchand (1901 and 1924), Maximow (1902 and 1926) and our own work, has mesenchymal potencies, can readily become phagocytic and forms a part of the so-called macrophage or reticulo-endothelial system. It is included in the "Gefässwandzellen" of Herzog (1916) and Marchand (1924).

In addition to the foregoing characteristic local cells, cells similar to, if not identical with, the circulating lymphocyte, monocyte and eosinophil occur in small numbers in normal connective tissue. Of these, the eosinophil and, to a somewhat lesser extent, the lymphocyte and monocyte are generally believed to come from the blood stream. In any case, they behave as do the blood leucocytes and will be considered with them. Lymphocytes in the tissue are often called lymphoid wandering cells.

Hematogenous cells

One of the first histological evidences of inflammation is a marked local hyperemia vascular leucocytosis which mainly involves the heterophil granulocyte (neutrophil in the species of monkeys under consideration, pseudoeosinophil in the canary, special or polymorphonuclear leucocyte) and the agranulocytes (lymphocyte and monocyte). Within 30 minutes after the introduction of an inflammatory stimulus, these leucocytes can be seen migrating through the vessel walls.

Although practically all investigators agree that the heterophil is the first hematogenous cell to appear in the field of inflammation, it must be stressed that both agranular and granular leuco-

cytes begin to migrate from the blood vessels at the same time. Actually, however, heterophils move much faster. They, therefore, spread more rapidly over the entire field and leave the nongranular leucocytes closer to the vessels in the earlier stages of the process. Once in the field of inflammation, the heterophil actively phagocytoses* certain kinds of particulate material and probably aids in the extracellular digestion and disposal of both foreign agents and local degenerating tissue. With hardly a dissenting report, all investigators agree that this cell is definitive or an end-cell type which cannot proliferate by mitosis and has a short life, i.e., dies in situ after 1 to 4 days.

The eosinophil varies in its response to local inflammation, but in general probably appears as a result of some immune or sensitizing reaction (cf. Stumpf, 1933 and Campbell, 1942 and 1943). Thus, it is found late in the process in normal animals and early in the process in actively or passively immunized animals. The actual function of the eosinophil in local inflammation is unknown but may be associated with the absorption or detoxication of foreign proteins. In any case this cell has the same general limitations of development and proliferation as noted for the heterophil.

The lymphocyte, which migrates into the field of inflammation from the blood vessel, quickly begins to hypertrophy, as evidenced by cytoplasmic and nuclear changes, into a phagocytic cell indistinguishable from the local macrophage. The transformation of the lymphocyte into a macrophage constitutes one of the most disputed questions in local inflammation, but is clear if studied soon enough. As early as 30 hours in the rat and even earlier in the monkey and bird after the introduction of an inflammatory stimulus, however, some lymphocytes have hypertrophied to such an extent that they can no longer be distinguished from histogenous macrophages. During this development, many lymphocytes pass through a monocyte stage. In subacute and chronic inflammation, lymphocytes accumulate around small blood vessels. This phenomenon is often called perivascular, round cell infiltration in pathological literature.

The monocyte migrates from the blood and has exactly the same phagocytopoietic potencies as the lymphocyte.

Polyblast includes all mononuclear exudate cells as found in inflamed areas, such as lymphocytes, monocytes, all transitional stages from these cells to macrophages, plasma cells, all free macrophages, and giant and epithelioid cells. Hematogenous polyblast includes all mononuclear exudate cells derived from the blood, such as lymphocytes, monocytes and their developmental stages through macrophages. Monocytoid lymphocyte designates stages intermediate between a lymphocyte and monocyte.

P. CATHEMERIUM IN CANARIES

Experimental material

The strain of *P. cathemerium* originally isolated by Hartman in 1927 was used in the present work. This strain has been passed continuously by blood inoculation and, as far as is known, does not produce exoerythrocytic stages. The particular inoculum was pooled from 3 canaries. It was drawn during the acute rise of the malarial infection when 14% of the red cells were parasitized and 6 hours before segmentation, i.e., at a time when parasites were large.

Comparative studies were made of the local inflammatory reactions following the subcutaneous injection of 0.05 ml of the malarial blood, as just described, into one leg and of 1% trypan blue into the other leg of 22 normal and 22 immune canaries.

* The heterophil in the canary does not readily phagocytose the particulate materials used in the present work.

The immune birds had recovered from an initial attack of malaria but still retained a latent infection as shown by the work of W. H. and L. G. Taliaferro (1929). One normal and 1 immune bird were killed at the following intervals: 5, 10, 15, 30 and 45 minutes; 1, 1.5, 3, 4, 6, 8, 10, 12, 19, 24, 30 and 36 hours; and 2, 4, 7, 11 and 14 days. The skin at the 2 sites of injection on each bird was removed, fixed and sectioned as previously described.

Normal skin and connective tissue in canaries

The epidermis and derma of the skin from the leg of the birds were thin compared to those of mammals. Consequently, our study of inflammation was largely limited to the hypodermis and underlying muscle. The loose connective tissue of the hypodermis was mainly composed of fat cells with comparatively few macrophages and fibroblasts. It was also relatively avascular and was loosely attached to the underlying muscle.

There were frequent localized areas of chronic inflammation in the derma and hypodermis in untreated birds. These involved the accumulation of nongranular leucocytes and small polyblasts in the tissue or around blood vessels. Because of their size and location, we encountered no difficulty in distinguishing these accumulations of cells from early stages of the experimentally induced inflammation. In addition, macrophages usually accumulated at the base of the feathers. Since these cells entered into any inflammatory reaction near them and thereby obscured the normal process, we did not describe inflammatory changes in the neighborhood of the feather germs.

General course of inflammation in the connective tissue of canaries after the subcutaneous injection of erythrocytes infected with P. cathemerium *or of trypan blue*

Although the intensity and progress of inflammation differed somewhat in various birds and in different parts of the affected area in the same bird, the general course of events was strikingly uniform. We could discover no uniform difference, either quantitatively or qualitatively, in the exudation of cells, the development of cells or the phagocytosis of malarial blood or trypan blue in either immune birds or previously uninfected birds. The surprising finding in the subcutaneous inflammation and resolution following the injection of malarial blood or trypan blue was the remarkable rapidity of the process as compared to similar processes in rats, rabbits and guinea pigs following the injection of aseptic irritants, as described by Maximow (1927). In the following description, we shall compare the stages of inflammation in the bird skins with the description of similar stages in rat skins after the injection of trypan blue or India ink, as reported by Maximow (1929).

At 5 minutes after the injection of malarial blood or trypan blue, a slight amount of edema was found and a few hematogenous blood cells, mainly small lymphocytes and monocytes, were beginning to migrate through the vessel wall in one small area. The parasites in the inoculum were half-grown schizonts and presegmenters.

There was more edema at 10 minutes than at the previous stage, the blood vessels were dilated, and lymphocytes and heterophils were migrating through the small blood vessels in the treated area.

The muscle was somewhat damaged at 15 minutes, and edema and the migration of hematogenous cells were more extensive. The adventitial cells were quiescent.

In all of the sections taken at 30 minutes from both normal and immune birds injected with either parasitized

red blood cells or trypan blue, leucocytes had migrated to a marked extent through the blood vessels, particularly through the venules, but were still close to the vessels. There was no phagocytosis of erythrocytes and no change in the adventitial cells.

At 45 minutes, the edema had become more marked. The migrating nongranular leucocytes had slightly hypertrophied. A few heterophil leucocytes were also migrating. In general, the histogenous macrophages were inactive, but a few adventitial cells were swollen. Some fibroblasts were degenerating in a manner similar to that found in rats 1½ hours after injection.

Early inflammation at 1 hour involved the marked accumulation of leucocytes in the blood vessels and the migration of tremendous numbers of lymphocytes and smaller numbers of heterophils. There was no phagocytosis in the immune or normal bird injected with malarial blood or trypan blue, but the lymphocytes were turning into polyblasts of intermediate size near the blood vessels.

At 1½ hours, the speed with which the lymphocytes were hypertrophying was incredibly rapid and in a few areas resembled the picture at 24 hours in rats injected with trypan blue. Many heterophils were migrating and some were beginning to degenerate. The histogenous macrophages as a rule had foamy cytoplasm and were sometimes difficult to distinguish from fibroblasts.

Microscopic fields in the area of injection at 3 hours were filled with enormous numbers of hematogenous polyblasts and heterophils, some plasma cells and occasional hematogenous myelocytes. Cells were migrating at such a rapid rate that some of the small blood vessels were obscured. Plasma cells, which do not occur in early acute inflammation in mammals, had infiltrated into the connective tissue of degenerating muscles. Little phagocytosis was found. In the sections thus far studied, the old inflammation about the feather germs could easily be distinguished from the early inflammatory changes resulting from the injection of malarial blood or trypan blue.

At 4 hours, the migration of leucocytes from the vessels was intense, as may be seen in plate 1, figure 1. This microscopic field occurred near the site of the injection of malarial blood in the normal bird killed at 4 hours. Here, it was easy to differentiate the tissue macrophages from the relatively small hematogenous polyblasts, but it was difficult in other areas. Some heterophils were degenerating (pl. 1, fig. 1). At the site of the injection, an occasional macrophage had phagocytosed a parasitized red cell, but the heterophils did not seem to be phagocytic. The parasites were chiefly presegmenters. In the tissues injected with trypan blue, many polyblasts were suggestive of plasma cells. Otherwise, the picture was the same as in tissues injected with malarial blood.

At 6 hours, the parasites in erythrocytes were mainly presegmenters and segmenters, but there were also some small extracellular stages derived from ruptured mature segmenters. A few of these parasites, especially the small extracellular stages, had been phagocytosed by hematogenous polyblasts (pl. 1, fig. 2). Many heterophils were present (pl. 1, fig. 2). Some were degenerating, but none seemed to be phagocytic. The inflammation had reached the same intensity in both the normal and immune bird after injection with either malarial blood or trypan blue.

Many hematogenous polyblasts were indistinguishable from tissue macrophages at 8 hours. Heterophils had increased in number, and an occasional adventitial cell was becoming basophil.

At 10 hours, some undoubted hematogenous polyblasts, as well as local macrophages, contained trypan blue or parasitized and unparasitized red blood cells. Many heterophils were disintegrating.

The migration of cells probably reached its climax at 12 hours. In some areas the heterophils were arranged in epithelial-like layers, but only an occasional one had phagocytosed a pigment granule. Many parasitized red cells, uninfected red cells and much trypan blue had been phagocytosed by macrophages.

Inflammation was intense at 19 hours. Many free parasites, parasitized red cells and unparasitized red cells were phagocytosed. Lymphocytes were still migrating from the vessels, and all stages of intermediate polyblasts from lymphocyte to macrophage were numerous. Some were even as large as macrophages of the spleen. Heterophils were numerous in the normal bird, but not in the immune bird after the injection of either parasitized blood or trypan blue. The reaction at this time in many places resembled that found in the rat 48 hours after the injection of foreign material. Less phagocytosis in the normal than in the immune bird occurred, but this finding was exceptional for the series as a whole. Many red cells contained single merozoites.

At 24 hours, inflammation continued to be intense and involved migrating lymphocytes, the development of macrophages from lymphocytes and the phagocytosis of parasites, parasitized red cells, normal red blood cells and trypan blue by the larger polyblasts (pl. 2). The nuclear and cytoplasmic changes of lymphocytes developing into macrophages are indicated by the cells labelled 1 through 5 in this plate. A few epithelioid macrophages with 2 or 3 nuclei were also found (pl. 2). These multinucleated macrophages probably represented the first stages in the formation of giant cell syncytia which were frequently found at this time. Only a few, if any, fibroblasts were transforming into macrophages, because many unchanged fibroblasts could be found throughout the inflamed area. As compared to the fibroblast, the nucleus of the macrophage was smaller and had a smaller nucleolus, a heavier membrane and more distinct chromatin masses, the granules of which were more regularly arranged (pl. 2). As neither macrophages nor fibroblasts were seen dividing, it is obvious that the increase in number of macrophages was mainly accounted for by the transformation of hematogenous agranulocytes into macrophages. In addition, adventitial cells were mobilizing into macrophages, as seen by their swelling and more basophil, well-defined cytoplasm (pl. 3, fig. 1; also cf. mobilizing with unmobilized adventitial cells in pl. 2). In some areas, many macrophages of hematogenous origin contained parasites and/or red cells in all stages of disintegration (pl. 3, fig. 2).

Heterophils and lymphocytes continued to migrate from adjacent blood vessels at 30 hours. Giant cells sometimes contained pigment and red cells. In the normal animal, areas were seen in which the inoculum was surrounded by masses of large macrophages.

At 36 hours, a few nongranular leucocytes continued to migrate from the blood vessels, and red cells, parasitized red cells and trypan blue were still being phagocytosed in the injected areas. The transformation of macrophages into fibroblasts, however, was beginning to dominate the picture in a manner corresponding to that seen in the rat 6 days after the injection of foreign material. In some areas, giant cell syncytia were prominent and some were phagocytic (pl. 4, fig. 1). Heterophils,

eosinophils and plasma cells were scarce. A dividing endothelial cell was seen.

The picture at 48 hours resembled that in the rat 18 days after the injection of trypan blue. The increase in cells in the tissue was due mainly to an increase in macrophages and transitional stages of macrophages into fibroblasts (pl. 4, fig. 2). Macrophages sometimes contained inclusions, but no free parasites or parasitized red cells were seen. A few lymphocytes and some degenerating heterophils were present. Around some blood vessels, perivascular accumulations of lymphocytes could still be found and an occasional adventitial cell appeared to be transforming into a large lymphoid cell.

At 4 days, macrophages occasionally contained large masses of yellowish green pigment. This material was probably hemosiderin, but some of it may have been malarial pigment. There were no phagocytosed parasitized red cells, free parasites or other evidences of the inoculum except parasitic debris in an occasional macrophage. Heterophils were rare.

Mosaics of large polyblasts occurred at 7 days, and a few of them were filled with greenish pigment. No inoculum as such was left, but here and there a macrophage contained malarial pigment and a rare parasite. In some areas, macrophages continued to transform into fibroblasts and there was some perivascular round cell infiltration adjacent to the injected area.

The connective tissue consisted mainly of fibroblasts and a few small polyblasts at 11 days. Four small areas of dense lymphocytic accumulations suggested lymphoid nodules.

The predominance of fibroblasts suggested early scarring at 14 days. Some unchanged lymphocytes were present. One mass of lymphocytes was organized like a lymphatic nodule. A few macrophages contained malarial pigment, but no heterophils were found.

P. BRASILIANUM IN CEBUS AND SPIDER MONKEYS

*Experimental material**

One strain of *P. brasilianum* was used for all injections of malarial blood in the present work. This strain has been extensively studied by W. H. and L. G. Taliaferro (1944; see monkeys 346A–378A) and consisted of 1 major and 2 insignificant minor broods of parasites, each with a quartan periodicity. In a given inoculum, therefore, the majority of the parasites were in the same stage of the asexual cycle and were usually intermediate or large trophozoites. The blood was drawn during the acute rise of infections at a time when 1 to 5% of the cells were parasitized.

The blood cells injected intracutaneously into the cebus and spider monkeys were always from a donor of the same species except that the blood cells for 13 of the injections into the red spider 297A were from a black spider monkey.

Local inflammatory reactions were studied following the intracutaneous injection of 0.1 ml of parasitized and/or normal blood into various parts of the skin of 3 immune and 9 normal cebus monkeys, 5 immune and 4 normal spider monkeys, and into 1 spider monkey during the acute rise of the infection. In addition, intracutaneous injections of trypan blue were made into 1 normal cebus monkey and into 1 immune spider monkey.

The data in Table 1 show the first and last interval at which each monkey was injected with a given material and the total number of injections, i.e., pieces of skin removed. In addition to a normal piece of skin from each monkey, pieces of skin were taken at all or most of the following intervals—15 and 45 minutes, 1.5, 3, 6, 12 and 18 hours, and 1, 2, 4 and 7 days from at least 2 normal and 2 immune cebus and 2 normal and 2 immune spider monkeys injected with malarial blood and with normal blood; and at the following intervals—15 and 45 minutes, 1.5, 3, 6 and 12 hours, and 1, 2 and 4 days from 1 normal cebus and 1 immune spider monkey injected with trypan blue. Pieces of skin were also taken from various monkeys at additional intervals, such as 30 minutes, 1 hour and 3, 9, 10, 14, 15, 18, 19 and 29 days. After the removal of the pieces of skin, they were fixed and sectioned as previously described.

* The authors are indebted to Dr. H. C. Clark of the Gorgas Memorial Laboratory for the facilities of the laboratory and the cebus and spider monkeys.

Since few monkeys were available, as compared to canaries, the standard procedure involved making 6 to 16 injections into 1 monkey at intervals and removing the tissues at one time. Thus, immune cebus monkey 346A was injected with malarial blood 7, 4, 2 and 1 days, 18, 12, 6, 3 and 1.5 hours, and 45 and 15 minutes, and with normal red blood cells 7 and 1 days and 18, 12, 6 and 1.5 hours before killing the animal and removing the pieces. In the process, this animal received 4 injections on the left as well as 4 injections on the right side of its abdomen, 2 injections on the left leg and 2 injections on the right leg, and 3 injections on the left side and 2 injections on the right side of its back. The pieces were removed in order beginning with the 15 minute piece, and a normal piece of skin was removed under the monkey's left arm.

To control the possibility that successive injections, as made in 14 of the monkeys, might produce a general leucocytosis or in other ways activate the animals—so that the inflammatory processes proceeded faster in the last sites injected, which would be the ones removed at the shortest intervals after injections, 7 monkeys were given only 1 or 2 injections and 2 were given only 3 or 4 injections.

The 237 pieces of skin removed from the various monkeys allowed us to study the inflammatory and reparative processes in immune cebus and spider monkeys beginning at 15 minutes after intracutaneous injections of malarial red cells, as collated with normal red cells and trypan blue, and to compare the foregoing processes with those in normal cebus and spider monkeys after similar injections. Thus, the cutaneous reactions in normal and immune monkeys were studied after injections of normal blood, as well as of malarial blood and trypan blue as studied in the canary.

TABLE 1.—*Details concerning the intracutaneous injection of various materials into immune and normal cebus and spider monkeys.*

Monkey's number	Injection of: Malarial blood — No. of injections	Malarial blood — Times of injections inclusive	Normal blood — No. of injections	Normal blood — Times of injections inclusive	Trypan blue — No. of injections	Trypan blue — Times of injections inclusive	Uninjected (Normal control) skin
			Cebus monkeys: immune				
345A	1	9 days					1
346A	11	15′— 7 days	6	1½ hr.—7 days			1
350A	11	15′— 7 days	6	1½ hr.—7 days			1
			Cebus monkeys: normal				
290A	12	15′— 4 days					1
293A	6	45′— 1 day					1
310A	13	15′—14 days	9	15′— 4 days			1
362A					16	15′—14 days	1
374A			15	15′—18 days			1
375A*			15	15′—14 days			1
188B			1	12 hr.			1
192B			1	45′			1
193B			1	3 hr.			1
196B			1	6 hr.			1
			Spider monkeys: acute rise of the infection				
357A	4	8 days—22 days					1
			Spider monkeys: immune				
297A	14	15′—19 days					1
339A					8	1½ hr.—4 days	1
342A**	10	15′— 7 days					1
342A	13	15′—29 days					1
441A			2	15′—6 hr.			1
194B			2	45′—3 hr.			1
			Spider monkeys: normal				
339A***	7	30′—14 days	7	30′—14 days			1
343A	8	1 hr.—10 days					1
357A***	10	15′— 7 days					1
359A	3	4 days—15 days					1

* Monkey 375A was tested with its own erythrocytes.
** Monkey 342A was tested at a later date; see next line.
*** Monkeys 339A and 357A were tested at a later date; see above.

Normal skin and connective tissue in cebus and spider monkeys

Except for its greater thickness, the skin of the spider monkeys did not appreciably differ histologically from that

of the cebus monkeys. In most animals, the histological picture was that of typical normal, uninflamed skin. The connective tissue contained pigment cells, which had exceedingly long processes filled with either green or brown pigment, in addition to macrophages, fibroblasts, adventitial cells and mast cells. The normal presence of the pigment cells had to be kept in mind in connection with the occurrence of malarial pigment in the macrophages in inflammation following the injection of malarial blood. Within blood vessels of the connective tissue, there were typical small and medium (the so-called large lymphocytes of the blood) lymphocytes, a few monocytoid lymphocytes and monocytes and granular leucocytes. The connective tissue itself contained practically no heterophils or eosinophils and extremely few lymphoid wandering cells. One exception to this was a rather frequent, low grade, chronic inflammation about the hair follicles as indicated by varying numbers of lymphoid cells around blood vessels or scattered through a limited portion of the tissue. In all of the present work, therefore, care was taken not to judge the extent of inflammation in the neighborhood of hair follicles. This chronic inflammation may have come from lice and/or from the incessant scratching indulged in by most monkeys.

Skin and connective tissue in immune cebus and spider monkeys

Skin and connective tissue from immune monkeys before injection of the various inoculums appeared similar to normal ones except that many of the circulating nongranular leucocytes possessed an appreciably larger amount of cytoplasm and frequently a looser chromatin structure in their nuclei. This development is similar to that found by Taliaferro and Klüver (1940). When such cells migrated from the blood vessels, they would already have developed somewhat toward the macrophage condition and, hence, would be expected to accelerate the process of inflammation to a certain extent. Study and comparison of immune with normal tissues, however, indicated that the acceleration was noticeable, if at all, only during the first few hours of inflammation.

An occasional parasitized red cell and a fairly frequent nongranular leucocyte containing pigment were also found in the circulating blood of the skin of immune animals.

General course of inflammation in the skin and connective tissue of cebus and spider monkeys after the intracutaneous injection of various materials including blood infected with P. brasilianum

The first question which had to be answered before the inflammatory responses in the monkeys could be properly evaluated was whether the multiple intracutaneous injections of some of the animals accelerated the process of inflammation. A comparison of serially injected monkeys with those that only received 1 to 4 injections indicated that repeated injections did accelerate the inflammatory process during the first few hours, but after 6 hours produced no noticeable effect. In the following account, therefore, it is likely that some of the initial hypertrophy of agranulocytes was abnormally fast during the first few hours, but the unexpected speed of the reactions as compared to trypan blue in the rat still holds because it was evidenced throughout the process.

As would be expected in examining such a mass of sections taken at such closely spaced intervals, the process of inflammation varied somewhat in comparable animals. This variation was un-

doubtedly due largely to differences in the amount of inoculum in a particular area, difficulties in sectioning every injected area through exactly comparable sites and movement of the inoculum into fresh sites. These discrepancies were counterbalanced by studying adjacent areas as well as the area injected. When the inoculum consisted of malarial blood, however, there were often slightly higher percentages of lymphocytes and especially of monocytes (cf. Taliaferro and Klüver, 1940) than when the inoculum consisted of normal blood. Such agranulocytes, being at the site of inflammation, could develop into polyblasts and expedite the process of inflammation—at least during the first few hours of inflammation. Since the amount of inoculum was only 0.1 ml, however, agranulocytes migrating from the blood vessels soon outnumbered and overshadowed the activity of the agranulocytes in the inoculum. After correcting for the foregoing discrepancies, inflammation still varied somewhat in extent, but, on the whole, exhibited remarkably consistent changes.

We found no difference in the rate or character of the inflammation which could be correlated with (1) the species of monkey or (2) the normal and immune monkey except as just noted. This similarity, particularly in the reaction in the skin of normal and immune animals to parasitized erythrocytes, indicates that the skin is not specifically immunized to the parasite. The general picture of inflammation found in these monkeys essentially followed the descriptions of inflammation in the skin after treatment with trypan blue, as given by Maximow (1927 and 1929).

As early as 15 minutes after injection, edema was noticed in the skin and varied from mild to marked. It usually increased in amount for several hours and persisted in varying amounts for 2 days. Many of the connective tissue mast cells had fewer granules and were slightly degenerated as early as 45 minutes after injection. They continued to degenerate during the first 18 hours and reappeared in about 9 days, i.e., when the healing process was well advanced.

At 15 minutes, the smaller blood vessels of the injected area, especially at the periphery of the inoculum, exhibited an intravascular leucocytosis involving an increase in number of all types of leucocytes, especially of heterophils, with a few leucocytes migrating through the walls of the capillaries and particularly the small veins. The migration of leucocytes and extravascular leucocytes increased rapidly within the next few hours (pl. 5, fig. 1). In fact, it was possible to trace the migration of the leucocytes away from the blood vessels into the inflamed tissue. Although both heterophils and nongranular leucocytes migrated from the blood vessels, heterophils migrated in such greater numbers that they were the predominant exudate cell in the field of inflammation during the first day, but were increasingly outnumbered by the nongranular leucocytes on the second day and thereafter. Later, the mononuclear exudate cells predominated—not as much because of their continued migration or local proliferation as because they were developing into macrophages while the heterophils were disintegrating and disappearing.

Heterophils with more (7 to 9) than the normal number of nuclear lobes and in various stages of degeneration appeared in noticeable numbers at 18 hours and reached striking proportions in some areas by 24 hours. These cells in whole or in part were phagocytosed by the macrophages and hematogenous polyblasts (pl. 6, fig. 1). After 3 days, they were not found in large numbers except when the inoculum moved into

new areas or when other conditions, such as superficial skin injuries, possibly from scratching, superimposed early acute inflammatory changes on later healing phases. Thus, the activity and degeneration of heterophils in the monkey occurred at about the same time intervals as in the rat.

The few monocytes and lymphocytes which were extravascular at 15 and 30 minutes were of approximately the same size as the cells within the blood vessels of a given monkey. Apparent increases in size of individual cells at this time were due to a flattening of the cells while migrating through the dense collagenous fibers. This conclusion was reached in consideration of the fact that the ratio of nucleus to cytoplasm of the cells was approximately unchanged and the heterophils, which do not appreciably increase in size, appeared enlarged to the same extent. The lymphocytes and monocytes were first noticeably larger in size 45 minutes after injection. At this time, the cytoplasm of some of the lymphocytes equalled the volume of the nucleus and many nuclei were intermediate between those of the typical lymphocyte or monocyte and macrophage.

The development of lymphocytes and monocytes into macrophages proceeded according to the description given by Maximow for rats and rabbits, but with greater rapidity in both normal and immune monkeys. For example, the changes in some areas in the monkey in 6 hours were comparable to those in the rat at 30 hours. In many areas at 12 hours and in most areas at 18 hours, there were all gradations between nongranular leucocytes, which in no way differed from the cells noticed 15 and 30 minutes after injection, to large cells, which in size and structure could not be distinguished from rounded up mobilized histogenous macrophages. This condition continued for the next 12 hours. Accordingly, [illegible] 1 day, all stages of intermediate [illegible]lyblasts from lymphocytes or [illegible]cytes to macrophages could be found (pl. 6, fig. 1 and 2). Throughout the earlier stages of inflammation when histogenous macrophages could easily be differentiated from hypertrophying agranulocytes, large hematogenous polyblasts were more actively phagocytic than the histogenous macrophages.

Local macrophages took comparatively little part in the early inflammatory process although they occasionally contained parasitized cells at 45 minutes. At 3 and especially at 6 hours, an occasional one could be seen in the process of mobilization, i.e., with prominent cytoplasm and a few inclusions. From 6 hours on, they were increasingly active phagocytically and were increasingly difficult to distinguish from the large hypertrophied hematogenous mononuclear exudate cells. Some adventitial cells were mobilizing at 24 hours and thereafter.

Macrophages, derived in small part from local macrophages and adventitial cells and in large part from hematogenous agranulocytes, increased in number and in size until at 4 days large areas in the field of inflammation appeared to be composed wholly of macrophages. These cells frequently assumed an epithelial arrangement.

It is interesting that the increase in number and activity of the macrophages should be associated primarily with a migration and mobilization of cells and not with division of cells either in situ or after migration. Few dividing cells were found in the skin in spite of intense cellular activities, and those that were found were limited to the later stages of repair.

In the normal uninflamed derma and subcutaneous fat tissue, fibroblasts

were easily differentiated from macrophages. This distinctness of structure continued during the first 4 days of inflammation. The fibroblasts neither took part in the early stages of inflammation nor were transformed into large macrophages, smaller polyblasts or granular leucocytes, but a few proliferated after the second day. No evidence of amitotic division was ever found. About the fourth day after injection, transitional stages between macrophages and fibroblasts appeared. These forms we interpret as a transformation of macrophages into fibroblasts as their occurrence was associated with a diminution of macrophages and an increase of fibroblasts. During later stages of repair, fibroblasts greatly increased in number. This increase could be accounted for by the continued transformation of macrophages (themselves derived from both local macrophages and adventitial cells, and hematogenous nongranular leucocytes) into fibroblasts (pl. 6, fig. 3).

Free parasites and parasitized erythrocytes were phagocytosed before uninfected red cells when the inoculum consisted of parasitized blood. Trypan blue seemed to be more irritating than red blood cells.

In both normal and immune monkeys, heterophils containing malarial pigment, free parasites and trypan blue were found in 45 minutes. The phagocytic activity by the heterophils of both free pigment and free parasites (occasionally of parasitized red cells) increased until it reached a peak at about 3 hours (pl. 5, fig. 3–9), continued to be intense through 18 hours and subsided as the heterophils degenerated.

Large hematogenous polyblasts and macrophages also phagocytosed parasites and parasitized erythrocytes as well as unparasitized red cells, tissue debris and trypan blue during this time and were usually more active than the heterophils after 6 hours (pl. 5, fig. 2). They continued to be actively phagocytic until all malarial material and tissue debris were cleared up, i.e., for 2 days or longer. In general, the size of the phagocytic cell could be correlated with the size and amount of material phagocytosed, although as mentioned previously, large hematogeneous polyblasts were more active than histogenous macrophages during early stages of inflammation. Large polyblasts were found containing as many as 24 to 30 parasitized red cells or as many as 12 parasitized red cells, 6 uninfected red cells, a heterophil and miscellaneous tissue debris.

After the inoculum had been cleared up, malarial pigment persisted in macrophages for a long time.

Beginning with the earliest biopsies at 15 minutes and continuing for several days, i.e., throughout the interval when the inoculum was present, many distended lymphatics were found in, at the periphery of, and near the inoculum. These were undoubtedly draining the area and frequently contained red blood cells and leucocytes. In neither the normal nor immune animals, however, was there any histological evidence of lymphatic blockage or that such blockage was functional in the localization of the inoculum. In a few lymphatics, small fibrin clots occurred and sometimes contained blood cells, but they never extended over a major portion of the lumen.

After the first week, the final stages of repair varied according to the extent of superimposed inflammation. In the uncomplicated picture when all the inoculum and tissue debris were phagocytosed and removed by the 4th to 6th day, the connective tissue generally contained increased numbers of both fibroblasts and macrophages and many intermediate stages between the two (pl. 6, fig. 3).

By the 9th day in uncomplicated areas, mast cells reappeared—generally in greater numbers than is characteristic of normal skin.

On the other hand, in areas in which the inoculum persisted or which were irritated by scratching, subacute inflammation continued and was superimposed on the healing process. In such areas, nongranular leucocytes, small polyblasts and plasma cells occurred simultaneously with macrophages, fibroblasts and stages intermediate between macrophages and fibroblasts. When the inoculum moved into new areas, acute inflammation was found. This condition differed, in general, from inflammation of longer standing by the presence of heterophils and the absence of plasma cells. As would be expected, the phagocytosis of tissue debris was pronounced in both of the superimposed processes.

P. KNOWLESI IN RHESUS MONKEYS

*Experimental material**

The strain of *P. knowlesi* is the one used by Coggeshall and Kumm (1937).

The malarial blood was obtained from 2 monkeys during the acute rise of their infection when about 30% of the red cells were infected with large trophozoites.

One immune, 1 normal and 2 hyperimmune monkeys were used.

The immune and normal monkeys were injected intracutaneously at 5 sites at the same time with 0.1 ml of the same freshly washed concentrated erythrocytes infected with *P. knowlesi*, and 1 site was removed from each monkey at the following intervals after treatment: 1, 3, 5, 24 and 51 hours. In addition, a normal piece of skin and connective tissue was taken from each monkey.

The 2 hyperimmune monkeys had been infected with *P. knowlesi* for 2 years and had been superinfected several times during that interval. Their serum was known to contain a relatively high concentration of antibodies. They were injected with malarial blood 1, 2, 3, 6 and 24 hours, respectively, before the simultaneous removal of the injected areas and an uninjected area. The material was stored on ice between injections. The possible injury to the parasites attendant on such a procedure was controlled by the different technic described for the monkeys in the preceding paragraph.

Normal and immune skin and connective tissue in rhesus monkeys

The histological appearance of the normal and immune skin and connective tissue in rhesus monkeys was in all essential respects similar to that in cebus and spider monkeys.

General course of inflammation in the skin and connective tissue of rhesus monkeys after the intracutaneous injection of blood infected with P. knowlesi

Since the reactions in the hyperimmune monkeys were essentially similar to those in the immune monkey, the reactions of the immune and hyperimmune monkeys will be considered collectively under the designation of immune monkeys.

In marked contrast to the previously described reactions in the skin of canaries and Central American monkeys injected with malarial blood, the subcutaneous reactions in immune rhesus monkeys differed significantly from those in the normal animal after the injection of red cells parasitized by *P. knowlesi*. These differences were probably all associated with a relatively high antibody titer and consisted in part of direct antiparasitic effects, such as agglutination and increased phagocytosis of the parasites, and indirect effects, such as heightened inflammation resulting probably from the toxicity of the antigen-antibody reaction.

In the tissues removed at 1 hour, large parasites were agglutinated in the skin of immune monkeys (pl. 7, fig. 1), but not in the normal monkey (pl. 7,

* The authors are indebted to Dr. L. T. Coggeshall, then at the Laboratories of the International Health Division at the Rockefeller Institute for Medical Research, for the material from the rhesus monkeys.

LEGENDS OF PLATES

PLATE 1.—Early inflammatory changes following the introduction of *P. cathemerium* blood in the subcutaneous tissue of canaries. In other areas much more pronounced hypertrophy of agranulocytes occurred. Both figures ×1200.

1. Blood vessel and surrounding tissue in a normal canary 4 hours after the subcutaneous injection of 0.05 ml malarial blood.

Many heterophils and agranulocytes (lymphocytes and monocytes) have accumulated within the blood vessel and are migrating through the vessel wall and surrounding tissue.

2. Subcutaneous tissue in an immune canary, 6 hours after the subcutaneous injection of 0.05 ml malarial blood.

Many heterophils, agranulocytes and hypertrophying agranulocytes (hematogenous polyblasts) are present among the normal tissue constituents (macrophages, fibroblasts and fat cells). Many of the heterophils, hematogenous polyblasts and macrophages are phagocytic.

PLATE 2.—Inflammatory changes in a nearby blood vessel and the surrounding subcutaneous tissue in an immune canary 24 hours after the subcutaneous injection of 0.05 ml blood parasitized with *P. cathemerium*. ×1200.

Many of the accumulated agranulocytes have hypertrophied. Also some adventitial cells are mobilizing into macrophages and some macrophages have formed early giant cells. Cells 1 through 6 are intermediate polyblasts between lymphocytes and macrophages as indicated by nuclear and cytoplasmic changes. Note in this plate the progress of the changes begun in pl. 1. Other areas in the same inflamed area are shown in pl. 3.

PLATE 3.—Inflammatory changes following the introduction of *P. cathemerium* blood in the subcutaneous tissue of the same immune canary as in plate 2 and at the same time. Both figures ×1200.

1. Blood vessel and subcutaneous tissue at the site of the injection of 0.05 ml malarial blood 24 hours earlier.

The same phenomena occur as in plate 2. In addition, several macrophages are actively phagocytic. Many adventitial cells are mobilizing.

2. Subcutaneous tissue at the site of the injection of 0.05 ml malarial blood 24 hours earlier.

Many parasites and parasitized red cells are unphagocytosed as well as in macrophages. The giant macrophages cannot be distinguished from the macrophages derived from hematogenous agranulocytes.

PLATE 4.—Inflammatory changes following the introduction of *P. cathemerium* blood in the subcutaneous tissue of immune canaries. Both figures ×1200.

1. Subcutaneous tissue at the site of the injection of 0.05 ml malarial blood 36 hours earlier.

Within the injected area, most of the parasites and parasitized erythrocytes have been phagocytosed by macrophages and some macrophages have coalesced to form giant cells. A few small hematogenous polyblasts occur which are not yet phagocytic.

2. Subcutaneous tissue near the site of the injection of 0.05 ml malarial blood 48 hours earlier.

Macrophages are changing into fibroblasts as indicated by the cells with the arrow.

PLATE 5.—Early inflammatory changes following the introduction of *P. brasilianum* blood in the subcutaneous tissue of a normal and of an immune cebus monkey. Fig. 1 and 2 ×1100 and fig. 3 through 10 ×1730.

1. Subcutaneous tissue in a normal cebus monkey at the site of the injection of 0.1 ml malarial blood 45 minutes earlier.

Heterophils and agranulocytes have accumulated in the inoculum and a few of the agranulocytes have hypertrophied to a slight extent. Unphagocytosed parasitized erythrocytes are numerous and a few heterophils, hematogenous polyblasts and tissue macrophages contain malarial pigment.

2. Subcutaneous tissue in an immune cebus monkey at the site of the injection of 0.1 ml malarial blood 6 hours earlier.

The same phenomena occur as in figure 1, but there are fewer unphagocytosed parasitized erythrocytes, the agranulocytes have hypertrophied more and the heterophils and small hematogenous polyblasts (no macrophage in the field) contain more malarial pigment. In other areas, the agranulocytes had hypertrophied to a greater extent.

3–9. Phagocytic heterophils in an immune cebus monkey at the site of the intracutaneous injection of 0.1 ml malarial blood 3 hours earlier.

The heterophils contain malarial pigment and a few contain malarial parasites.

10. Phagocytic hematogenous polyblast in an immune cebus monkey at the site of the intracutaneous injection of 0.1 ml malarial blood 18 hours earlier.

The polyblast contains 2 malarial parasites.

PLATE 6.—Inflammatory changes following the introduction of *P. brasilianum* blood in the subcutaneous tissue of an immune spider and immune cebus monkey. All figures ×1100.

1 and 2. Subcutaneous tissue in an immune spider monkey at the site of the injection of 0.1 ml malarial blood 1 day earlier.

The agranulocytes have hypertrophied to small and intermediate polyblasts and some of the hematogenous polyblasts and a tissue macrophage contain malarial material. One polyblast contains a degenerated heterophil. The tissue macrophages cannot be distinguished from the macrophages derived from hematogenous agranulocytes.

3. Subcutaneous tissue in an immune cebus monkey at the site of the injection of 0.1 ml malarial blood 6 days earlier.

Many macrophages contain malarial pigment and some are transforming into fibroblasts as indicated by the cells with the arrow.

PLATE 7.—Early antibody effects following the introduction of *P. knowlesi* blood in the subcutaneous tissue of an immune rhesus monkey. Figures 1 and 2 photomicrographs ×900 and Figures 3 and 4 drawings ×1100.

1. Subcutaneous tissue in an immune rhesus monkey at the site of the injection of 0.1 ml malarial blood 1 hour earlier.

The parasitized erythrocytes are agglutinated into clumps to such an extent that adjacent areas are composed mainly of unparasitized erythrocytes.

2. Subcutaneous tissue in a normal rhesus monkey at the site of the injection of 0.1 ml malarial blood 1 hour earlier.

The parasitized erythrocytes are more or less evenly distributed over the entire field.

3. Higher magnification of the same phenomenon in an immune monkey as shown in figure 1.

The large parasites are agglutinated into several clumps. Also, many of them do not seem to be in erythrocytes, and many erythrocytes containing parasites seem hemolyzed.

4. Higher magnification of malarial blood in a normal monkey as shown in figure 2.

The parasitized erythrocytes are evenly distributed and normal in appearance.

PLATE I

(Legends appear on pp. 124 and 125.)

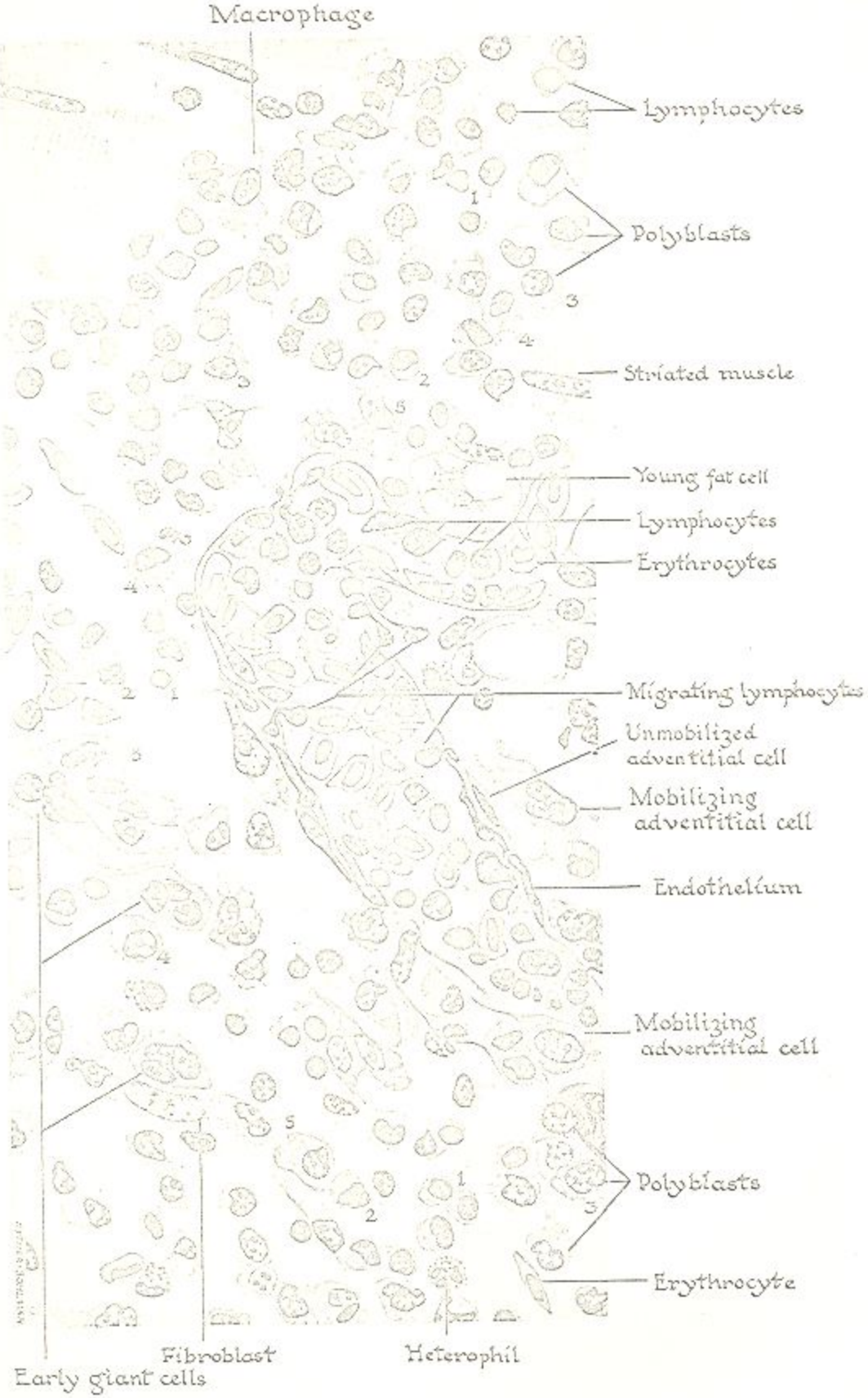

PLATE 2

(Legends appear on pp. 124 and 125.)

PLATE 3

(Legends appear on pp. 124 and 125.)

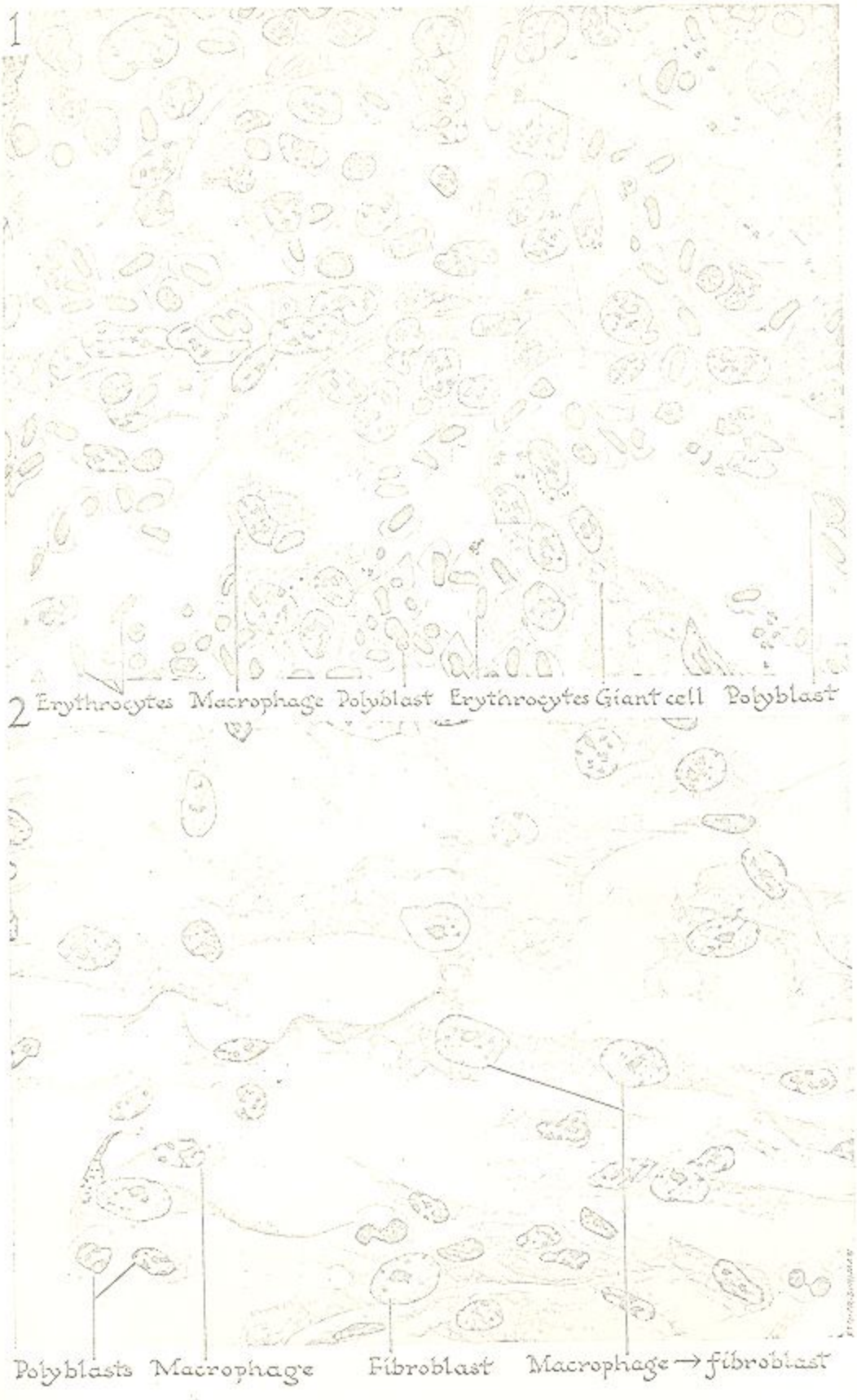

PLATE 4

(Legends appear on pp. 124 and 125.)

PLATE 5

(Legends appear on pp. 124 and 125.)

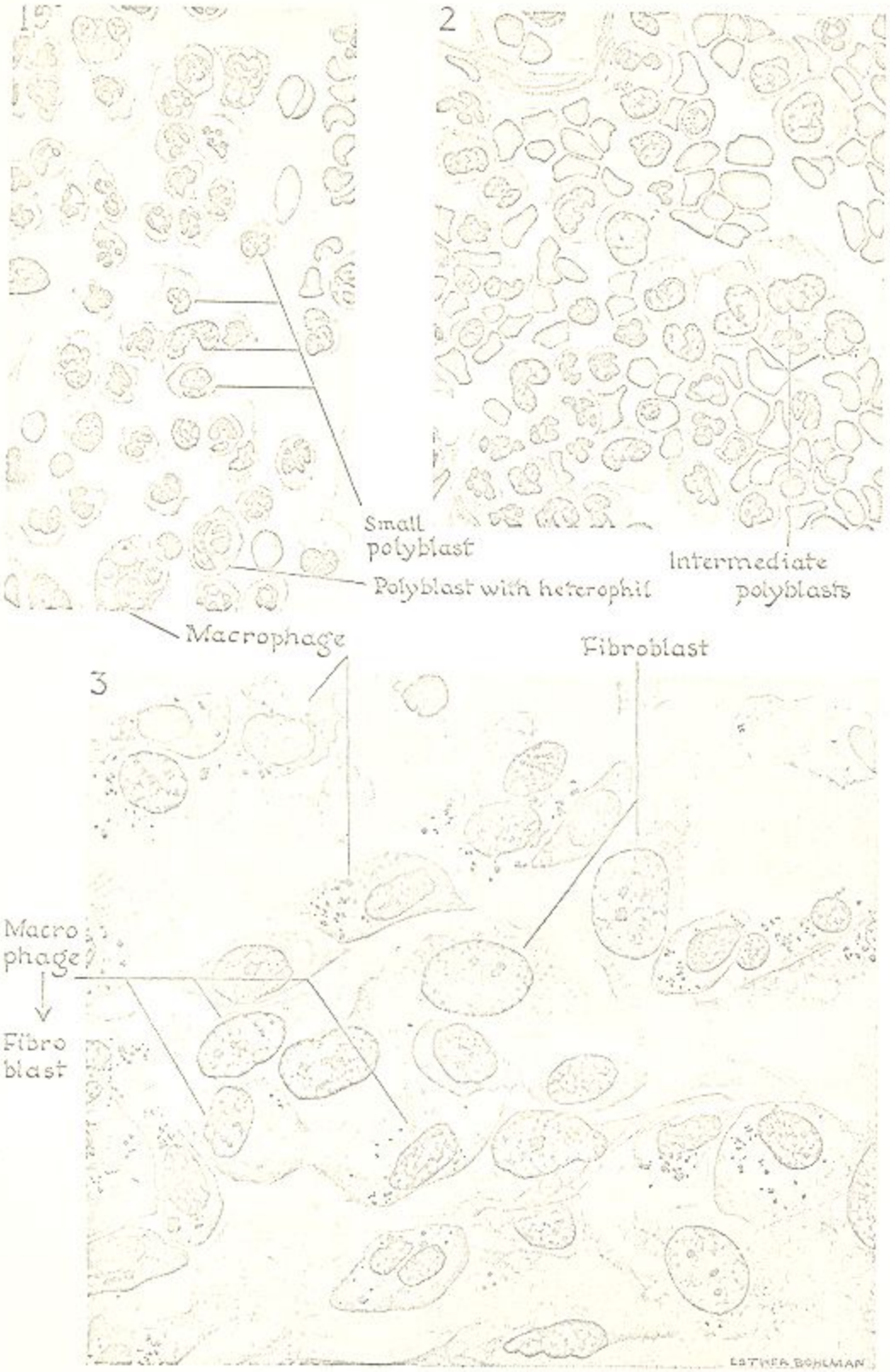

PLATE 6

(Legends appear on pp. 124 and 125.)

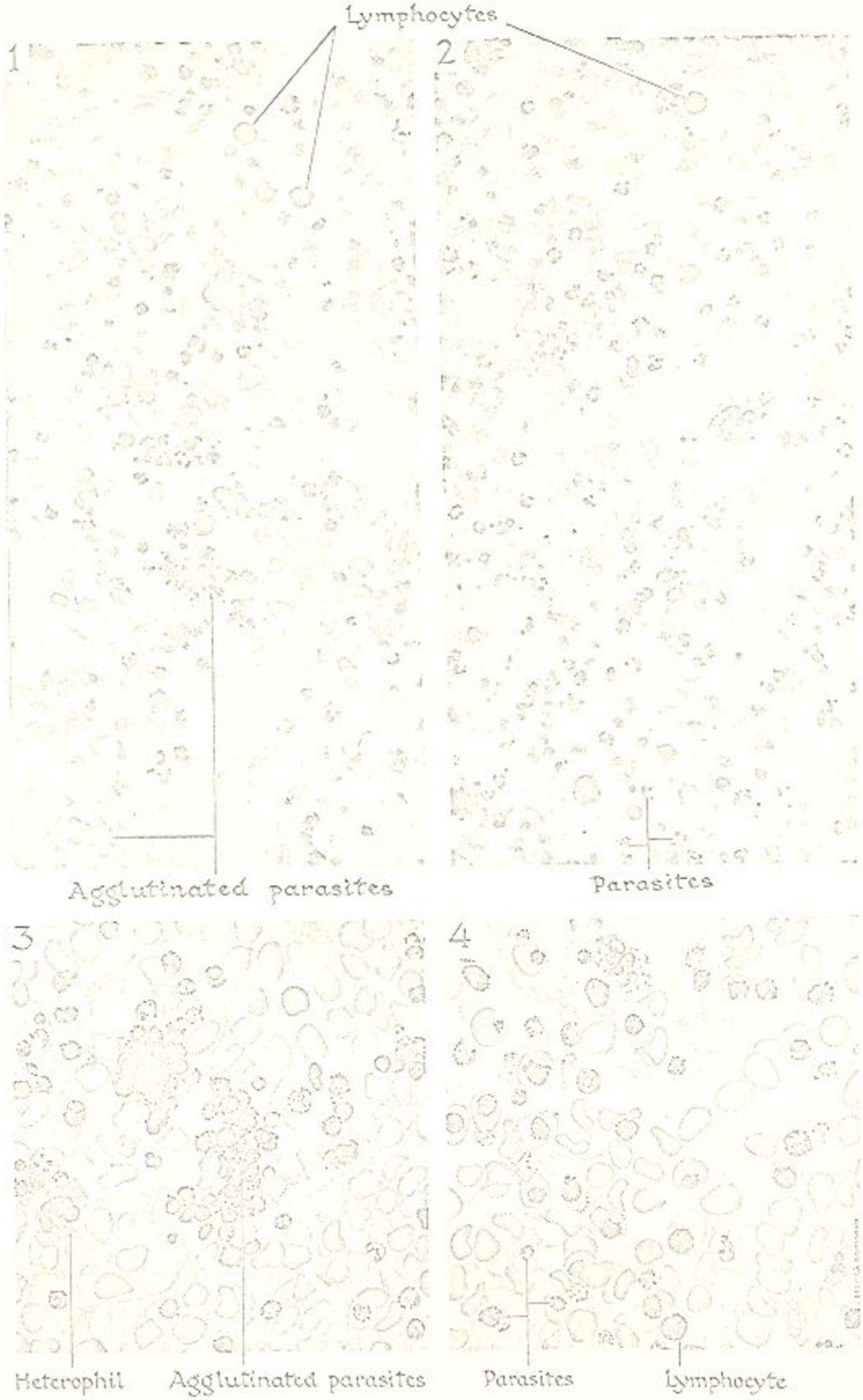

PLATE 7

(Legends appear on pp. 124 and 125.)

fig. 2). Many parasites in the agglutinated masses had apparently become freed of their surrounding red cells (pl. 7, fig. 3), while the red cells containing parasites often appeared hemolyzed. The uneven distribution of large parasites in the inoculum in the immune animals as compared to their more or less even distribution in the inoculum in the normal animal is shown by the detailed drawings in plate 7, figures 3 and 4. The appearance of the agglutinated plasmodia was in every way similar to the description of *P. knowlesi* agglutinated by immune serum in vitro, as given by Eaton (1938) and confirmed by Ray, Mukerjee and Roy (1941). This agglutination was still apparent at 3 hours in the immune animal, but disappeared thereafter because such agglutinated masses were avidly phagocytosed.

Inoculum had to be carefully distinguished from apparent inoculum. Apparent inoculum consisted of many blood cells and few parasites and was probably hemorrhagic in origin, i.e., extravasated blood due to the intense inflammatory reaction in the experimental animal itself.

In general, inflammation was more pronounced in the immune animals than in the normal animal. At 1 hour, the differences consisted of the following.

(1) More edema was undoubtedly present in the immune than in the normal animal.

(2) Migration of leucocytes, especially of heterophils, was more intense.

(3) The proportion of large polyblasts was greater and, although most of them could be identified as having originated from the blood, a few forms were indistinguishable from fully developed macrophages.

(4) In the immune monkey, many heterophils were engorged with pigment and a few with parasites; polyblasts obviously of hematogenous origin contained all types of malarial material; and a few mobilized histogenous macrophages contained both parasites and malarial pigment. In the normal monkey, phagocytosis was less prominent.

(5) The lymphatics were more distended.

Aside from the foregoing differences, inflammation was the same in all monkeys and involved edema, the accumulation of granular and nongranular leucocytes in and their migration through small blood vessels, the beginning hypertrophy of agranulocytes, the degeneration of mast cells, and the phagocytosis of malarial material by heterophils, polyblasts and local macrophages. The adventitial cells had not begun to mobilize. In both normal and immune animals, the lymphatics were distended and draining the inoculated area, but no lymphatic blockage was found. It is interesting to note that a few parasitized red cells were found in the lymphatics of one of the immune animals.

At 3 hours, all the differences noted at 1 hour were greater. In the immune monkeys as contrasted to the normal monkey, edema was markedly more pronounced, agglutination of parasites was intense, heterophils and agranulocytes occurred in larger numbers, hypertrophy of agranulocytes was greater, and phagocytosis per heterophil, hematogenous polyblast and macrophage was more active. Even 1 fibroblast was found containing 2 parasites in an immune monkey. In addition, the transformation of agranulocytes toward macrophages in the immune monkeys at 3 hours resembled that in the rat 30 hours after injection with trypan blue, whereas in the normal rhesus at 3 hours it resembled that in the rat 18 hours after injection with trypan blue. In the normal and immune animals, the lymphatics were distended and occasionally

contained a few parasites.

At 5 to 6 hours, inflammation was still more intense and, in certain respects, more rapid in the immune monkeys than in the normal animal with all of the activities, such as edema, exudation of cells, polyblast development and phagocytic activity, steadily continuing. On account of the greater phagocytic activity in the immune animals, fewer unphagocytosed parasites and parasitized erythrocytes were found in the immune animals than in the normal monkey. A few fibroblasts containing malarial pigment were now found in the normal animal as well as in the immune ones. In addition, in both normal and immune animals, a few adventitial cells were beginning to mobilize, as ascertained by their more basophil and better defined cytoplasm, and a few of the pigment cells, found normally in monkey skin, contained undoubted malarial pigment. Digestion of the parasites and parasitized erythrocytes within the local macrophages, which had begun at 3 hours, was well-advanced at this time. In the normal and immune monkeys, the lymphatics were distended and contained parasites.

At 1 day, in the normal monkey as compared to the immune monkeys, less of the inoculum was cleared up, polyblasts were smaller, and phagocytosis was less and that which was taking place was less specific, i.e., involved more unparasitized erythrocytes. For example, macrophages in the normal monkey often contained uninfected erythrocytes, as well as parasitized erythrocytes and parasitic debris, whereas macrophages in the immune monkeys were frequently surrounded by uninfected erythrocytes and engorged with parasitized erythrocytes and parasitic debris. In one of the immune monkeys, a large lymphocyte contained parasites and parasitized erythrocytes. In both normal and immune animals, heterophils, many of which had been phagocytic, were dying, and the lymphatic vessels were distended and contained red cells and infected red cells.

At 2⅛ days, the more effective, earlier activity of the immune animals was evident. In other words, the immune animals had disposed of the parasites so expeditiously, i.e., within 24 hours, that only a few parasites remained and segmented, i.e., multiplied by 10 or more times, whereas the normal animal had to cope with this second generation of parasites. In the normal animal at 2⅛ days, there were tremendous sheets of macrophages of all sizes, most of which contained malarial pigment and/or an occasional parasite. At this time, however, the normal animal was itself controlling the infection because only a few unphagocytosed parasites could be found. The lymphatics were distended and contained small polyblasts, but no free parasites or parasitized erythrocytes.

DISCUSSION

The outstanding finding in the present work is the different reactivity of the immune host to *P. knowlesi* as compared to *P. brasilianum* or *P. cathemerium*. This difference involved an increased rate of inflammation, an increased rate of phagocytosis by heterophils and macrophages, and the agglutination of parasitized red cells, in immune rhesus monkeys injected intracutaneously with *P. knowlesi* as compared to normal rhesus monkeys similarly injected. On the other hand, the immune canaries and cebus and spider monkeys reacted essentially similarly to normal canaries and monkeys when injected subcutaneously and intracutaneously with *P. cathemerium* and *P. brasilianum*, respectively. The difference in reactivity of the immune host to *P.*

knowlesi as contrasted to *P. brasilianum* and *P. cathemerium* took place despite the fact that previous work indicates that phagocytosis and presumably antibody manifestations increase tremendously in the spleen, liver and bone marrow of immune animals during infection of all 3 malarial infections. The difference seems best explained by assuming that there is sufficient antibody mobilized locally in the skin to influence the reactivity of the immune rhesus monkey to *P. knowlesi* but not of the immune Central American monkeys to *P. brasilianum* or of the immune canary to *P. cathemerium*.

Antibodies are believed by some investigators to be mobilized locally because of one or both of 2 conditions. In the first place, when an antigen is repeatedly injected into a local site, more and more antibody is produced locally by the local macrophages. In the second place, when an antigen is introduced into the blood stream, it is generally removed by the macrophages of the filter organs (spleen, liver and bone marrow) which in turn produce antibodies that circulate in the blood. Such circulating antibodies may diffuse into a local area because of changes in the permeability of the vessel walls which occur during local inflammation. In the present experiments, the antigen was not introduced at first either intracutaneously or subcutaneously. In the canaries and Central American monkeys, the initial infections were started intravenously with parasitized red cells, and in the rhesus monkeys, they were started intramuscularly with parasitized red cells. It seems, therefore, that the difference in the mobilization of antibodies in the skin, in the present experiments, must be explained, not on any difference in the local production of antibodies since antibodies were not produced locally, but entirely on differences in the diffusion of circulating antibodies into the area.

The question then arises as to why antibodies diffuse more readily from the circulation into the local area in infections of *P. knowlesi* than in the other two. Of 2 factors which might be expected to influence such a diffusion, viz., a greater permeability of the vessels following a more intense inflammation and a higher concentration of circulating antibodies, the second one seems to be responsible in view of the following facts.

P. knowlesi does not produce a more intense inflammation and, hence, give rise to greater permeability of the vessel than the other 2 species of malaria, because the speed and degree of inflammation in the *normal* rhesus monkey (the only place where the degree of inflammation is uncomplicated by possible antigen-antibody reactions) was no greater than in the South American monkey. In considering the question of the intensity of the inflammation, attention has to be limited to the normal rhesus because the initiation of the process—with which we are concerned—cannot be differentiated in the immune rhesus from stages when antibodies have diffused into the tissue. Obviously, in such stages an antigen-antibody reaction has taken place in the local tissues which itself produces more intense inflammation and more diffusion of antibody.

The assumption that circulating antibodies are higher in titer in the rhesus monkey than in Central American monkeys or canaries is substantiated in part by experimental evidence. Previous work as far as it goes, indicates that there is not as much circulating antibody in infections of *P. cathemerium* and *P. brasilianum* as in infections with *P. knowlesi*.

In *P. cathemerium* in the canary, appreciable amounts of antibody have

not been demonstrated by ordinary techniques. Working with infections of *P. cathemerium*, W. H. and L. G. Taliaferro (1929) were not able to obtain clear-cut protection with single doses of 2 ml of immune serum (equivalent to about 10 ml per 100 gm) or with 3 doses of 0.3 ml (equivalent to about 1.5 ml per 100 gm) on 3 consecutive days. In addition, no sensitization of the parasites could be demonstrated after contact of about 5½ hours at 5 C. Moreover, Hegner and Eskridge (1938) and Hegner and Dobler (1939) could obtain little or no evidence of the protective action of serum from birds immune to *P. cathemerium*.

In line with the negative results in avian malaria, W. H. and L. G. Taliaferro (1934) found no circulating antibodies against *P. brasilianum* in Central American monkeys. Single doses of from 2 to 6.8 ml of serum (equivalent to about 0.5 to 2 ml per 100 gm) from immune animals failed to influence the infection. These experiments, however, should be repeated with multiple doses of serum.

Positive results with *P. knowlesi* have been reported by Coggeshall and his coworkers. Coggeshall and Kumm (1937) obtained partial protection against *P. knowlesi* with immune serum, as evidenced by a lengthening of the life of the monkey or by a low parasitemia and survival of the monkey. In general, the first effect was produced with a comparatively large initial dose of immune serum (25 ml per monkey or 1.25 ml per 100 gm monkey) given at the time the parasites were given, whereas survival of the monkey was obtained with a comparatively large initial dose and several smaller daily doses (30 ml initially and 12 to 24 ml per monkey, i.e., 1.5 ml and 0.6 to 1.2 ml per 100 gm, respectively). In the following year, they (1938) obtained complete or almost complete suppression of the parasitemia with 11 daily doses of 2.5, 5 or 10 ml (equivalent to 0.125, 0.25 or 0.5 ml per 100 gm monkey) of hyperimmune serum started at the time parasites were given. In addition, Coggeshall and Eaton (1938) found that the smaller the inoculum, the less the quantity of immune serum required to prevent the death of the animal. Mosna (1938) confirmed some of this work in the same year, and Coggeshall in 1940 demonstrated protective antibodies in human beings infected with *P. knowlesi* when tested in rhesus monkeys. In most of this work, 10,000 parasites were incubated at 37 C for 30 minutes with 2 ml of serum before injection into a test monkey by the method developed by Coggeshall and Eaton (1938).

There have been 2 main ideas as to why it is difficult in some infections to transfer protective antibodies passively. Taliaferro and Cannon (1936) suggested that the antibodies were formed during infection in the spleen, liver and bone marrow in sufficient quantities to be active in those sites, but were insufficient in quantity after dilution in the blood stream to be passively transferred. Coggeshall (1940), on the other hand, believed that antibodies may be high in titer, but may not be able to act effectively against the parasite because the parasite is protected by the red cell. He based his suggestion on the fact that Eaton (1938) had previously demonstrated that *P. knowlesi* antiserum agglutinated red cells containing full grown parasites but not those containing smaller parasites. There are many reasons for accepting Coggeshall's (1940) suggestion as an explanation of part of the difficulty in demonstrating circulating agglutinating and opsonizing antibodies, but it cannot explain the differences in our experiments because large parasites of all 3 species of malaria

were used as inoculums. The lower concentrations of antibodies in *P. brasilianum* and *P. cathemerium* must, therefore, be explained in part by some such reason as suggested by Taliaferro and Cannon.

One of the outstanding findings in the present work is the unexpected speed of certain of the inflammatory reactions in the monkeys and canary—in particular the hypertrophy of agranulocytes and the related activities of phagocytosis, formation of macrophages and giant cells, and the transformation of macrophages into fibroblasts. This speed may be exemplified by the following 2 stages which lend themselves to comparison more readily than others. At about 30 hours after the injection of trypan blue in the rat, it becomes impossible to differentiate large mobilized histogenous macrophages from many macrophages which have developed from hematogenous agranulocytes (Maximow, 1929). This stage is reached in some areas in the monkey in about 6 hours. In the canary, it is reached in some areas in about 3 hours. Later, at about 18 days after the injection of trypan blue in the rat, there has been a distinct advance toward scar formation as evidenced by the transformation of large numbers of macrophages into fibroblasts (Maximow, 1929). This stage occurs in the monkey at about 7 days and is even further advanced in the canary at 48 hours.

SUMMARY

This work involved the histological study of the skin and subcutaneous tissue of normal and immune canaries and monkeys following the subcutaneous (canary) or intracutaneous (monkey) injection of small amounts of malarial blood. *Plasmodium cathemerium* was used for the canary, *P. brasilianum* for cebus and spider monkeys and *P. knowlesi* for rhesus monkeys. For comparative purposes, similar injections of small amounts of normal blood and of trypan blue were also studied.

The data are conclusive in indicating that the immune host reacted more strenuously to *P. knowlesi* than to *P. brasilianum* or *P. cathemerium*. Thus, red cells containing large stages of *P. knowlesi* were agglutinated and localized in the skin of immune rhesus monkeys, but were not agglutinated at all in normal rhesus monkeys. In addition, inflammation, including phagocytosis by heterophils and macrophages, was more pronounced in the skin of immune rhesus monkeys than in the skin of normal rhesus monkeys. In marked contrast, inflammation was similar in extent in the skin of normal and immune canaries and normal and immune cebus and spider monkeys, and agglutination of the plasmodia did not occur at all. It is suggested that this difference is due to a greater content of circulating antibody in *P. knowlesi* infected animals than in *P. cathemerium* or *P. brasilianum* infected animals which, thereby, allows effective amounts of antibody to leave the blood and enter the inflamed area. In this connection, the origin and interrelations of local and general immunity are discussed.

Inflammation was not only more intense, but also proceeded more rapidly in certain respects in the host immune to *P. knowlesi* than to *P. brasilianum* or *P. cathemerium*. In addition, certain activities proceeded in the skin in normal rhesus monkeys, in normal and immune Central American monkeys, and especially in normal and immune canaries injected locally with either malarial blood or trypan blue at a far greater speed than they proceeded in the skin in rats injected intracutaneously with trypan blue, as reported by the v. Möllendorffs and Maximow. For example, the development of hematogenous

polyblasts in the skin of the canary 3 hours or in the monkey 6 hours after injection resembled those of the rat about 30 hours after injection. Later on, the transition of macrophages into fibroblasts in the skin of the canary 2 days or in the monkey 7 days after injection resembled those of the rat 18 days after injection.

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