LEUCOCYTIC SECRETIONS.

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I.

INTRODUCTION.

Leucocytes may be regarded as mobile unicellular glandular bodies which set free their secretions in the humors of the organism. But little is known of the nature and functions of the substances they secrete. In the following experiments, leucocytes were cultivated *in vitro* and a study was made of certain modifications of the medium caused by their secretions.

II.

Technique.

The leucocytes were obtained from hen blood and cultivated in Gabritschewski dishes. The serum was extracted from the culture medium and the variations of its inhibiting action on homologous fibroblasts, and of its lytic action on foreign erythrocytes, were measured.

1. Preparation of the Leucocytes.—The blood was taken from young adult chickens which had fasted for 24 hours. The animals were bled from the carotid artery or from one of the veins of the wing, through an oiled cannula or syringe. The blood was received in cold paraffin-coated tubes and centrifuged in ice for 10 minutes. After the plasma had been removed, a few drops of Ringer solution containing a small amount of embryo juice were placed at the surface of the corpuscles and the tubes were allowed to stand at room temperature for about 15 minutes. Then the film of leucocytes, which covered the erythrocytes, became solid, and could be removed to a watch-glass filled with Ringer solution. The film was washed almost free of erythrocytes and cut in small fragments of about equal size.

2. Preparation of the Medium.—The medium consisted of chicken plasma and embryo tissue juice. Its composition was generally as follows:

Chicken plasma	5.00 volumes.
Embryonic tissue juice	0.25 volume.
Tyrode solution	2.75 volumes.
Distilled water	2.00 volumes.

The medium was rendered hypotonic on account of the evaporation which takes place in the Gabritschewski dishes. In some cultures, casein was added to the medium. 1 gm. of casein was dissolved in a diluted solution of sodium hydroxide, and dialyzed against tap water for 3 days. Then it was again brought into solution by the addition of a little alkali, and rendered isotonic by a proper amount of NaCl. The pH of the solution was about 7.8. The amount of the solution used in the medium varied from 0.1 to 1 volume of a 1 per cent casein solution; that is, the concentration of casein was from 0.1 to 1 per 1,000.

3. Preparation of the Cultures.—The cultures were made on Gabritschewski dishes, the covers of which had been replaced by mica plates. The fragments of leucocytic films were suspended in 5 volumes of Tyrode solution, distilled water, and embryo juice in the concentrations previously described. The same number of fragments was used for each culture. Then 5 volumes of plasma were spread on the mica plate, with the necessary precautions to prevent bacterial contamination from the air. Afterwards, the leucocytic suspension was dropped from a pipette into the plasma, and thoroughly mixed with it. Care was taken to distribute the fragments of leucocyte film evenly. Their number was about thirty to each dish, in order that the colonies might grow freely around each fragment, without being in contact with the edges of the neighboring colonies before 48 hours. Controls were prepared consisting of the medium without leucocytes. Both cultures and their controls were incubated for 48 hours at 38°C. Other cultures and controls were placed in the refrigerator for the same time.

4. Preparation of the Serum from the Cultures.—After 48 hours, the cultures were examined. Around each fragment, a large colony of leucocytes had grown. The edges of the colonies were generally in close contact. Microscopic examination showed whether the cells

were living and in active condition, and whether bacterial contamination had occurred. Then the boxes were opened and the coagulum was sliced with fine scissors. The fluid and the fragments of coagulum were centrifuged at high speed for 10 minutes. The supernatant fluid was removed and its H ion concentration ascertained by the technique described by Felton.[1] The serum of the cultures of leucocytes always became slightly acid after 48 hours in the refrigerator. When the colonies had grown actively in the incubator for 48 hours, the acidity of the medium was much more marked. The pH of the serum from the cultures was brought to about that of normal serum by adding a small amount of sodium hydroxide.

5. *Action of Serum on Chicken Fibroblasts and on Foreign Erythrocytes.*—The sera from cultures with and without leucocytes, incubated or kept in the refrigerator, were compared from the standpoint of both their hemolytic effect on sheep or rabbit erythrocytes and their inhibiting power on chicken fibroblasts. The hemolytic action is expressed in the tables by the amount of serum required for a given degree of hemolysis. The action on chicken fibroblasts was ascertained by the rate of growth of a 10 year old strain of fibroblasts in a medium composed of 2.5 volumes of chicken plasma, 5 volumes of serum from leucocyte cultures, 2.2 volumes of Tyrode solution, and 0.3 volume of embryonic juice. In the tables, the rate of growth is indicated by the relative increase of the fragment of fibroblasts in 48 hours. The quotient of the average rate of growth of the experiment by the average rate of growth of the control expresses the action of serum on homologous fibroblasts.

III.

RESULTS.

The experiments consisted in measuring the inhibiting effect on chicken fibroblasts and the hemolytic power on sheep erythrocytes of the serum from media without leucocytes and of the serum from similar media with leucocytes, after the colonies had been allowed to grow for 48 hours. Other experiments were made in order to study the action of temperature on serum and cells. A culture medium without

[1] Felton, L. D., *J. Biol. Chem.*, 1921, xlvi, 299.

TABLE I.

Action of Serum from Cultures of Chicken Leucocytes on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (*C*).	With leucocytes (*E*).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
					per cent	*per cent*	*per cent*
1	1410	1.50	3.00	2.00	0	0	0
2	24858–1	2.65 2.85 2.74	3.00 3.84 2.56	1.13	0	0	0
3	24858–2	2.19 3.27 2.82	4.60 4.10 3.44	1.51	0	0	0
4	25171	1.94 2.36 1.86	3.05 3.65 3.17	1.60	0	0	0
5	25213	2.53 2.94 2.66 3.40	3.97 4.46 4.28 5.43	1.57	0	0	0
6	25391	2.77 2.40 2.02 3.28	3.90 3.35 3.64 4.00	1.45	0	0	0
7	28616	2.59 3.00 2.57	4.57 4.94 4.16	1.71	0	0	0
8	28801	3.07 3.19	5.41 4.37	1.56	0	0	0
9	28684	1.62 2.13	3.40 4.09	2.00	70 50 30	0 0 0	40 20 5
10	28641	2.66 4.13	4.57 4.29	1.37	70 50 30	0 0 0	45 25 5

TABLE I—*Concluded.*

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
					Amount of serum.	Amount of hemolysis by serum from cultures.	
		Without leucocytes (*C*).	With leucocytes (*E*).			Without leucocytes.	With leucocytes.
					per cent	*per cent*	*per cent*
11	28770	6.18 3.38	7.00 7.68	1.70	70	0	0
12	29263	4.41 2.57 2.60	9.07 6.30 5.29	2.18	80 40 20	7.00 5.00 2.50	10 10 4
13	28801	3.07 3.19	5.41 4.37	1.56	70	0	0
14	28886	2.38 3.10	1.70 4.66	1.10	70 50 30	0 0 0	40 30 8
15	28926	0	0		80 60 20 10	5.00 2.50 2.50 2.50	40 20 5 2.50
16	29019	0	0		80 60 40 20	10.00 8.00 5.00 2.50	20 15 8 3
17	29162	1.21 3.20 2.26	6.77 5.15 6.38	3.33	80	0	0
18	29400	0	0		80 60 40	60.00 50.00 30.00	75 55 30
19	29059	1.87 3.86 3.38	4.14 6.70 6.80	1.98	80	0	0

leucocytes kept in the refrigerator for 48 hours was compared with a similar medium incubated for the same length of time. The sera from culture media with and without leucocytes, kept in the refrigerator instead of being incubated, were also compared.

1. Serum from Media Incubated for 48 Hours, with and without Leucocytes.—In every case, the colonies grew actively and were still living after 48 hours. In seventeen experiments (Table I), the serum from the media with leucocytes was more favorable to the activity of fibroblasts. The quotient of the rate of growth in serum from leucocyte cultures divided by the rate of growth in serum from media without leucocytes averaged 1.73.

TABLE II.

Action of Serum from Culture Media, Incubated and Refrigerated, on Homologous Fibroblasts and Foreign Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from culture media.		Ratio, $\frac{E}{C}$.	Hemolytic action on foreign erythrocytes.		
					Amount of serum.	Amount of hemolysis by serum from culture media.	
		Incubated (*C*).	Refrigerated (*E*).			Incubated.	Refrigerated.
					per cent	*per cent*	*per cent*
1*	29094	1.25	6.53	4.49	80	8	80
		1.68	6.33		20	5	50
					10	0	25
2†	29339	1.04	2.38	3.20	70	15	80
		0.87	3.73		50	8	80
		1.34	4.10		30	3	70
3†	29436	0	0		80	55	75
					40	40	70
					10	10	20

* In Experiment 1, sheep erythrocytes were used.

† In Experiments 2 and 3, rabbit erythrocytes were used.

The hemolytic power of the serum on sheep or rabbit erythrocytes was measured in eleven experiments. In four cases (Table I), both sera were inactive. In seven cases (Table I), the sera from cultures without leucocytes were inactive, while the sera from cultures with leucocytes were hemolytic for sheep or rabbit corpuscles. After the serum had been heated at 56°C., its hemolytic action disappeared completely.

(*a*) *Serum from Culture Media without Leucocytes, Incubated and Refrigerated.*—The effect of heat at 38°C. for 48 hours on media without leucocytes was examined (Table II). The natural hemolytic action of the serum on sheep or rabbit corpuscles decreased after the medium had been incubated, while it remained normal when the medium was kept in the refrigerator. At the same time, the serum from the incubated medium became much less favorable to the growth of chicken fibroblasts (Table II).

TABLE III.

Action of Serum from Cultures of Chicken Leucocytes, Refrigerated, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (*C*).	With leucocytes (*E*).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
					per cent	*per cent*	*per cent*
		1.33	2.14		70	40	60
1	28684	1.92	2.03	1.31	50	20	20
		1.91	2.50		30	5	10
2	28641	5.20	3.83	0.73	70	70	80
					30	10	30
3	28770	4.83	4.82	0.99	50	100	100
		7.93	7.85		30	80	35
		4.06	4.55		60	35	35
4	29263	2.59	5.16	1.91	40	8	20
		2.85	7.47		20	2.5	10
					10	2.5	5

(*b*) *Serum from Culture Media with and without Leucocytes, Refrigerated for 48 Hours.*—In every experiment (Table III), the hemolytic power of the serum from media without leucocytes remained normal, while that from media with leucocytes was slightly increased. At the same time, there was no marked difference in the average rates of growth of fibroblasts in both sera.

2. Serum from Media with and without Leucocytes, to Which a Foreign Protein Was Added.—When 1 per 1,000 casein was added to a culture medium containing no leucocytes, the serum became more inhibiting

for the growth of fibroblasts. It was less toxic when the medium contained only 0.1 per 1,000 casein. A comparison of both sera from cultures of leucocytes with and without 1 per 1,000 casein showed that the presence of casein increased the amount of leucocytic secretion. The juice of the cultures containing casein was always a better medium for homologous fibroblasts than that of the cultures without casein, in spite of the toxic action of the casein (Table IV).

TABLE IV.

Action of Serum from Cultures of Chicken Leucocytes, without and with Casein, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from leucocytic cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
			Without casein (*C*).	With casein (*E*).		Amount of serum.	Amount of hemolysis by serum from leucocytic cultures.	
							Without casein.	With casein.
						per cent	*per cent*	*per cent*
1	28801	1 per 1,000	4.49 4.16 7.90	8.80 7.70 10.50	1.71	70	0	0
2	28886	1 " 1,000	4.81 2.75	5.35 4.23	1.34	70 50 30	40 30 8	40 30 8
3	1410	Dog serum.	1.00	2.50	2.50	0	0	0
4	28988	0.1 per 1,000	2.05 1.54 1.81	2.10 1.32 2.21	1.03	80	0	0
5	29059	0.1 " 1,000	6.71 5.31	6.70 7.25	1.17	80	0	0

The quotient of the rates of growth of fibroblasts in serum from casein media with and without leucocytes was higher than the quotient of the rates of growth of similar cultures containing no casein (Table V). There was little or no difference in the action of the sera when the concentration of the casein in the culture media was 0.1 per 1,000 (Table V).

TABLE V.

Action of Serum from Cultures Containing Casein, without and with Chicken Leucocytes, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
			Without leucocytes (*C*).	With leucocytes (*E*).		Amount of serum.	Amount of hemolysis by serum from cultures.	
							Without leucocytes.	With leucocytes.
						per cent	*per cent*	*per cent*
1	28801	1 per 1,000	1.00 0.95 1.13	4.04 4.84 4.63	4.40	70	0	0
2	28886	1 " 1,000	1.00 1.00 1.14	1.94 2.00 2.10	2.03	0	0	0
3	28926	0.5 per 1,000	0	0		80 60 40	5 3.5 2.5	50 30 15
4	29019	0.5 " 1,000	0	0		80 60 20	10 8 2.5	25 20 5
5	29162	0.5 " 1,000	2.88 1.25 1.69	10.00 5.76 8.66	4.39	80	0	0
6	29400	0.5 " 1,000	0	0		80 60 40	60 50 30	75 65 40
7	29059	0.1 " 1,000	6.80 4.81 2.61	12.77 10.35 5.68	2.06	80	0	0
8	29435	0.5 " 1,000	0.65 1.19	2.93 4.90	4.34	0	0	0

No increase of the lytic action on foreign erythrocytes of the serum from culture media containing casein was observed.

IV.

DISCUSSION, SUMMARY, AND CONCLUSIONS.

In the present condition of the technique of cultivation of tissues, the only possible way of studying leucocytic secretions was to grow colonies of leucocytes in a medium of known properties and to examine the modifications of these properties under the influence of the living cells. The method was far from perfect, because the secretions were mixed with serum and accumulated for 48 hours in a medium where they probably underwent partial destruction. But an approximate idea of certain of the qualities of the secretions, although not of their quantity, could be derived from the experiments. In the fluids extracted from the cultures, we attempted to detect the presence of the leucocytic secretions through their physiological effects on homologous and foreign cells. Two kinds of substances were sought, those which act on homologous cells, and those which destroy foreign erythrocytes.

The secretion by leucocytes of substances necessary to the nutrition of other cells was considered as probable long ago. Renaut[2] thought that the main function of the white blood corpuscles was to bring to the fixed cells of the tissues the food material which they need. While the existence of physiological relations between leucocytes and tissue cells could be considered as almost certain, their nature had remained practically unknown. It was probable, however, that the substances secreted by leucocytes were analogous to the growth-activating and unstable substances which are found in embryonic tissues, leucocytes, and certain adult tissues.[3] When connective tissue was aseptically inflamed, or when an aseptic peritoneal exudate contained many leucocytes, aqueous extracts of both connective tissue and peritoneal exudate were found to have acquired the power of stimulating cell proliferation.[4] These experiments showed that leucocytes could bring to the tissues some activating substances. But it remained to be ascertained whether leucocytes, while they are alive, could secrete similar substances either spontaneously or under the stimulus of a foreign factor.

[2] Renaut, J., Traité d'histologie pratique, Paris, 1893, i, pt. 2, 968.
[3] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14; xviii, 287.
[4] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.

Leucocytes are supposed to be, as is well know, the origin of the substances which protect the organism against infection.[5] Although the problem of the origin of alexin and antibodies has been investigated by many experimenters, it is not yet completely solved. It was of interest, therefore, to ascertain whether leucocytic secretions could increase the natural hemolytic effect of hen serum on sheep or rabbit erythrocytes, and whether these secretions would become more active under the influence of a foreign protein. The substances which destroy foreign cells are not necessarily different from those which act on homologous cells. The word substance is used for simplicity of description and may be taken as meaning only a given property of an unknown substrate.

A comparison was made of certain properties of sera extracted after 48 hours incubation from media containing leucocytes and from media containing no leucocytes. The serum from the leucocytic cultures was always found to be more favorable to the growth of homologous fibroblasts than the serum from the culture media incubated without leucocytes. The natural hemolytic power of the serum on sheep erythrocytes was found to be increased in about 50 per cent of the experiments.

In other experiments, we found that when two culture media free of cells were placed, one in an incubator at +38°C. and the other in a refrigerator at +5°C. for 48 hours, the serum from the incubated medium partly lost its hemolytic action on sheep or rabbit erythrocytes, while that from the refrigerated medium remained normal; at the same time, the inhibiting action of the incubated medium on homologous fibroblasts had increased very much. This effect of incubation indicates that certain unstable constituents of serum are destroyed by heat. Then the changes found in the properties of the serum from cultures of leucocytes are due to the fraction of the activating substances which has not been destroyed by incubation at 38°C. A quantitative study of the secretions is, therefore, impossible with the present technique, which can furnish only qualitative indications about the substances set free by the leucocytes.

[5] Metchnikoff, E., Immunity in infective diseases, translated by Binnie, F. G., Cambridge, 1905.

We have ascertained also whether a medium containing leucocytes and kept in the refrigerator undergoes any change under the influence of the cells while they are in a condition of latent life. Gabritschewski dishes with and without leucocytes were placed in a refrigerator at a temperature of about +5°C. After 48 hours, the hemolytic power on sheep erythrocytes of the serum from the leucocytic cultures had increased slightly and its inhibiting action on the growth of homologous fibroblasts had decreased. Then certain substances favorable to the growth of homologous cells and toxic for heterologous cells were diffused by the leucocytes into their medium. But the action of these substances was weaker than in the case of the cultures kept in the incubator. This experiment showed that leucocytes under certain conditions diffuse alexin or natural hemolysins which originate from them at the same time as the substances which activate homologous cells. In other experiments, although leucocytes were frozen at −10°C., treated with distilled water, or extracted with saline solution, they did not yield any hemolysin.

To summarize: Leucocytes, cultivated in plasma, always secreted substances which increased the rate of growth of homologous cells. Less frequently, they set free substances which hemolyzed foreign erythrocytes.

The growth-promoting substances are analogous to those contained in embryonic tissues, and probably represent some of the foodstuffs brought to fixed tissue cells by leucocytes. They may possess the function of rejuvenating cells which have ceased to multiply when the cicatrization of a wound or the repair of a fracture requires a resumption of tissue activity.[4] According to this hypothesis, the leucocytes brought to the surface of a wound by the process of inflammation would not only oppose bacterial invasion, but also bring to the tissues the material necessary to cell multiplication. It seems that in some cases regeneration is started by substances brought to the tissues by other cells. Loeb thinks that in *Tubularia*, when endodermic cells gather at the end where a new polyp is about to be formed, the substances given off by these cells are responsible for polyp formation.[6] There may be

[6] Loeb, J., The organism as a whole from a physicochemical viewpoint, New York and London, 1916, 170.

an analogy between this phenomenon and the secretion by leucocytes of growth-activating substances at the surface of a wound.

If we assume that leucocytes *in vivo* set free their secretions in the blood stream, certain variations of the growth-inhibiting action of normal serum can be better understood. The rate of proliferation of homologous fibroblasts is much slower in the serum of an old chicken than in that of a young one.[7] When the serum is heated at 56° and 70°C. for ½ hour, it becomes still more inhibiting.[8] A substance favorable to cell activity has disappeared. It is therefore permissible to suppose that the growth-inhibiting power of serum and its variations are due to the antagonistic action of two substances, one growth-promoting and thermolabile, and the other growth-inhibiting and thermostable, the activating substance being always weaker in its effect than the inhibiting one. We know that activating substances can be extracted from embryonic tissue, from muscle and gland tissues, and from leucocytes of the adult animal, and that they are thermolabile and very unstable. Leucocytic secretions seem to have some of the properties of leucocytic extracts. It is probable that the activating substances which disappear from the heated serum are secreted by leucocytes and other cells. An increase of these secretions, then, would diminish the inhibiting action of serum on homologous fibroblasts. On the contrary, a decrease of the secretions in the serum would increase its inhibiting effect on homologous cells. The strong inhibiting action of serum in old age would be due partly to a reduction in the amount and activity of the substances secreted by leucocytes and tissue cells in the humors of the organism.

Leucocytes also secreted *in vitro* substances which were toxic for foreign cells. Although the results were not constant, the serum appeared to become slightly more hemolytic for sheep or rabbit erythrocytes, under the influence of the leucocytes. The hemolysis of rabbit corpuscles by hen serum is due, according to Hyde,[9] to a complex sensitizer alexin, and not merely to alexin, as Bordet thought.[10]

[7] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.
[8] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.
[9] Hyde, R. R., *Am. J. Hyg.*, 1921, i, 358.
[10] Bordet, J., quoted by Hyde,[9] p. 358.

When a foreign protein was added to the culture medium, the leucocytic secretions increased, as was shown by the action on homologous fibroblasts of sera taken from cultures of leucocytes with and without casein. The presence in the medium of the cultures of leucocytes of only 0.1 per 1,000 casein did not markedly modify the action of their serum on the proliferation of fibroblasts. When the concentration of casein in the leucocyte cultures reached 1 per 1,000, the growth of chicken fibroblasts in the serum extracted from the Gabritschewski dishes became more rapid. But there was no parallel increase of the hemolytic action of the serum upon sheep erythrocytes.

We found that chicken serum containing 0.1 per 1,000 casein was barely toxic for homologous fibroblasts, while it became markedly inhibiting when the casein concentration reached 1 per 1,000. Probably, there is a relation between the toxicity of the medium, the increase of leucocytic secretions, and the time of the increase. The change brought about by casein in the equilibrium of the system composed of the cells and their medium determines the secretion by the leucocytes of substances which increase the activity of homologous cells and oppose the inhibiting effect of the foreign proteins. This reaction of the leucocytes is immediate, and may represent the first defense of the organism against a factor which disturbs its equilibrium. Possibly it differs from the specific cell reaction which leads to the production of antibodies. It is known that antibodies develop more slowly. Hemolysins were detected in cultures of bone marrow 4 days after the addition of antigen.[11] The immunization of fibroblasts against foreign proteins has been shown by Fischer[12] to begin after 4 days. If leucocytes behave in the organism as they do *in vitro*, we may assume that before the appearance of antibodies, they respond to the presence of an antigen by setting free growth-promoting substances and possibly alexin. This immediate reaction of the leucocytes against a disturbing factor, and the resulting production of substances which increase the activity of homologous cells, might be partly responsible for the results observed in the treatment of certain diseases by the injection of foreign proteins.

[11] Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.
[12] Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 535.

It may be concluded that, under the conditions of the experiments:

1. The serum obtained from cultures of leucocytes is less inhibiting for homologous fibroblasts than the serum from media without leucocytes. In some experiments, its hemolytic action on sheep or rabbit erythrocytes is also increased.

2. The addition of casein to leucocytic cultures brings about a decrease in the inhibiting effect of the serum on homologous fibroblasts.

3. The increase in the activity of homologous fibroblasts in serum obtained from leucocytic cultures is probably due to growth-promoting substances secreted by the leucocytes. The presence of a foreign protein under certain conditions determines a more abundant leucocytic secretion.