VERVET MONKEY DISEASE

EXPERIMENTAL INFECTION OF GUINEA-PIGS AND MONKEYS WITH THE CAUSATIVE AGENT

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BRIEF accounts (Smith, Simpson, Bowen and Zlotnik, 1967; Seigert, Shu, Hsiu-Lu, Slenczka, Peters and Muller, 1967) have described the isolation of the causative agent of a fatal human disease associated with handling blood and tissues from vervet monkeys (*Cercopithecus aethiops*). The growth of the agent in tissue culture has been described in detail (Zlotnik and Simpson, 1968; Zlotnik, Simpson, Bright, Bowen and Batter-Hatton, 1968). This paper describes the clinical, haematological and pathological findings in guinea-pigs and monkeys infected with the disease agent.

MATERIALS AND METHODS

Animals.—Dunkin-Hartley strain guinea-pigs were used. Weaned animals weighing 250–300 g. have been used in the experiments. Four rhesus (*Macaca mulatta*) and 9 vervet monkeys (*Cercopithecus aethiops*) were used.

Inocula—(i) Whole blood from 2 patients taken during the acute stages of illness. (ii) Suspensions of post-mortem brain and kidney from 1 fatal case. Suspensions (10 per cent) were prepared in Ten Broeck grinders using a pH 7·2 phosphate buffered saline containing 0·75 per cent bovine albumin (Armour Fraction V). (iii) Whole heparinised guinea-pig blood taken during the febrile stages of the disease at various passage levels. (iv) Whole heparinised vervet monkey blood taken during the febrile stages of the disease. (v) Suspensions of infected rhesus liver prepared as above.

Guinea-pigs were inoculated i.p. with 0·1 ml. amounts or i.c. with 0·02 ml. amounts. Monkeys were inoculated i.p. with 0·5 ml. amounts.

Haematological examination.—Erythrocyte sedimentation rates (ESR) and packed cell volumes were estimated using Wintrobe tubes whilst platelet counts were calculated by the method of Brecher and Cronkite (1950) using 1 per cent ammonium oxalate as diluent. For leucocyte estimations 3 per cent acetic acid was used as diluent. Unfixed blood smears were stained with May Grunwald Giemsa or Leishman. Whole blood clotting times were measured by the method of Lee and White (1913).

Histological examination.—Guinea-pigs were killed with ether, monkeys with intracardiac nembutal and organs were removed immediately after death. Brain, lung, heart, kidneys, adrenals and testes were fixed in 10 per cent formol/saline solution. Spleen and mesenteric glands were fixed in Susa solution. Formol-fixed tissues were generally blocked after 3–5 days. Paraffin sections were cut at 5 and 7 μ and stained with haematoxylin and eosin. Susa blocks were fixed for only 18–24 hr. Brains from monkeys were generally fixed for 4 weeks, cut into blocks and then post-fixed for 24 hr. in saturated corrosive sublimate. The following additional staining methods were used: Giemsa, Machiavello, iron-haematoxylin, phloxin tartrazine, Feulgen, Von Kossa, periodic acid Schiff, Sudan IV and Sudan black.

CLINICAL OBSERVATIONS

Guinea-pigs inoculated with original human material developed a febrile illness after an incubation period ranging from 4–10 days. The febrile stage, with temperatures reaching 105° F. (40·5°) lasted for 6 days and during this

period the animals ate and drank very little, lost weight and remained hunched up and immobile in their cages. Following the febrile stage they slowly recovered. Whole, heparinised blood taken from febrile guinea-pigs was inoculated into further guinea-pigs. The incubation period shortened to 3 days and several animals died after 13–15 days. By the 8th passage the incubation period was 2–3 days and all infected guinea-pigs died 7–9 days after infection, their temperatures in the febrile stages often reaching 106° F. (41·1°). Occasionally blood taken immediately before death failed to clot.

Rhesus and vervet monkeys developed almost identical illnesses following inoculation. The incubation period varied from 2–5 days after which a febrile illness developed with temperatures of 104–105° F. (40–40·5°). The febrile stage continued until immediately before death which occurred 6–9 days after infection. None of the monkeys displayed any overt signs of illness until the day before death when they became apathetic, difficult to rouse and failed to eat or drink. Loss of weight was noted as early as 4 days after inoculation. One monkey had diarrhoea on the day before death whilst another was noted to be bleeding *per rectum* 5 days after infection. A mild skin rash, petechial in type, was occasionally noted in the terminal stages of illness on the flexor surfaces of the arms and thighs. On a few occasions blood taken during the later stages of disease failed to clot.

Blood from monkeys in the febrile stages produced the typical fatal illness when inoculated into guinea-pigs.

HAEMATOLOGICAL FINDINGS

Guinea pigs

The sedimentation rate scarcely varied throughout the course of disease being 0–1 mm./hr. in normal guinea-pigs and rising to 2 mm./hr. in the terminal stages of illness. The packed cell volume fell from 49 per cent to 44 per cent but the clotting time increased from an average of 157–425 sec. Platelets and leucocytes were not studied.

Monkeys

Similar changes were found in both rhesus and vervet monkeys. The typical changes for a rhesus monkey are summarised in the table.

Generally the sedimentation rate remained low until 5 days after infection but even in the terminal stages the rate was not greatly increased. The haematocrit fell by 5–6 per cent during the first 3 days and remained fairly constant at 33–35 per cent until death. The whole blood clotting time scarcely changed until the 7th day and even then was only modestly increased. However not all

TABLE.—*Showing Typical Blood Changes in a Rhesus Monkey Experimentally Infected with the Vervet Disease Agent*

Days after infection	ESR	PVC	Clotting time (sec.)	Total WBC	Neutr	Eos	Lymph	Mono	Platelets
0	2	39	203	4500	2580	290	1360	280	380,000
3	2	34	—	4700	2120	230	2210	140	435,000
4	2	35	201	4500	2970	40	1400	90	340,000
5	4	33	—	4500	3420	90	900	90	ND*
6	4	33	206	3700	2800	70	680	150	340,000
7	8	33	226	5800	5280	—	490	30	205,000

* ND — not calculated.

infected monkeys were tested and as already noted blood taken in the advanced stages of illness sometimes failed to clot.

The total leucocyte count in uninfected monkeys showed considerable variation but generally the total count declined after infection. Neutrophils usually decreased in number during the first few days and then increased; lymphocyte numbers increased markedly (up to 170 per cent of the pre-infection level) by the 3rd day and then fell. The final lymphocyte count in some animals was only 10 per cent of pre-infection levels. Eosinophils became less common with the course of disease more so in vervet monkeys. In animals *in extremis* eosinophils were less than 50/cu. mm.

In vervet monkeys, but not in rhesus, there was a progressive increase in the number of cells containing basophilic granules, the numbers rising from less than 5/cu. mm. early in the infection to over 300/cu. mm. by Day 7. These cells measuring about 30 μ were larger than neutrophils and the nucleus was large, usually reniform and with a less dense chromatin than polymorphs. Discrete basophilic bodies were visible in the cytoplasm. These granules varied in size but were generally 0·2–0·4 μ in diameter. The cells resembled monocytes rather than polymorphs.

The total number of platelets remained within normal limits for 5–6 days and then declined to about 50–80 per cent of pre-infection levels.

PATHOLOGY

Macroscopic Lesions

Guinea-pigs

Irrespective of the stage of disease the most striking feature was splenomegaly, the spleen being sometimes 3 times its normal size. The capsule was dull and often showed bright red effusions. On section the pulp was hard and slightly friable and very dark in colour ranging from dark brown to black. The second organ regularly affected was the liver which often was soft, mottled, light yellow to reddish brown in colour. The lungs showed varying degrees of consolidation with hard dark red foci. Occasional haemorrhages were found within the bronchi while pleural effusions were regularly found, but no fluid was present in the cavities. Brains were congested and the kidneys although mainly normal in appearance sometimes showed definite haemorrhages.

EXPLANATION OF PLATES

FIG. 1.—Guinea-pig spleen showing complete destruction of lymphocytic elements, nuclear debris, hypertrophied reticulo-endothelial cells and 2 megakaryocytes. H and E. × 375.

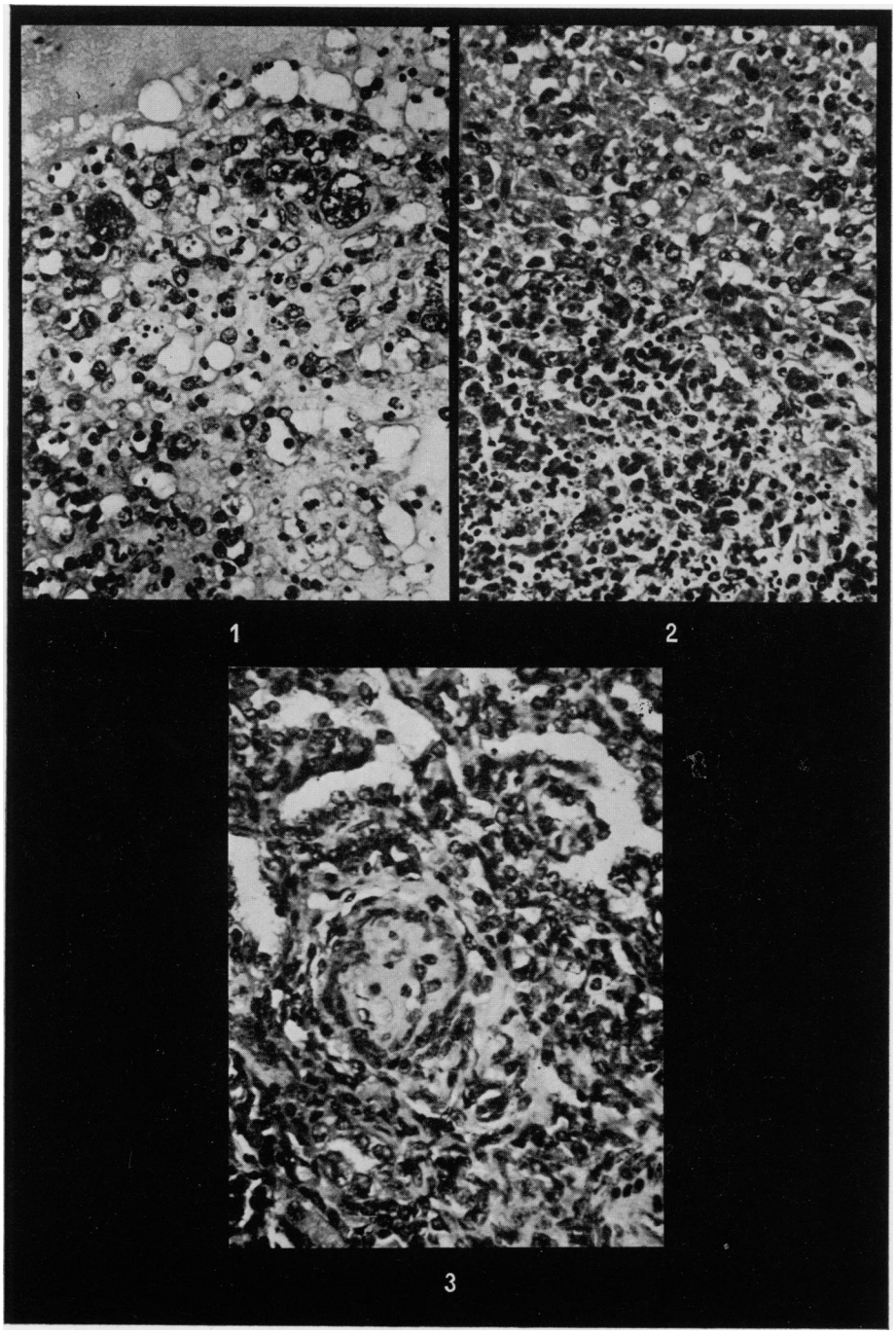
FIG. 2.—Monkey spleen showing severe destruction of lymph elements in the white pulp. H and E. × 375.

FIG. 3.—Guinea-pig lung showing interstitial pneumonitis. Note cuboidal metaplasia and occlusion of a small arteriole. H and E. × 410.

FIG. 4.—Monkey lung showing interstitial pneumonitis. Note hypertrophy of the arteriolar wall and intimal changes. H and E. × 600.

FIG. 5.—Guinea-pig liver. Note the degeneration of liver cells and appearance of intracytoplasmic bodies. H and E. × 720.

FIG. 6.—Monkey liver. Note the widespread degeneration and necrosis of liver cells. H and E. × 410.



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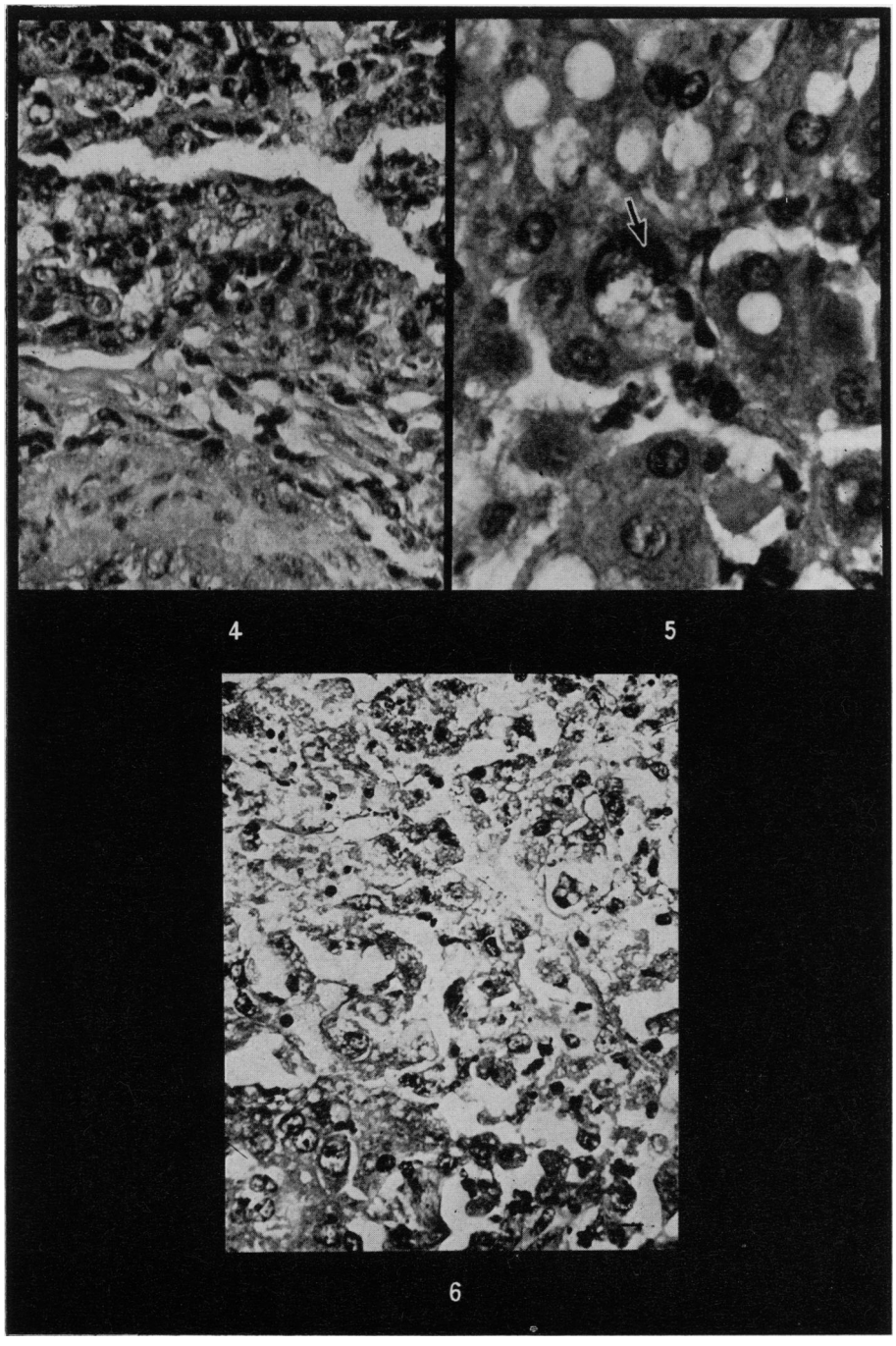

4 5

6

Monkeys

The liver was greatly enlarged, congested and very friable. On section blood poured out freely and left the whole organ a homogeneous light yellow colour. The spleen was moderately enlarged with an uneven surface dark bluish-purple in colour. On section the cut surface was rough and protruded beyond the capsule. The follicles were hardly visible but occasional small infarcts were present in some animals. Some enlargement in the mesenteric lymph nodes was noted in most animals. Apart from some congestion no gross abnormality was seen in the gastro-intestinal tract, pancreas, adrenals, kidneys, bladder or testes. In the thorax small, firm plum-coloured zones of consolidation often bilateral and involving half a lobe were found in the lungs. On section these zones were found to be well demarcated. The pleura appeared normal apart from some fibrous adhesions and no excessive pleural fluid was noted. The heart appeared normal although a small amount of clear pericardial fluid was often found. The brain appeared normal apart from congested vessels and cerebro-spinal fluid was crystal clear.

Histology

Guinea-pigs

Brain.—The only changes were in the vascular system irrespective of the route of inoculation. The endothelial cells of small venules and capillaries were hyperplastic and the lumina were either packed with RBC and occasional macrophages or filled with coagulated homogeneous albuminous material.

Lung.—Focal or diffuse interstitial pneumonitis without any peribronchial lymphocytic infiltration was found in all animals. There was marked cuboidal metaplasia of the capillary walls and increasing numbers of free macrophages were found in the alveoli. Many of these macrophages had a foamy appearance and partly digested RBC were frequently found in their cytoplasm. The bronchi occasionally contained blood and desquamated bronchial epithelium. Most of the arteries were tightly closed and some of the veins contained masses of coagulated blood (Fig. 3).

Spleen.—In the earliest passage in guinea-pigs hyperaemia, proliferation of reticulo-endothelial cells, the appearance of large numbers of macrophages packed with cellular debris and occasional normoblasts were the most striking features. The lymphoid tissue was little affected at this stage.

In later passages, however, and especially in animals killed *in extremis*, changes were usually found in both lymphoid and reticulo-endothelial tissue. The amount of lymphoid tissue was generally greatly reduced and remaining cells had the appearance of very primitive lymphoblasts. The red pulp was packed with RBC, masses of degenerating leucocytes and other cell debris. The sinuses were all filled with macrophages, coagulated serous material and degenerating reticulo-endothelial cells. At this stage the number of reticulo-endothelial cells was reduced and individual cells were usually very hyperplastic. Large clusters of megakaryocytes were seen in conjunction with the reticulo-endothelial cells (Fig. 1).

Liver.—In the first passage, no obvious degenerative or inflammatory changes were seen. Occasional single cells or groups of 2 or 3 cells could be found, however, scattered throughout the liver. These cells contained varying amounts of granules either clumped together to fill the whole cytoplasm or as small elongated

structures which were either discrete and sharply defined or arranged in clumps or ringlets. They were generally basophilic staining dark-purple with haematoxylin and eosin while in Giemsa preparations they appeared as reddish-purple granules. With Machiavello's method the granules appeared bright-red and in Feulgen preparations similarly situated positive material was found in the cytoplasm of the liver cells. The granules were also PAS positive and stained brown with Von Kossa's method. After treating sections with N HCl for 10 min. at 60° Von Kossa's reaction was negative but when the same sections were counterstained with haematoxylin and eosin the granules were found apparently undamaged within the cells and staining dark-purple. However they appeared invariably slightly smaller in size than in sections not treated with N HCl. In cells packed with large numbers of granules there seemed to be a gradual deposition of calcium around the minute structures resulting in a complete obliteration of the cell by an amorphous mass. In 3rd and 4th passage guinea-pigs very small focal necrotic lesions could be found in the livers but in some cases these necrotic areas were confluent and formed sharply circumscribed areas. On 2 occasions the centre of the necrotic mass was filled with coagulated blood. As a rule no granules were found in the centre of these necrotic areas but comparatively large numbers of granules were found in cells at their periphery. In cells undergoing early degeneration without nuclear changes, the cytoplasm appeared to contain only small numbers of discrete granules.

Livers of guinea-pigs that developed the disease as a result of inoculations with material from monkeys (previously passed 9 times in guinea-pigs and 3 times in rhesus monkeys) showed very advanced lesions. Degeneration of liver cells was very widespread and necrotic cells were so frequent that about 50 per cent of these cells were undergoing eosinophilic degeneration and necrosis. In addition, about one third of the liver cells were found to contain clumps of basophilic bodies whilst free macrophages and Kupffer cells also contained large numbers of the minute intracytoplasmic bodies (Fig. 5). These bodies were very discrete in the less affected parenchymatous cells while in necrotic cells they appeared to be fused by a homogeneous substance staining darkly with haematoxylin. As in earlier passages the granules were pleomorphic and mostly slightly elongated with a few round bodies. The round bodies measured 0·3 μ and the elongate up to 0·7 μ.

The remaining organs showed no characteristic lesions except that there was marked proliferation of the reticulo-endothelial cells in lymphoid tissue.

Monkeys

Brain.—In general, changes were confined to the capillaries and the small venules and arterioles and resembled those already described in guinea-pigs.

Lung.—Interstitial pneumonitis was present in all infected animals but the degree of lung involvement varied considerably. In animals with a shorter clinical history only foci of cuboidal metaplasia of alveolar cells were observed. In other cases large areas of lung were severely affected resulting in a mass of epithelium-like cells with macrophages often laden with blood pigment in the luminia of air sacs. The muscular layer of all arterioles was very hyperplastic and it was not uncommon to see small arteries where this hyperplasia had caused complete obliteration of the lumen (Fig. 4).

Spleen.—The only spleens examined were from animals in advanced stages

of disease. Severe changes were observed in the red pulp in all cases but lesions in the white pulp varied from almost complete destruction of all lymphoid elements to only a depletion of lymphoid cells, with necrotic areas in the central part of lymphoid germinal centres, and the appearance of large groups of hyperplastic phagocytic reticular cells. The red pulp was usually engorged with RBC and products of cellular degeneration including masses of fragmented nuclear matter. The splenic cords were completely devoid of lymph cells while many reticulo-endothelial cells were destroyed. Other reticulo-endothelial cells appeared to be greatly hypertrophied and the number of free macrophages was visibly increased (Fig. 2).

Liver.—Widespread degeneration of liver cells was a constant feature in all the affected monkeys. However, while some cells showed only parenchymatous degeneration an increasing number of liver cells were necrotic; or large foci of cells were noted in various stages of necrosis or complete disintegration. Such cells stained strongly with eosin and had only small remnants of nuclear matter in their cytoplasm. They either retained the shape of liver cells or resembled a mass of granular material. Kupffer cells, in various transitional stages, were often very enlarged and contained basophilic material or products of RBC disintegration. These cells were very obvious and the number of free macrophages containing similar basophilic material was much increased. Free macrophages were seen inside the sinusoids especially around the periportal spaces. In some livers large accumulations of monocytic cells were noticeable in the periportal spaces, but in other monkeys showing very advanced liver necrosis no such mononuclear infiltrations could be found. So far the intracytoplasmic basophilic bodies described above in the livers of infected guinea pigs have not been seen in any of the monkey livers (Fig. 6).

DISCUSSION

The disease in guinea-pigs and monkeys is very similar in many respects except that not all monkeys develop the degree of interstitial pneumonitis seen in guinea-pigs and that intracytoplasmic basophilic bodies within the liver cells were not seen in monkeys. Although light microscopy did not reveal any such bodies in the livers of affected monkeys, preliminary electron microscopic examination shows the presence of intracytoplasmic structures somewhat similar to those of guinea-pigs (unpublished). The differences in the visibility of these bodies which may possibly constitute the pathogen in monkeys and guinea-pigs can be explained on the basis that the actual infective agent in both animals is too small to be seen by the light microscope. In the guinea-pig liver, however, the presence of the pathogen provokes deposition by the host of a substance around the bodies which increases their size and improves their staining properties, bringing them within the resolution of the light microscope. Further deposition of this matrix-substance causes fusion of large numbers of these bodies into granular intracytoplasmic masses and subsequent calcification of the matrix produces the positive Von Kossa's reaction. The facts that the large calcified clumps are broken down after incubation at 60° in N HCl; and the individual intracytoplasmic bodies, when stained with haematoxylin, appear smaller, supports this hypothesis.

In previous contributions the authors (Zlotnik and Simpson, 1968; Zlotnik *et al.*, 1968) described characteristic inclusion bodies in infected BHK tissue culture cells and showed that the supernate of cultures after freezing and thawing

produce the same disease in guinea-pigs irrespective of whether the tissue cultures were infected with material from guinea-pigs or monkeys. The disease is thus caused by the same agent, and the great similarity of lesions in lung, spleen and liver shows that the actual disease is identical in the guinea-pig and in the monkey.

The results of the examination of the monkeys' blood, although not entirely conclusive, show that the lymphocyte depletion observed in the spleen is accompanied by a severe drop in the lymphocyte count in the peripheral blood after an initial increase. There appears to be a decrease in the number of platelets in the later stages of disease but the decrease appears insufficient to explain the terminal bleeding tendency found in some cases both in monkeys and guinea-pigs.

SUMMARY

In both the monkey and the guinea-pig the principal pathological changes are confined to the liver, spleen and lung. However, not all the monkey lungs are equally affected. The disease has a destructive effect on the reticulo-endothelial system and causes depletion of the lymphocytic elements in tissues and peripheral blood.

The lungs of guinea-pigs show various degrees of interstitial pneumonitis with increased formation of free macrophages. In monkeys, on the other hand, only about two-thirds of cases had lung lesions resembling those in the guinea-pig. In the remainder, apart from oedema and congestion, no other major changes could be seen.

The liver in both animals is equally affected by a degenerative necrotising process without giving rise to a cellular reaction. In guinea-pigs varying numbers of liver cells (and macrophages in later passages) were found to contain clusters of minute, deeply staining, basophilic intracytoplasmic bodies.

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