CELLULAR REACTIONS TO A DYE-PROTEIN WITH A CONCEPT OF THE MECHANISM OF ANTIBODY FORMATION

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It has long been known that antibodies are almost invariably associated with the globulin fractions in the serum. An example of this association in the case of antipneumococcus serum was presented some years ago by Avery (1). Furthermore, within the last decade, evidence that antibodies are themselves protein has been steadily accumulating. Especially convincing have been the studies of Felton and his collaborators (2, 3) on the concentration and purification of the antibodies in antipneumococcus horse sera and the extension of these studies by Chow and Goebel (4). However, without quantitative micro methods for the estimation of antibodies in absolute terms the identification of antibodies as modified serum globulins would necessarily have remained uncertain. The development of such methods by Heidelberger and his collaborators (5) and the extension of their theoretical studies to the preparation of more highly purified antibody than had previously been obtainable led to accurate investigations on the physical-chemical properties of antibodies by the ultracentrifugal (6–9) and electrophoretic (10, 11) methods. Since the molecular weights and electrical mobilities found were those characteristic of proteins, the identification of antibodies as modified serum globulins may be considered accomplished. A chemically satisfactory theory of antibody formation has been put forward by Breinl and Haurowitz (12) and was restated by Mudd (13).

All of this chemical work signifies that the cellular mechanisms which give rise to antibodies must be concerned in their normal functions with the synthesis of globulin. For some years evidence has been presented implicating the cells of the reticulo-endothelial system in the formation of antibodies. The present report seeks to make our understanding of the mechanism of this function more definite.

Studies of the reactions of the phagocytic mononuclear cells stem from the work of Ehrlich and Metchnikoff. It is known that when Ehrlich was experimenting with dyes, he suggested to his friend Goldmann that he use pyrrol blue for observations on these cells. Goldmann (14) then made a survey of all the cells in the body which will phagocytize azo dyes given in the form of particulate matter. His work was followed by that of Aschoff (15) and of Kiyono (16) who used carmine particles, and by that of Evans and his associates (17–19) who employed trypan blue. Aschoff formulated the concept

of the reticulo-endothelial system. Though this name has been used in different ways, the idea is based on the fact that certain specific endothelia and certain mononuclear cells of the tissues have high phagocytic power and react toward the same materials. With these studies as a basis there has grown up an extensive literature implicating the reticulo-endothelial system in the formation of antibodies. It includes observations involving the spleen (20), the lymph nodes (21), and macrophages or clasmatocytes (22–24). A critical review of this literature has been given by Zinsser, Enders, and Fothergill (25).

In studies with chemical fractions from tubercle bacilli it was found that certain lipids, notably a phosphatide (26) and wax-like substances (27), could be identified within the phagocytic cells, the phosphatide because it assumed the form of myelin figures, and the wax because of its acid fastness. This made it possible both to identify with assurance the cells which had reacted to these materials and to follow to some extent the ability of these cells to deal with them. The wax was seen to become surrounded by monocytes which fused into giant cells to engulf it; within these giant cells the wax soon lost its acid fastness and was then slowly degraded until the cytoplasm of these cells returned to the normal state, the giant cells splitting into the original monocytes (27). On the other hand, single monocytes engulfed the phosphatide without any fusion of cells; occasional multinuclear forms were induced by the multiplication of nuclei without cell division. The degradation of the phosphatide appeared to be only partial for an epithelioid cell was formed which never returned to the size and appearance of the monocyte. These epithelioid cells then died either singly or *en masse*, bringing about a form of caseation (26). These examples illustrate two methods of dealing with phagocytized material, the one instance showing what may be called complete digestion of the material, the other, a partial digestion with permanent storage of some intermediate product. Still another mechanism involving the phagocytosis of an antigen will be considered.

Many recent studies on the origin of the serum proteins have also been pointing, in our judgment, toward the reticulo-endothelial system. Most of these studies have implicated the liver, but have also suggested that the liver is not the sole source of these proteins. The involvement of the liver is due, it would seem, to the presence of the Kupffer cells, a part of the reticulo-endothelial system. Observations on the liver have been made largely after damage of the organ by chloroform, or the use of an Eck fistula, or after total extirpation of the liver (28–37). Other observers have specifically implicated other tissues as well as the liver (38, 39).

In 1912, Downey and Weidenreich described and illustrated as a characteristic of lymphocytes a shedding of parts of the surface films or exoplasm of these cells, which caused no permanent damage to the cells. They demonstrated this phenomenon in lymphocytes within the sinuses of lymph nodes and also in lymphocytes throughout the follicles (40). Later Downey (41) studied the phenomenon further and showed that the shed bits of cytoplasm were not platelets, since they were devoid of the granules which characterize platelets. He observed that they disintegrated rapidly in the thoracic duct.

This phenomenon has been observed in our laboratory as a characteristic of monocytes (macrophages or clasmatocytes) after they have been stimulated and it has been illustrated after injections of tuberculo-protein (Fig. 8 in reference 42). As a matter of fact, it occurs in the normal reactions of monocytes or macrophages and was observed many years ago by Ranvier (43). He described such shedding of exoplasm from certain

connective tissue cells in the frog and on account of this property named the cell "clasmatocyte." Since that time the terms macrophage ("big eater") and clasmatocyte (shedding of exoplasm) have been used synonymously and thus have been recorded two of the properties of the same cell.

The synthesis of a dark red dye-protein, R-salt-azo-benzidine-azo-egg albumin, by Heidelberger and Kendall in 1930 (44–46) provided a material which can also be positively identified within cells, in this instance by its color. Moreover, this material has the added important property of being a good antigen. They employed the substance in a study of the quantitative relations in the antigen-antibody precipitate, since the amount of antigen could be determined colorimetrically both in the precipitate and in the supernatant. In synthesizing this antigen Heidelberger had also in mind that it might be an effective material for following the cellular reactions involved in sensitization and in immunity.[1] It has been proven that the dye-protein is also taken up by the cells of the reticulo-endothelial system and the following pages will describe what may be observed concerning the reactions of the phagocytic cells to this antigen.

In 1920 the author published studies on the origin of blood vessels in the classic material of the chick blastoderm, using the method of mounting the living blastoderm in a hanging drop preparation and watching the development of blood vessels for some hours. In such preparations of chicks of the 2nd day of incubation it was possible to see the origin of a vessel by the liquefaction of the central part of a mass of angioblasts while the peripheral cells flattened out to make endothelium (see Fig. 23 in reference 47). This process had been inferred years before from a study of sections by Klein (48). The phenomenon may be stated in physiological terms as follows: Since for a vessel to function as such, it is essential for the plasma to contain some protein to maintain the balance of intra- and extravascular fluid, the original transformation of a solid mass of angioblasts into a vessel involves the sacrifice of some cytoplasm to make the serum proteins. In the present studies some evidence will be presented in support of the concept that throughout life the serum proteins, certainly as far as globulin is concerned, come from the sacrifice of a part of the cytoplasm of cells. This process has no relation to the function of secretion, in which case a material, formed by the cytoplasm but clearly distinct from it, appears within the cell and is cast out of it with no loss of cytoplasm whatever. If it be correct that the Kupffer cells of the liver sinuses play a part in this process, it is interesting to record that Kupffer cells develop in the embryo long before blood islands have entirely disappeared. This ob-

[1] We are indebted to Dr. Heidelberger not only for giving us the material for our experiments but also for making all of the quantitative estimations of the antibody titre in the sera and for help and advice throughout the work.

servation was checked in sections of a monkey, perfectly preserved, at the Carnegie Institution of Embryology, through the courtesy of Dr. George L. Streeter (monkey embryo 479, 12 mm., ovulation age, 35 days).

RESULTS

The dye-protein was used in the form of an alum precipitate, since Heidelberger, Kendall, and Soo Hoo (44–46) had found that with this form the antibody titre was higher than after the same material given in solution. It was a suspension of purplish red particles which settled quickly on standing and were in aggregates large enough to be readily visible. The material was introduced by four routes, namely, the intraperitoneal, intravenous, intradermal, and subcutaneous. After intravenous injections the dye-protein-alum particles were found in the Kupffer cells of the liver, in the macrophages of the splenic pulp, and, to a minor extent, in the macrophages (the so called adventitial cells) which lie along the border of the sinusoids in the bone marrow. After intraperitoneal injection the dye-protein was found in the macrophages of the milk spots of the omentum and in the corresponding cells of the peritoneal walls, as well as in the endothelium lining the lymphatic sinuses of the retrosternal nodes and in the free macrophages of sinuses and follicles of these nodes. After intradermal and subcutaneous injections the dye-protein was in the local macrophages and in the regional lymph nodes. After a single subcutaneous injection macrophages engorged with dye were not found in the subcutaneous tissues but only in the regional lymph nodes.

The dosage, route, and spacing of the injections, together with the cellular reactions and the antibody titre of the serum, are presented in Table I. Many additional subcutaneous injections into the tissues of foot pads and lips were made but the results are not recorded in this table.

The intraperitoneal route of injection stimulates tissues that lend themselves most readily to the study of the living cell in films of the omentum, in peritoneal exudates, and in the tissues of the retrosternal nodes. In Fig. 1 is shown a part of a milk spot of the omentum of rabbit R 5109, 24 hours after a final injection of 2 mg. of the dye-protein. As will be noted in Table I, this animal had received 50 mg. of the material intraperitoneally, the last injection having been made 24 hours before the tissues were studied. The macrophages of the milk spots were heavily loaded with particles of the dye-protein, as demonstrated by their color in the photograph. These cells were studied while alive and with no stain whatever, so that the dye-protein could be positively identified. The film was then fixed and the nuclei were stained in hematoxylin only, care being taken that

all other fluids used did not contain any counterstain. All of the red granules in the photograph are due, therefore, to the material injected. In the macrophages in Fig. 1 the numerous particles of dye-protein are plainly visible, but there are also one or two larger vacuoles which in the living cell were translucent and smooth in contour, as if of fluid, in contrast to the smaller, irregular, opaque particles of the dye-protein-alum-salt. These larger vacuoles are interpreted as containing dye in solution which the cell had been able to separate from the dye-protein and had segregated into a few vacuoles by itself. The particles of the residual dye-protein varied in the density of color according to the varying amounts of dye which had been extracted from each. This variation in the density of the color is imperfectly recorded in the photograph. In the lower macrophage of the figure are three or four blue dots which are the residue of the nucleus, probably nucleic acid of a neutrophile which had been phagocytized. These cells illustrate the first reaction of the phagocyte toward the engulfed material. Such materials are segregated into vacuoles of digestion, with a barrier of fluid between the ingested material and the cytoplasm. This barrier of fluid can be made visible by treating the living cell with neutral red, which procedure stains the digestive fluid and then conceals the phagocytized particle. The first reaction of the cell was to separate some of the dye from each dye-protein aggregate. Besides being in the macrophages of the milk spots of the omentum, there was some dye-protein in a part of the fibroblasts between the milk spots. No fibroblast was as heavily loaded with the dye-protein as were the macrophages, but they also showed an occasional vacuole containing what has been interpreted as dye alone.

The intravenous injections provided material that was especially instructive. Rabbits H-668, 669, and 670 received 88 mg. of the dye-protein intravenously and were then tested after an interval of 1 week for antibodies in the serum. H-669 showed a relatively high titre, namely, 0.63 mg. N per cc., while H-668 had an intermediate titre, 0.30 mg., and H-670 a lower one, 0.27 mg. To this rabbit (H-670) was given a final intravenous injection of 8 mg. of dye-protein and the tissues were studied 6 hours later. The dye-protein was in all instances in the Kupffer cells of the hepatic sinuses, in the macrophages of the splenic pulp, and, to a minor extent, in the adventitial cells along the sinuses of the bone marrow. In rabbit H-670 many of the Kupffer cells along the portal border of the lobules of the liver were enlarged and engorged with the dye-protein, as revealed in Figs. 2 to 4. In Fig. 2 is shown a long Kupffer cell which contained, besides much dye-protein, a neutrophile, a red cell, and some nuclear debris. In one place the cytoplasm of this Kupffer cell stretches completely across the

TABLE I

Correlation between Cellular Reactions and Antibody Titre after Injections of an Alum-Precipitated Dye-Protein into Rabbits

Animal No.	Number, spacing, amount, and route of injections	Total amount of dye-protein	Cellular reactions	Antibody titre of serum
		mg.		
R 5108* R 5413 R 5414† R 5415†	12 and 14 daily 0.1 cc. of 5 per cent solution intradermally		The dye-protein remained visible through the skin and there was no sensitization	—
R 2714	4 daily 1 mg. intraperitoneally. Studied 4 days later	4	Omentum: Dye-protein in a few macrophages; some in neutrophiles. Neutrophiles containing dye within macrophages Retrosternal nodes: Dye-protein within lymphatic endothelium; monocytes and macrophages loaded with dye-protein	None
R 2817 R 2818	3 daily 1 mg. intraperitoneally. Studied 24 hrs. later	3	Omentum: Small amount of dye-protein in macrophages. Many neutrophiles Retrosternal nodes: Endothelium and macrophages filled with dye-protein. Some neutrophiles containing dye-protein. Dye-protein in small, irregular, opaque masses. Some macrophages had one round, red globule darker than the dye-protein	R 2817 none R 2818 not tested
R 2808 R 2433	3 every 4 days 1 mg. intraperitoneally. Studied 4 days later	3	Omentum: Some macrophages contained small amount of dye-protein Retrosternal nodes: Same The monocytes and macrophages of omentum and peritoneal exudates which no longer contained visible dye-protein showed shedding of surface films (Fig. 5)	R 2808 0.1 mg. precipitin per cc. R 2433 0.16 mg. precipitin per cc.
R 2765	4 daily 0.1 mg. intraperitoneally 5 daily 0.1 mg. intraperitoneally. Interval of 11 wks. 3 daily 1 mg. intraperitoneally. Studied 4 days later	3.9	Omentum: Traces of dye-protein in macrophages Retrosternal nodes: A few macrophages containing dye-protein and dye only	After first 2 courses too little precipitin for accurate estimation. 4 days after final injection 0.47 mg. precipitin per cc.
R 2503	4 daily 10 mg. intraperitoneally. Studied 2 days later	40	Omentum: Macrophages loaded with dye-protein in large aggregates. Many cells contained dye within neutrophiles Retrosternal nodes: Same	

* These are serial numbers used in this laboratory over a term of years.

† Guinea pigs.

TABLE I—*Concluded*

Animal No.	Number, spacing, amount, and route of injections	Total amount of dye-protein	Cellular reactions	Antibody titre of serum
		mg.		
R 2636	2 mg. foot pads. Studied 4 hrs. later	2	Foot pads: Dye stained the fibers of the tissues. No dye-protein found in macrophages Popliteal nodes: Macrophages containing dye-protein	—
R 5109	6 weekly series 4 days each 2 mg. intraperitoneally. Interval of 12 days 2 mg. intraperitoneally. Studied 24 hrs. later	50	Omentum: Macrophages loaded with dye-protein. Some showed 2–3 large, round vacuoles darker in color (Fig. 1). Some dye-protein in fibroblasts Retrosternal nodes: Dye-protein in endothelium of sinuses. Macrophages loaded with it	Antibody titre weak after 3rd and 4th series. 11 days after 6th series, 0.07 mg. antibody N per cc. 24 hrs. after final injection, 0.04 mg. antibody N per cc.
R 5110	3 weekly series 4 days each 2 mg. intraperitoneally 3 weekly series 4 days each 2 mg. intravenously. Interval of 12 days 2 mg. intraperitoneally. Studied 24 hrs. later	50	Omentum: Macrophages contained some dye-protein Retrosternal nodes: Macrophages contained considerable dye-protein Liver: A few Kupffer cells contained little dye-protein Spleen: Many large macrophages with dye-protein and neutrophiles	Antibody titre weak after 3rd series. 6 days after 6th series, 0.13 mg. antibody N per cc. 24 hrs. after last injection, 0.07 mg. antibody N per cc.
H-668 H-669	2 mg. intravenously Interval of 2 days 2 mg. 1 day 3 mg. 3 days. Interval of 3 days 4 mg. 1 day 5 mg. 3 days. Interval of 2 days 5 mg. 4 days. Interval of 2 days 6 mg. 2 days 8 mg. 2 days. Interval of 2 days 8 mg. 1 day. Studied 7 days later	88	Liver: Some dye-protein in Kupffer cells throughout lobule. All Kupffer cells normal size. Less dye-protein in cells of H-669, in which it could be found only with oil immersion lenses (Fig. 6) Spleen: Some dye-protein and debris of neutrophiles in macrophages—less in H-669 Bone marrow: Traces of dye-protein in adventitial cells	H-668: 0.30 mg. antibody N per cc. H-669: 0.63 mg. antibody N per cc.
H-670	Same as above, except a final 8 mg. intravenously. Studied 6 hrs. later	96	Liver: Kupffer cells along portal border enlarged and engorged with dye-protein. They contained also some neutrophiles, themselves containing dye-protein. Some monocytes containing dye in vessels Spleen: Macrophages engorged with dye-protein and neutrophiles. Monocytes and neutrophiles containing dye Bone marrow: Traces of dye-protein in adventitial cells Blood vessels: Monocytes and neutrophiles containing dye-protein	0.27 mg. antibody N per cc.

lumen of the sinus. In addition to this endothelial cell is a monocyte engorged with dye-protein within the vessel. In Fig. 3 are shown two Kupffer cells chosen to demonstrate the folds of the surface films of these cells that serve as guy-ropes which fasten themselves to the opposite wall and keep these surface films floating in midstream to perform their function of phagocytosis. The surface films of the Kupffer cells, which are the most highly developed of any of the cells of the reticulo-endothelial system, have been shown in the isolated cell by Rous and Beard (49, 50). In Fig. 4 is revealed a multinucleated Kupffer cell containing dye-protein with a long film of cytoplasm which appears to be separated from the main mass by the plane of the section. These very large Kupffer cells were all near the portal border, while smaller ones were found more deeply placed, as in Fig. 5. This cell was chosen to show two engulfed neutrophiles which themselves contain dye. In this animal many of the neutrophiles in the vessels, for example, in the vessels of the kidney, contained dye. This phenomenon was especially prominent in the tissues of the spleen. The sections of the spleen demonstrate many neutrophiles, monocytes, and macrophages filled with the dye-protein within the pulp. The macrophages for the most part contained both dye-protein and neutrophiles. This reaction may be seen in Fig. 7.

The tissues of rabbits H-668 and 669 contained dye-protein in the cells and showed the same distribution of it except that it was far less in amount since they had not received a recent injection. These two animals had some residual dye-protein in both liver and spleen in inverse proportion to the antibody titre of their sera. The difference in the amount of dye-protein is sufficiently marked so that the sections may be discriminated without reference to their labels, for in the one, H-669, the dye could be located only with the oil immersion lens, while in rabbit H-668 it could still be discerned with low power, that is, with a 16 mm. lens. Traces of the dye-protein were visible in cells throughout the liver lobule, even in the Kupffer cells that are nearest to the central vein. The amount of dye remaining can be seen in Fig. 6, taken from the liver of rabbit H-669, in the cells at the extreme right and left of the photograph.

This marked reduction in the amount of dye-protein in some of the cells and its disappearance from most of the phagocytic cells were correlated with the appearance of antibodies in the serum. This correlation was present also in rabbit R 2433. This animal had received three spaced injections of 1 mg. of the dye-protein and the tissues were studied 4 days after the third injection. The mononuclear cells of the peritoneal exudate and the macrophages of the omentum showed a marked shedding of parts of

the surface films or exoplasm. Some of the macrophages had some residual dye-protein but it was striking that it was not these cells that presented the shedding phenomenon but rather those without visible dye-protein particles. This observation was quite clear in the preparations of the living cells studied without any accessory stain; the use of neutral red accentuated somewhat the phenomenon of shedding. Two cells from the peritoneal exudate of this animal stained in neutral red are shown in Fig. 8. The magnification at which the photograph was taken (× 1,800) proved to be too high to demonstrate the processes of exoplasm to the greatest advantage; they are better illustrated at a magnification of 1,000 in the omentum of a rabbit obtained 7 days following an injection of tuberculoprotein (Fig. 8 in reference 42). It should be made clear that this process of the shedding of parts of the exoplasm has not been observed within the living animal, and thus not from the Kupffer cells, but only in living cells removed from the animal. When the omentum or a drop of peritoneal fluid is first mounted on a slide, all the cells are rounded, but on standing for a short time the reaction of the shedding begins by the pushing out of such processes of cytoplasm as are photographed in Fig. 8. These then separate from the cell and break into globules. It should be noted that the peritoneal exudates are mounted in their own fluid and the preparations are sealed immediately, so that there is little chance for any change in the surrounding fluid medium to produce this reaction. The omentum can only be mounted with the addition of saline. The one tissue in which the shed globules can be seen immediately upon removal from the body is that of scrapings from the regional lymph nodes, in which case, at the proper functional stage, the fluid of the sinuses is filled with these particles.

DISCUSSION

The data presented in this paper may thus be summed up as follows. The use of a "marked antigen," such as an alum-precipitated dye-protein, makes it possible to identify the cells by which it is phagocytized. The material is placed in the vacuoles of digestion by the cell and altered first by the removal of the dye. After removal of the dye the solid particles of protein disappear and it is assumed that the protein has been rendered into soluble form (possibly, as Heidelberger suggests, with amino groups replacing the original RN:N linkages) and passed into the cytoplasm. It should be stated here that Heidelberger has found that in the case of this antigen the antibodies are not oriented to the hapten group. Coincident with the time when the dye-protein is no longer visible within these cells, and when there are antibodies in the serum, there is a marked shedding of

the surface films of the macrophages without damage to them. The phenomenon of shedding of surface films is characteristic of the normal monocyte or macrophage; it occurs also after the phagocytosis of non-antigens, but it is much accentuated at certain periods after the ingestion of antigens. Thus these mononuclear cells function first as macrophages and then as clasmatocytes. The hypothesis which may be formulated from these observations is that the cells of the reticulo-endothelial system take up foreign materials which may be classified into two groups, namely, antigens and non-antigens. Both kinds of material are first taken into the vacuoles of the cells, indicating that a cell guards its basic cytoplasm from the immediate entrance of foreign substances. The vacuoles are the cellular organs of digestion; the cytoplasm is the zone of syntheses. In turn, the synthesis of cytoplasm is usually from normal food substances. If the material phagocytized is an antigen, it is rendered into suitable soluble form within the vacuole and then passed into the cytoplasm itself. There its presence in some way increases the synthesis of globulin and modifies some of it into antibody globulin (*cf.* 11, 12). With the shedding of parts of the surface films of these cells, both normal globulin and antibody globulin are carried into the blood plasma, since immunologists have discovered that both usually increase at the same time. Thus an antigen may be defined as a substance which can specifically modify the synthesis of cytoplasm. This process is the evolution of a change in cytoplasm in response to environment. It may be possible that the cell which has formed a new kind of globulin and still retains it in the cytoplasm is sensitized, meaning that it would react differently from the normal cell in the presence of the original antigen. If this be true, then certain phases of sensitization and immunization are founded on the same mechanism.

Certain details concerning the action of the phagocytic mononuclear cells illustrated in these experiments seem worthy of mention. A question has been raised concerning the name reticulo-endothelial system (24) *versus* the use of some term such as the macrophage system or the functions of the phagocytic mononuclear cells. The data obtained in this study illustrate the fact that it is not possible to introduce particulate matter by any of the four usual routes without involving both certain specific endothelial cells and free macrophages. After intravenous injection the endothelial cells are the Kupffer cells of the liver and the macrophages are those of the spleen and bone marrow. Following any kind of interstitial injection, intraperitoneal, subcutaneous, or intradermal, the material introduced is phagocytized by both the local macrophages and the lymphatic endothelium of the regional lymph nodes, together with macrophages in these nodes.

There is a marked contrast between the dermis and the subcutaneous levels in the abundance of macrophages in the one and their paucity in the other; macrophages may, however, be induced in the subcutaneous level. It may thus be seen that the reticulo-endothelial system is widespread and that phagocytic endothelium, either vascular or lymphatic, is always called into play together with free macrophages. Macrophages or their less mature form, monocytes, arise throughout the connective tissues of the body. From these observations it may be concluded that the reticulo-endothelial system has an enormous factor of safety in the sense discussed by Meltzer (51). This is clear also from the fact that after repeated intravenous injections not all of the Kupffer cells had engulfed the dye-protein and the same was true of the cells of the milk spots of the omentum. Moreover, it is well known from experiments with benzidine dyes (15, 16) that after repeated intravenous injections the endothelium of splenic and marrow sinuses becomes phagocytic. A further reservoir of phagocytic cells is brought about by the ready multiplication of monocytes in the tissues. This phenomenon was striking in experiments with tuberculo-protein (42) but there was no evidence of multiplication of cells following administration of dye-protein.

It is thus clear that for the experimental production of antibodies one may call into action either the tissues of the liver and spleen by employing the intravenous route of injection, or local macrophages and the endothelium and macrophages of the regional lymph nodes. As has been said, the production of antibodies within lymph nodes was proved by McMaster and Kidd in 1937 (21). In the present experiments a higher titre of antibodies was produced by the intravenous route with the use of the dye-protein. This is illustrated in the comparison of the antibody titres of rabbits R 5109 and 5110 (Table I). The total amount of antigen given was the same for these two animals, but R 5110 received 24 of the 50 mg. intravenously and had the higher titre. On the other hand, Hurwitz and Meyer (38) reported a more rapid rise in globulin after intraperitoneal injections of bacterial antigens.

The observation that there was some phagocytosis of the dye-protein by fibroblasts between the milk spots of the omentum is important in connection with the subject of sensitization. All of the studies made with carmine and the benzidine dyes (15–19) demonstrated that fibroblasts phagocytize particulate matter as well as macrophages but always in less amount. Experiments with the method of tissue culture have demonstrated that fibroblasts can be sensitized. In 1932 Rich and Lewis (52) showed that in tissues from the spleen of tuberculous guinea pigs the fibro-

blasts were sensitive to tuberculo-protein, although always to a less degree than the neutrophiles and macrophages. This finding was found to be true by Moen and Swift (53).

These studies serve to explain the function of the special form of circulation discovered by Knisely (see Fig. 2 in reference 54) as characteristic of the spleen. He showed, by watching the circulation in the living organ, that the sinusoids frequently collapse through a valve-like action at either end, which traps the red cells and forces a flooding of the plasma from the vessel into the pulp spaces. In the experiments carried out in this laboratory the plasma thus flooded out of the vessels carried free antigen and neutrophilic leucocytes loaded with antigen to surround the monocytes and the macrophages of the splenic pulp. This special vascular mechanism may, therefore, be explained by stating that it serves to give the phagocytic mononuclear cells of this organ time to act.

In all of the experiments with the dye-protein carried out in this laboratory there has been evidence that the neutrophilic leucocytes play a rôle in bringing this antigen into the macrophages. Of course many of the macrophages took up the dye-protein without the aid of the neutrophiles and in their normal function the cells of the spleen are constantly phagocytizing the old neutrophiles; but the presence of the dye-protein in so many of these phagocytized neutrophiles in the spleen, as well as the phagocytosis of neutrophiles containing dye-protein by the Kupffer cells, indicates that the neutrophiles helped to bring this antigen into the reticuloendothelial cells. The present data thus provide an example of a functional reaction of both neutrophiles and monocytes within the blood stream, whereas it is true that they function for the most part in the tissues, using the blood stream for transport.

These experiments throw some light on the importance of dosage and spacing of the injections of antigens as developed in the experience of immunologists. The cells require time for two processes, namely, the preparation of the antigen for introduction into the cytoplasm and the synthesis of the new globulins. The element of time is therefore of prime importance. The final injection of antigen in rabbit H-670 served to engorge only the Kupffer cells along the portal border of the lobule. A study of the sections of the three rabbits, H-668, 669, and 670, suggests that four or five injections would be required to fill a majority of the Kupffer cells throughout the lobules to the edge of the central vein. Massive doses, on the other hand, such as were given to rabbit R 2503, served to load the cells with much larger aggregates of the dye-protein which, presumably, could not be taken up by the cell as easily as the smaller particles. Thus these data suggest a

justification for the practice of small, divided doses, in that several daily injections of moderate amounts of antigen serve to bring into action a sufficiently large number of phagocytic cells to assure an effective production of antibodies. Studied from 6 to 24 hours after such a series of injections, the cells were found to be filled with antigen in relatively unchanged state and there were no antibodies in the serum. An interval of from 4 to 7 days allows for the production of antibodies and reveals the cells with only a small amount of residual visible antigen. As an illustration of the significance of the time element in these reactions, it is interesting to compare rabbit R 2817 with R 2808 and R 2433. All three animals received the same amount of the dye-protein, namely, 3 mg., but R 2817, which had three daily injections and was studied 24 hours later, had much antigen in the cells and no detectable antibodies, while the other two, which had received spaced injections and were studied 4 days after the last injection, had minimal residual dye in the cells and antibodies in the serum. However, animals vary somewhat in the speed with which they deal with an antigen, for the two rabbits (H-668 and H-669) which had received the same amount of antigen varied in the amount of residual visible antigen in their cells in inverse proportion to the amount of antibodies in the serum.

CONCLUSIONS

1. The use of an antigen which can be seen within cells demonstrates that one may stimulate the phagocytic cells either of the liver and spleen or of the tissues and lymph nodes to produce antibodies.

2. The appearance of antibodies in the serum correlates with the time when the dye-protein is no longer visible within the cells and with the phenomenon of a partial shedding of their surface films.

3. It is thus inferred that the cells of the reticulo-endothelial system normally produce globulin and that antibody globulin represents the synthesis of a new kind of protein under the influence of an antigen.

4. An antigen is a substance which can specifically modify the synthesis of the cytoplasm of the cells of the reticulo-endothelial system.

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EXPLANATION OF PLATE 7

FIG. 1. Photograph of macrophages filled with the alum-precipitated dye-protein (red in color) from a milk spot in a film of omentum of rabbit R 5109, 24 hours after a final injection of 2 mg. of dye-protein intraperitoneally (see Table I). Stained in hematoxylin only so the red granules are due to the material injected. Each of the two macrophages contains one large, round, red globule (lower border of the upper cell and upper right border of the lower cell) which is interpreted as dye separated from the dye-protein and segregated into a single vacuole. The small blue dots in the lower macrophage are remnants of the nucleus (probably nucleic acid) of a neutrophile which had been phagocytized previously. The large, pale nucleus in the center of the figure belongs to a serosal cell. × 1,000.

FIG. 2. Monocyte and Kupffer cell containing dye-protein (red in color) near the portal border of a lobule of the liver from rabbit H-670, 6 hours after a final injection of 8 mg. of the alum-precipitated dye-protein intravenously (see Table I). The long Kupffer cell contains much dye-protein, an unstained neutrophile, and a fragment of a red cell. Near this fragment is a round blue dot from the nucleus (nucleic acid) of a phagocytized cell, probably a neutrophile. Stained in hematoxylin only. × 1,000.

FIG. 3. Kupffer cells containing dye-protein (red in color) near the portal border of the lobule of the liver in the same rabbit as that shown in Fig. 2. These cells show the folds of the surface films of the Kupffer cells which attach themselves to the opposite wall and keep the surface films stretched across the lumen of the vessel for their function of phagocytosis. Stained in hematoxylin only. × 1,000.

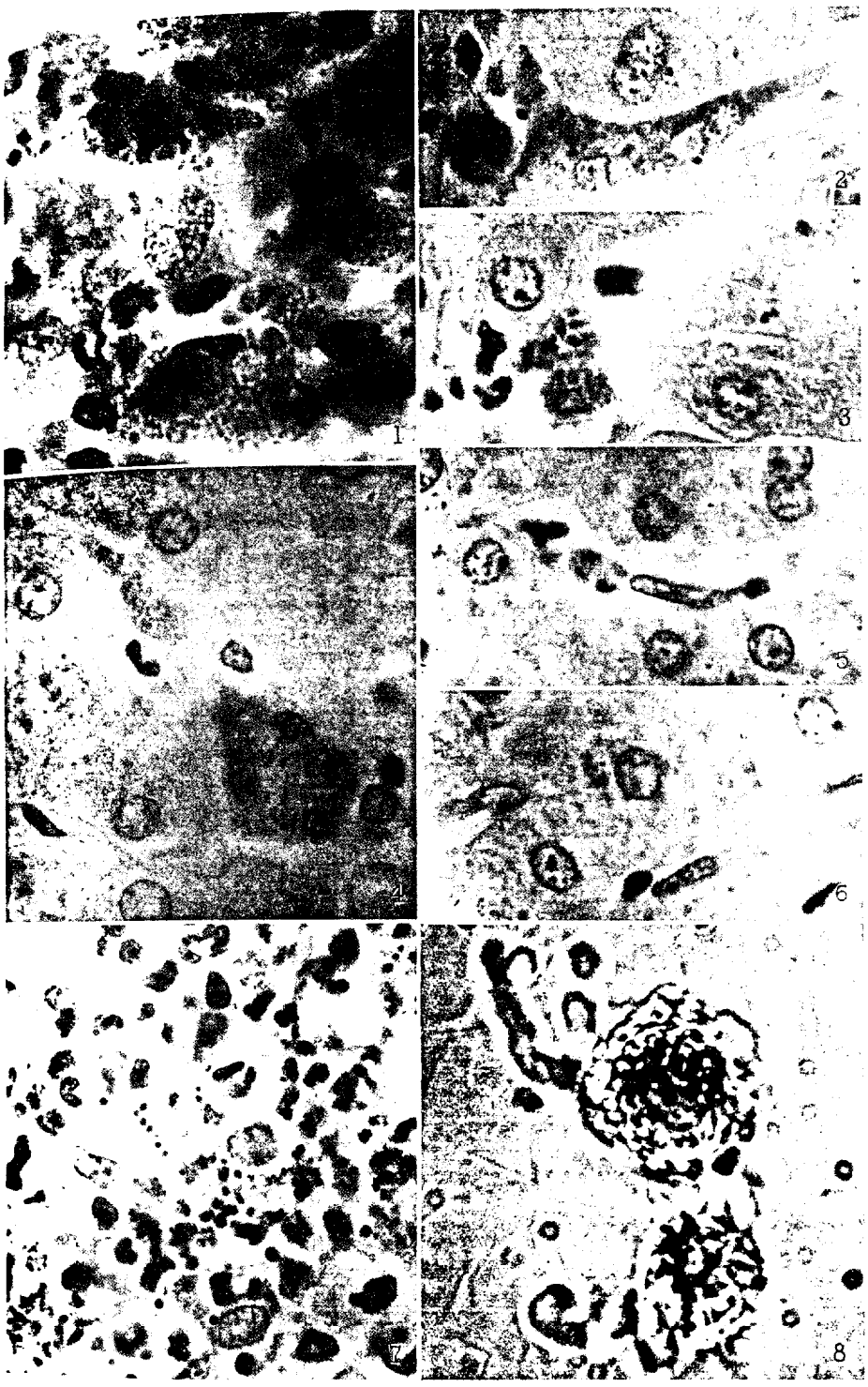
FIG. 4. Multinucleated Kupffer cell containing dye-protein (red in color), not far from the portal border of a lobule of the liver of the same rabbit as shown in Figs. 2 and 3. Stained in hematoxylin only. × 1,000.

FIG. 5. Kupffer cell from the liver of the same rabbit as shown in Figs. 2, 3, and 4, to demonstrate two phagocytized neutrophiles which themselves contain dye-protein, red in color. Stained in hematoxylin only. × 1,000.

FIG. 6. Two Kupffer cells, showing the maximum amount of dye-protein (red in color) found in any one cell in the liver of rabbit H-669, 7 days after a final injection of 8 mg. of the alum-precipitated dye-protein, when there was a relatively high antibody titre in the serum (see Table I). Stained in hematoxylin only. × 1,000.

FIG. 7. Section of the spleen showing a pulp cord from the same rabbit as in Figs. 2, 3, 4, and 5. It contains monocytes (center), macrophages, and a neutrophile (left border) filled with dye-protein, red in color. A large macrophage just below the central monocyte and another in the lower left corner of the photograph show both the red dye-protein and much nuclear debris of phagocytized neutrophiles. Stained in hematoxylin only. × 1,000.

FIG. 8. Two macrophages from the peritoneal exudate of rabbit R 2433, 4 days after an injection of 1 mg. of the alum-precipitated dye-protein (see Table I). These cells were stained with neutral red and were photographed while living. Each one shows a long process of the surface film of the cell about to be shed from the cytoplasm. × 1,800.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