Identity of the Filterable Hemolytic Anemia Agent of Sacks with *Haemobartonella muris*

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ABSTRACT

MOORE, D. H. (The Rockefeller University, New York, N. Y.), R. N. ARISON, H. TANAKA, W. T. HALL, AND M. CHANOWITZ. Identity of the filterable hemolytic anemia agent of Sacks with *Haemobartonella muris*. J. Bacteriol. **90**:1669-1674. 1965.—In 1960 a new hemolytic agent in rats was reported. It was thought to be a filterable, nonsedimentable, replicating infectious agent, different from *Haemobartonella muris*. Rats which recovered from the infection developed a resistance to several kinds of transplantable tumors. It is here shown that this agent has the same properties as those reported in the literature and reconfirmed by us for *H. muris*. The size of the agent is approximately 500 m μ as determined by correlating bioactivity with Gradocol membrane filtrates and fractions from a diffusion cell, and its density is about midway between that of whole serum and distilled water (approximately 1.020) as determined by the sedimentation of bioactivity in an ultracentrifuge. Diffusion at 30 C indicated a lack of motility by the agent bodies. Rats were found to be protected against infection with the agent by daily administration of chlortetracycline and by prior infection with *H. muris*. The agent bodies were indistinguishable from *H. muris* in both the light and the electron microscope.

Sacks, Clark, and Egdahl (1960) and Sacks and Egdahl (1960a, b) described a transmissible replicating agent in rats that produced an active hemolytic anemia, tumor immunity, and other biological manifestations related to the neoplastic process. It was claimed that rats which recovered from infection with this agent developed resistance to the growth of standard transplantable rat tumors and that rats which were carrying tumors were particularly susceptible to the agent. Six different types of tumor were tested. The infectious agent was designated as a filterable hemolytic anemia (FHA) agent and was believed to be different from *H. muris*, a minute blood parasite of rats.

To find out more about the physical and biological properties of the FHA agent, we have compared it with *H. muris*, studied its sedimentation and density in the ultracentrifuge, and correlated its bioactivity with Gradocol membrane filtrates and with fractions taken from a diffusion cell.

A preliminary report of these studies (see also following paper by Tanaka et al., 1965) was read at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology held in April, 1965 (Moore et al., Federation Proc., **24**:403, 1965).

MATERIALS AND METHODS

Rats infected with the FHA agent were kindly provided by Jerome Sacks. The rats were bled, and this initial supply of whole blood was stored at -70 C.

Pen-bred Sprague-Dawley rats of both sexes were used in all of our experiments. Rats of the Amsterdam strain were found to be susceptible to the agent, but were not used in the several tests reported here. In collecting blood samples, heparin or acetic-dextrose solutions were sometimes used to prevent clotting. Infections were usually made by inoculating 0.1 or 0.2 ml of whole blood, serum, or dilutions thereof intraperitoneally into animals that had been splenectomized 5 to 15 days previously. Intact rats are not satisfactory for bioassays because, although they demonstrate the hemolytic syndrome, its duration is usually short and may be missed. Infection of splenectomized rats almost always resulted in death within 14 days, the time required depending on the titer of the inoculum.

RESULTS

Ultracentrifugation. To determine whether or not the active agent could be sedimented in the ultracentrifuge, infectious serum, undiluted and at various dilutions, was subjected to centrifugation in a Spinco swinging-bucket rotor (SW 39) run at 39,000 rev/min for various periods of

TABLE 1. Assays of ultracentrifuge fractions of serum

Fraction	No. of rats inoculated	No. of rats showing hemoglobinuria				
		6th day	7th day	8th day	9th day	10th day
Undiluted serum, centrifuged for 4 hr at 39,000 rev/min						
Top	2			1	1	
Middle	2		1		1	
Bottom	2		—	—	—	—
Diluted 1:2 with phosphate-saline buffer, centrifuged for 4 hr at 39,000 rev/min						
Top	2				1	1
Middle	2			1		1
Bottom	2		1	1		
Diluted 1:4 with phosphate-saline buffer, centrifuged for 4 hr at 39,000 rev/min						
Top	2		2			
Bottom	2	1		1		
Diluted 1:4 with distilled water, centrifuged for 16 hr at 39,000 rev/min						
Top	3		—	—	—	—
Bottom	3				1	2
Top fractions after centrifugation for 5 hr at 39,000 rev/min						
Undiluted serum	2		2			
Serum diluted 1:2 with water	2				1	1
Serum diluted 1:5 with water	2					1

time, as indicated in Table 1. After each run, the ultracentrifuge was slowly stopped, and the contents of each tube were divided into five 1-ml samples. The first (top), third (middle), and fifth (bottom) samples were assayed (Table 1) in splenectomized rats by intraperitoneal injection of 0.2 ml.

When undiluted serum was ultracentrifuged for 4 hr, the bottom 1 ml contained no assayable agent, whereas the middle and top fractions did. When diluted with 2 parts or 4 parts of phosphate-buffered saline (PBS; 0.02 M sodium phosphate; 0.15 M sodium chloride, density 1.008 g/cm³), activity neither sedimented nor rose. However, when the serum was diluted with 4 parts distilled water and centrifuged for 16 hr, no activity was found in the top fraction. After 5 hr of centrifugation, the top fraction of undiluted serum killed in 7 days the two test animals inoculated; for serum diluted with an equal volume of water (1:1), it took 9 and 10 days, respectively, to kill the rats, but at a 1:4 dilution with water, only one of the rats died (10th day). Thus, the density of the agent seemed to be about midway between that of distilled water and whole serum, because it slowly sedimented in the former and tended to float in the latter. The densities of a test serum at the three levels in the tube after centrifugation for 15 hr were 1.007, 1.020, and 1.108 g/cm³ for the top, middle, and bottom

fractions, respectively. The density of the serum before centrifugation was 1.031.

Gradocol filtration. Undiluted serum separated from clotted blood taken from rats during the acute phase was filtered through Gradocol membranes (Bernard and Elford, 1932; Elford, 1933) of various pore sizes, obtained from the National Institutes of Health, Bethesda, Md. The filtrations were carried out in a cold room (4 C). Into the 3-cm diameter filter chamber was placed 0.5 ml serum, and 10 psi of positive pressure from a N₂ cylinder were applied until 0.2 ml of filtrate was collected below; 0.5 ml of Eagle's medium was then added to the filter chamber, and the pressure was again applied until the total volume of filtrate was 0.4 ml. The filtrates were assayed by injecting 0.1 ml intraperitoneally into splenectomized rats. The results of these experiments are recorded in Table 2. Membranes with pores smaller than 460 m μ did not pass active filtrates. A membrane with 480-m μ pores gave a filtrate that killed two of three inoculated rats and one with 800-m μ pores gave a filtrate that killed three of four. The infectious particle, therefore, has a diameter in agreement with the bodies seen in the electron microscope (see succeeding article).

Diffusion and motility. Another procedure used to determine the size of the infectious particle was free diffusion (Moore et al., 1959; Breese and Trautman, 1960). The cell used in these experi-

ments consisted of a glass block containing a 5 by 5 mm square "well" 2 cm deep and five sliding ground glass plates, each 3 mm thick and with a 5 by 5 mm square hole in its center, the surfaces being lubricated with silicone grease. Fresh, undiluted, infectious serum was placed in the "well," and the channels of the sliding section were filled with phosphate-buffered saline (pH 7.2) in experiments 1 and 2 and with a diluted culture medium in experiments 3 and 4. The diluted culture medium contained 0.37% tryptone (Difco), 12.5% fresh rabbit serum, 0.00005% ascorbic acid, and 0.0002% glutathione (Ford and Murray, 1959). After the cell had come to equilibrium temperature at either 4 or 30 C, a boundary between serum and overlay was formed by aligning the column for periods of 18, 22, or 48 hr. The plate compartments were then segregated by disaligning the column, and the contents of each compartment were diluted with 3 parts of fresh overlay fluid (PBS or culture medium) for assay in splenectomized rats. The results of these experiments are presented in Table 3. The conclusions indicated by these experiments are that the bioactivity of Sacks' agent is carried by a relatively large particle and that the particle has no detectable motility of its own, even at 30 C and in a culture medium reported to support replication of *H. muris* (Ford and Murray, 1959). If the activity were carried by a small virus, as was thought by Sacks et al., the disease would have been transmitted by the contents of cell sections above the first 3-mm section overlaying the "well" of the diffusion cell. Some activity was detected in this first section in all experiments, but this activity may have been restricted entirely to the first 1-mm layer. Since we do not know the titer of the activity at various levels within this first 3-mm section above the original boundary, we cannot calculate the exact diameter of the assumed spheres responsible for the bioactivity. However, in no experiment could they be smaller than about 200 m μ (see Trautmann and Breese, 1960).

Since the filtration data (Table 2) and the electron microscopy (see following paper) established the size of the agent at approximately 500 m μ , the diffusion data indicate that the agent is essentially nonmotile. Otherwise, activity would have been found at a higher level in the cell. In experiment 4 (Table 3), where the temperature was 30 C and the "well" was overlaid with culture medium, activity was found in the well after 18 hr but very little in the first section above it (see following paper for further discussion of motility).

Chlortetracycline sensitivity. In this experiment all of the rats were splenectomized, adult males

TABLE 2. Assays of filtrates passing through Gradocol membranes

Sample no.	Pore size of filter	No. rats dying/ no. inoculated
1	m μ	
	110	0/3
	130	0/3
	Unfiltered	2/3
2	110	0/3
	130	0/3
	Unfiltered	3/3
3	240	0/3
	330	0/3
	Unfiltered	3/3
4	480	2/3
	Unfiltered	2/3
5	350	0/4
	460	1/4
	525	1/4
	800	3/4
	Unfiltered	4/4

weighing about 300 g. Each of 15 rats was inoculated intraperitoneally with 0.2 ml of freshly collected citrated blood drawn from a rat at the height of anemia crises. Treatment with chlortetracycline was begun immediately in 10 of the rats, the other 5 serving as controls. Into each of the 10 rats 15 mg of chlortetracycline in 1 ml of saline were injected subcutaneously twice daily. One half of the animals received 7 injections and the other half received 13. The five controls received seven subcutaneous injections of isotonic saline. All five of the control animals exhibited the usual symptoms of anemia and died on the 3rd or 4th day after inoculation. One of the chlortetracycline-treated animals died on the 1st day of the experiment, but Giemsa-stained blood smears showed no erythrocyte lesions, and neither the serum nor the urine showed evidence of hemolysis. Since there were no signs of anemia and since FHA has never been known to cause death in less than 48 hr, it was assumed that this rat died of other causes. On the 3rd day when the Giemsa-stained smears of all five of the control animals were highly positive, the blood smears of the chlortetracycline-treated animals were negative. With the noted exception, all of the experimental animals survived until the ninth day when two more died, again without any of the signs of the FHA syndrome. At 2.5 weeks after the beginning of the experiment, four of the seven survivors were challenged with a second inoculation of FHA. These rats all exhibited the ane-

TABLE 3. Assays of fractions from diffusion cell

Expt no.	Diffusion time	Temp	Location of sample and dilutions	No. of rats inoculated	No. of rats showing hemoglobinuria						Total dead/inoculated
					7th day	8th day	9th day	10th day	12th day	13th day	
1	22	4	3-6 mm	3							0/3
			0-3 mm	3				1	1		2/3
			Well 10 ⁻¹	4	2	2					4/4
2	48	4	6-9 mm	3							0/3
			3-6 mm	3							0/3
			0-3 mm	3			1		1		2/3
			Well 10 ⁻¹	3		3					3/3
			Well 10 ⁻²	3			1	2			3/3
			Well 10 ⁻³	3				3			3/3
			Well 10 ⁻⁴	3							0/3
			Well 10 ⁻⁵	3							0/3
3	18	4	3-6 mm	2							0/2
			0-3 mm	2							1/2
			Well, undiluted	2	1			1		1	2/2
			Well 10 ⁻¹	2		1	1				2/2
			Well 10 ⁻²	2				1			1/2
4	18	30	6-9 mm	2							0/2
			3-6 mm	2							0/2
			0-3 mm	2							1/2
			Well, undiluted	4	1	3				1	4/4

mia syndrome and died within 4 days. It is concluded that FHA infection is inhibited by chlortetracycline, as is *H. muris* (Mayer, 1949; Wigand and Peters, 1950).

H. muris. Even though Sprague-Dawley rats are reportedly free from agents resembling *H. muris*, as a precaution against the possibility that some might be carriers, all rats were watched during the 1st week after splenectomy for hemoglobinuria. The value of this precaution became evident when hemoglobinuria was observed in one rat 4 days after splenectomy. Blood was collected from this animal and injected intraperitoneally into two healthy, splenectomized rats. Both of these rats contracted a fatal anemia with all the symptoms of FHA and of *H. muris* infection, as described in the literature. To test whether the new agent (*H. muris*) would cause an immunological response that would protect against FHA or conversely FHA would protect against *H. muris*, one group of six intact rats was inoculated with FHA (intact animals seldom succumb to FHA infection, whereas splenectomized ones almost always do) and a second group of six with the new agent (*H. muris*). At 2 weeks after the inoculations, the animals of both groups were splenectomized. At the end of a second 2-week period none of the animals developed anemia, indicating

that they had already passed through the syndrome before they were splenectomized. Three of the six rats that had received FHA were then challenged with the *H. muris*, and three of the six that had received *H. muris* were challenged with the FHA agent. None of the animals developed anemia, indicating that the two agents caused a similar immunological response.

DISCUSSION

The group of microorganisms classified under family Rickettsiales and which includes *H. muris* and *Eperythrozoon coccoides* has received relatively little attention. Recent findings (Niven et al., 1952; Sacks et al., 1960; Arison, Cassaro, and Shonk, 1963) suggest that these organisms are of more than taxonomic interest. Both *H. muris* and *E. coccoides* are widely prevalent among laboratory animals, and their presence may alter experimental findings. An awareness of the occurrence of these agents and an increased knowledge concerning their morphology and physical properties should contribute to the proper evaluation of experimentally induced disease processes.

The assays of filtrates passing through calibrated Gradocol membranes indicated that the bioactivity in FHA-infected blood is carried by particles about 0.5 μ in diameter, in agreement

with the size of the bodies seen in close association with erythrocytes in the electron microscope, and that no infectious activity is carried by bodies very much smaller than 0.5μ .

The density of these bodies is lower than that of most infectious agents, because they do not sediment when subjected to ultracentrifugation in serum. Both viruses and bacteria have specific gravities that permit sedimentation in serum, and most mycoplasmas are also readily sedimentable. But the infectious anemia agent has an unusually low specific gravity.

The diffusion data do not permit an accurate calculation of size, because in none of the experiments did activity diffuse above the first plate (0 to 3 mm above the original boundary). Some bodies should be found at this location (0 to 3 mm), irrespective of size, because there is always some mixing in the immediate neighborhood of the boundary as it is made by aligning the plates with the "well." The detectable decrease in bioactivity in this first section and the lack of activity further up in the cell do indicate, however, that the bodies responsible for the bioactivity are greater than $200 m\mu$ in diameter. Likewise, the lack of bioactivity in the upper sections even at 30°C and in a favorable culture medium rules out the possibility of the agent's possessing active motility.

As to the relationship of this hemolytic agent (FHA) to *H. muris*, all of the data suggest that they are identical. Sacks and Egdahl (1960a) listed five characteristics of their agent that they thought differed from reported characteristics of *H. muris* (Weinman, 1944; Peters and Wigand, 1955). These were (i) that FHA passed through filters of small pore size, (ii) that FHA infection produced a high degree of immunity which was humoral, (iii) that there were no relapses of the hemolytic disease in animals that recovered from FHA infection, (iv) that mature and immature rats are equally susceptible to FHA, and (v) that 10^{-5} ml of FHA-containing serum could produce the disease in mature intact rats. Our study showed that, although the FHA agent is filterable, the pore size of the filters must be much larger than that reported by Sacks et al. (1960). The particle size indicated by the Gradocol filtrations (Table 2) is in agreement with the size of *H. muris* reported in the literature (Peters and Wigand, 1955). A careful study of the published reports on *H. muris* and on FHA does not justify the differences claimed in the other four statements. Permanent immunity with no relapses for FHA was not clearly shown by Sacks and co-workers. In some of the early work by others, rats were used which were not entirely free of *H. muris*; therefore, it is likely that many of the adult animals

carried an immunity resulting from a previous infection, thus accounting for the difference in susceptibility of mature and immature rats. The titer of FHA infectious serum varies greatly from one animal to another, depending on the individual response of the animal and the stage of the infection at the time the serum is taken. Often a low-titer serum is found for FHA, as was reported for *H. muris* (Weinman, 1944; Rudnick and Hollingsworth, 1959).

When our rat spontaneously developed the syndrome after splenectomy without being exposed to Sacks' agent, the infectious blood was transferred to other rats which, if not splenectomized, survived and became immune to infection with either the new agent (*H. muris*) or Sacks' agent. After a rat had recovered from the syndrome, splenectomy did not cause it to reoccur. We do not know, however, whether immunity is permanently conferred and that animals could not be infected again many months later.

Although the spontaneous *H. muris* isolated in our laboratory effectively cross-reacted with Sacks' agent with respect to resistance to reinfection, the possibility of small immunological differences which were not detectable in this test should not be excluded.

Although *H. muris* cells in the peripheral blood were larger than Sacks' agent in peripheral blood, they were within the size range of the latter in bone marrow (see Fig. 12 in the following paper). Their ability to produce permanent cross-immunity, their morphological similarities (see Fig. 2 and 7 in the succeeding paper), and the ability to protect against infection of Sacks' agent with chlortetracycline indicate that they are the same infectious agent.

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