HAEMATOLOGICAL CHANGES IN ADJUVANT DISEASE IN THE RAT

I. PERIPHERAL BLOOD AND BONE MARROW AFTER REPEATED INJECTIONS OF FREUND'S ADJUVANT

BY

MARIA MIKOŁAJEW, ZOFIA KURATOWSKA, MARIA KOSSAKOWSKA,
MARIA PŁACHECKA, AND MARIA KOPEĆ

From the Institute of Rheumatology and Institute of Nuclear Research, Warsaw, Poland

Freund's adjuvant (FA) injected intracutaneously into the rat is known to induce a systemic disease (Pearson, 1956; Pearson and Wood, 1959; Pearson, Waksman, and Sharp, 1961; Ryzewska and Ryzewski, 1965). Its most common manifestation is polyarthritis, which can be accompanied by skin changes, genito-urinary lesions, diarrhoea, and eye involvement. These changes resolve slowly but the joint lesions may become chronic or recurrent. Repeated injections of FA are followed by fresh attacks of disease (Pearson and others, 1961; Ryzewska and Ryzewski, 1965; Flax and Waksman, 1963).

On the basis of personal experience and data from the literature, Pearson and others (1961) analysed the similarities and differences between FA disease in the rat and human connective tissue disease and hypersensitivity syndromes. They concluded that the experimental disease in rats "presents individual features of rheumatoid arthritis, erythema nodosum, rheumatic fever, Reiter's syndrome, and sarcoidosis, but cannot be regarded as an acceptable experimental model of any of these human diseases because it is distinct in respect of each of them in the constellation of findings".

Very little is known about the haematological manifestations of FA disease in the rat, and the purpose of the present study was to investigate the peripheral blood and bone marrow changes after repeated FA injections.

Material and Methods

3 month-old Wistar rats were used. They were kept in roomy cages in groups of six to ten, and purina laboratory chow and water were given *ad libitum*.

Freund's adjuvant FA containing 3 mg. dried, heat-killed *Mycobacterium phlei* in 0.1 ml. (?) paraffin oil was injected intracutaneously into the right hind footpad of 58 experimental animals. Forty control animals of the same age and similar weight did not receive FA. Three experimental groups received FA once (19 rats), twice (18 rats), or three times (21 rats). After the first injection of 0.1 ml. FA daily observations were made of the inflammatory changes which appeared in the joints of the other legs (usually after 18 to 28 days). The second injection of 0.05 ml. FA was given when the visible polyarthritic changes induced by the first dose of FA had subsided, and observations were made when the second attack of arthritis appeared (usually after 33 to 44 days). The third injection of 0.05 ml. FA was given during a second remission of joint symptoms, and, observations were made during the third attack of inflammatory joint changes, usually 54 to 64 days after the first FA injection. The animals were then anaesthetized by ether and killed by exsanguination.

Standard methods were used for the serial haematological examinations (Dacie, 1958).

Haemoglobin was determined as cyanmethaemoglobin. Haematocrit values of heparinized blood were determined according to the method of McGovern, Jones, and Steinberg (1955), using an International Micro-Capillary centrifuge, Model MB, and an International Micro-Capillary Reader, Model CR. Bone marrow was obtained from the femur. The smears of the cells suspended in serum were stained with May-Grünwald-Giemsa. The percentage of nucleated cells was calculated after counting 1,000 cells.

Results

After the first injection of FA, the haemoglobin concentration fell significantly as compared with the control group, (Fig. 1, overleaf). A further drop in haemoglobin level was observed after the second

Fig. 1.—Haemoglobin.

Fig. 3.—Haematocrit.

injection. The frequency of significant anaemia in the three experimental groups is shown in Fig. 2, a haemoglobin less than 12.8 g. per cent. taken as significant anaemia. Only 10 per cent. of the animals injected once showed pronounced anaemia, whereas 60 per cent. of the second group and 65 per cent. of the third group became anaemic. All

Fig. 2.—Percentage of rats with marked anaemia (Hb < 12.8 g. per cent.).

rats of the control group showed a significantly higher haemoglobin level. Fig. 3 shows that a significant drop in mean haematocrit was observed in all three experimental groups of rats, and that this was more pronounced after repeated injections of FA. Table I shows that much lower haematocrit values were found in the animals with low haemoglobin levels.

Further studies were carried out in the third group of rats with the most pronounced frequency and degree of anaemia.

Fig. 4 shows that the erythrocyte count decreased markedly in the animals with low haemoglobin levels. Table II shows that, after repeated injections of FA, the anaemia became macrocytic and hypochromic. A significant increase in mean corpuscular volume (MCV) or erythrocytes and a decrease in mean corpuscular haemoglobin concentration (MCHC) was found in the whole experimental group. Macrocytosis was more pronounced in the animals with low haemoglobin levels and low MCHC.

The changes in the reticulocyte count are illustrated in Fig. 5. The count was high in the rats with marked anaemia but values similar to those in

Group	Controls	First	Whole Group
		Whole Group	
Number of Rats	40	19	18
Haematocrit	49.4 ± 1.87	45.6 ± 1.95	41.19 ± 8.57
P		<0.001	<0.001

Fig. 4.—Mean value of erythrocyte counts in the control rats and after three FA injections.

Fig. 5.—Reticulocytes.

the controls were observed when the haemoglobin level exceeded 12.8 g. per cent.

Table III shows that the thrombocyte count in the experimental rats did not differ from that in the controls irrespective of the degree of anaemia.

The behaviour of leucocytes in the peripheral blood is shown in Table IV and Fig. 6 (overleaf). A high mean leucocytosis $24 \times 10^3/\text{cu. mm.}$ was found in the experimental group as compared with $8.65 \times 10^3/\text{cu. mm.}$ in the controls.

TABLE II
MCV AND MCHC IN 10 CONTROLS AND IN 22 RATS AFTER THREE INJECTIONS OF FA

Group	Controls	Rats after FA Injection		
		Whole Group	Hb < 12.8 g. per cent.	Hb > 12.8 g. per cent.
Number of Rats	10	22	13	9
MCV μ^3	59.96 ± 8.7	73.3 ± 17.4	79.9 ± 17.7	61.1 ± 5.7
P		<0.01	<0.001	>0.7
MCHC per cent.	33.39 ± 1.09	29.0 ± 2.87	28.1 ± 1.95	30.97 ± 1.81
P		<0.001	<0.001	<0.01

TABLE III
THROMBOCYTES IN 10 CONTROLS AND IN 23 RATS AFTER THREE FA INJECTIONS

Group	Controls	Rats after FA Injection		
		Whole Group	Hb < 12.8 g. per cent.	Hb > 12.8 g. per cent.
Number of Rats	10	23	15	8
Thrombocytes $1 \times 10^3/\text{mm}^3$	932.1 ± 226.4	970.2 ± 276.0	915.2 ± 276.4	$1,080.1 \pm 259.1$
P		>0.5	>0.8	>0.2

OCRIT

FA Injection				
Second		Third		
Hb < 12.8 g. per cent.	Hb > 12.8 g. per cent.	Whole Group	Hb < 12.8 g. per cent.	Hb > 12.8 g. per cent.
12	6	21	14	7
38.0 ± 8.6	47.5 ± 3.74	40.0 ± 6.26	36.3 ± 6.4	46.8 ± 1.4
<0.001	>0.1	<0.001	<0.001	<0.01

TABLE IV
LEUCOCYTES IN 10 CONTROLS AND IN 23 RATS
AFTER THREE FA INJECTIONS

Group	Number of Rats	Leucocytes $1 \times 10^3/\text{mm}^3$	Per cent. Granulocytes	Per cent. Lymphocytes
Controls	10	8.65 ± 2.25	18.8 ± 6.29	70.5 ± 7.36
Rats after FA injection	23	24.6 ± 5.3	57.56 ± 8.2	31.8 ± 7.85
P		<0.001	<0.001	<0.001

Fig. 6.—Absolute number of white blood cells in the control rats and after three FA injections.

Quite different was the ratio between granulocytes and lymphocytes. In the controls the mean physiological lymphocytosis was 70.5 ± 7.36 per cent. but in the experimental group 57.56 ± 8.2 per cent. of white blood cells were granulocytes.

The absolute numbers of granulocytes, lymphocytes, and other blood cells are illustrated in Fig. 6. The white blood cells of every type increased in the experimental rats, but this increase was irregular; the granulocytes were seven times more than in the controls, whereas the rise in lymphocytes was moderate.

Bone marrow smears showed marked hypercellularity in the experimental rats as compared with the controls. The differential counts of bone marrow nucleated cells of normal rats and of rats injected twice with FA are presented in Table V. In the experimental group there are more erythroblasts with an arrest of maturation at the polychromatophilic stage; the slightly higher percentage of granulocytes was mainly due to the increase of more mature forms—metamyelocytes and polymorphonuclears. The percentage of lymphocytes was much lower.

TABLE V
MEAN BONE MARROW DIFFERENTIAL COUNT
PER CENT.

Cells	Controls*	After 2 FA Injections†
Normoblasts	15.2 ± 9.5	40.1 ± 1.33
Pronormoblasts	0.6 ± 0.81	1.7 ± 0.83
Basophilic-normoblasts	1.5 ± 1.44	8.8 ± 3.96
Polychromatic-normoblasts	6.1 ± 4.61	22.5 ± 8.64
Orthochromatic-normoblasts	7.0 ± 4.29	7.1 ± 5.17
Granulocytes	33.9 ± 6.33	44.8 ± 3.83
Myeloblasts, pro-myelocytes	4.0 ± 2.92	3.8 ± 1.5
Metamyelocytes	18.4 ± 1.55	23.0 ± 8.27
Polymorphonuclears	11.5 ± 4.77	18.0 ± 6.31
Lymphocytes	36.5 ± 6.38	6.5 ± 2.86
Plasmacytes	1.5 ± 1.23	1.5 ± 1.29
Reticulocytes	11.3 ± 6.60	6.8 ± 3.51

*mean values from ten animals.

†mean values from nine animals.

Discussion

Pronounced anaemia and increased leucocytosis are found in rats with adjuvant-induced polyarthritis. The anaemia is hypochromic and macrocytic and is accompanied by rather high reticulocytosis. Leucocytosis is due mainly to an increase in polymorphonuclear granulocytes, but the absolute number of lymphocytes is also augmented in contrast to the lymphopenia observed in mice in the early stages of adjuvant-induced disease (Morton and Siegel, 1966). The thrombocyte counts do not differ from those in the controls. This blood picture partly resembles the human anaemia of chronic inflammatory states (Cartwright, 1966) particularly the severe anaemia observed in some cases of rheumatoid arthritis (Mikolajew, Plachecka, Piotrowska, Gałajowa, and Kopeć, 1966). Lukens, Cartwright, and Wintrobe (1967) have demonstrated that moderate anaemia observed after a single injection of FA is hypochromic and microcytic, and is accompanied by sideropenia and lowering of serum iron-binding capacity. Hypochromia is known to be characteristic for the human anaemia of inflammatory states, but normocytosis and even a tendency to microcytosis are sometimes seen (Partridge and Duthie, 1963; Mikolajew and others, 1966). The pronounced degree of macrocytosis observed in our rats is uncommon in the human anaemia of inflammatory states. Macrocytic anaemia with megaloblastic marrow responsive to folic acid treatment has been observed in some cases of rheumatoid arthritis (Gough, McCarthy, Read, Mollin, and Waters, 1964). It is possible that the gastro-intestinal changes with diarrhoea which often occur in adjuvant disease (Pearson and others,

1961) may lead to folic acid deficiency and macrocytosis.

"Skipping erythropoiesis" may be another explanation of this macrocytosis. This term was introduced by Borsook, Lingrel, Scaro, and Millette (1962) for the shortened process of maturation of the red cell precursors which occurs when there is an increased demand for erythrocytes. In these conditions erythroid cell precursors, *e.g.* polychromatic erythroblasts, may skip the mitosis stage and become reticulocytes directly, omitting the orthochromatic erythroblast stage. This phenomenon causes defective reduction in cell size and an increase in the macrocytes in the peripheral blood. Marked erythroid hyperplasia of the bone marrow, with arrest of maturation, and lack of megaloblastosis and macrocytosis in myelograms as well as a high degree of reticulocytosis in the peripheral blood, may be considered as supporting this possibility. These signs suggest that haemolysis and/or chronic blood loss may assist in the pathogenesis of anaemia after repeated injections of adjuvant. Attempts to elucidate these questions are the subject

of further studies (Mikołajew, Kuratowska, Kossakowska, Plachecka, and Kopeć, 1969).

Thrombocytosis has been observed in iron-deficiency anaemia (Gross, Keefer, and Newman, 1964; Shloesser, Kipp, and Wenzel, 1965) and in chronic inflammatory disease, particularly rheumatoid arthritis (Pazdur, Szpilman, and Stachurska, 1965), but we have not observed this symptom in adjuvant arthritis in the rat.

Summary

Changes in the peripheral blood and in the bone marrow were observed after repeated intracutaneous injection of Freund's adjuvant into the foot-pad of rats. Pronounced hypochromic and macrocytic anaemia, reticulocytosis, and increased leukocytosis were found. Bone marrow smears showed marked hyperplasia and an increased percentage of erythroblasts, the maturation of which was inhibited. The total white blood cells increased in experimental rats, but the lymphocytes : granulocyte ratio decreases. This blood picture had some features in common with human anaemia in chronic inflammatory states.

REFERENCES

- Borsook, H., Lingrel, J. B., Scaro, J. L., and Millette, R. L. (1962). *Nature*, **196**, 347 (Synthesis of haemoglobin in relation to the maturation of erythroid cells).
- Cartwright, G. E. (1966). *Semin. Hemat.*, **3**, 351 (The anemia of chronic disorders).
- Dacie, J. V. (1958). "Practical Haematology", 2nd ed. Churchill, London.
- Flax, M. H., and Waksman, B. H. (1963). *Int. Arch. Allergy*, **23**, 331 (Further immunologic studies of adjuvant disease in the rat).
- Gough, K. R., McCarthy, C., Read, A. E., Mollin, D. L., and Waters, A. H. (1964). *Brit. med. J.*, **1**, 212 (Folic-acid deficiency in rheumatoid arthritis).
- Gross, S., Keefer, W., and Newman, A. J. (1964). *Pediatrics*, **34**, 315 (The platelets in iron-deficiency anemia. I. The response to oral and parenteral iron).
- Lukens, J. N., Cartwright, G. E., and Wintrobe, M. M. (1967). *Proc. Soc. exp. Biol. (N.Y.)*, **126**, 346 (Anemia of adjuvant-induced inflammation in rats).
- McGovern, J. J., Jones, A. R., and Steinberg, A. G. (1955). *New Engl. J. Med.*, **253**, 308 (The hematocrit of capillary blood).
- Mikołajew, M., Kuratowska, Z., Kossakowska, M., Plachecka, M., and Kopeć, M. (1969). *Ann. rheum. Dis.*, **28**, (in press) (Haematological changes in adjuvant disease in the rat. II. Iron metabolism and ⁵¹Cr erythrocyte survival).
- , Plachecka, M., Piotrowska, D., Gałajowa, W., and Kopeć, M. (1966). *Reum. pol.*, **4**, 209 (Anaemia in the course of rheumatoid arthritis).
- Morton, J. I., and Siegel, B. V. (1966). *Vox Sang.*, **11**, 570 (Hematological changes in mice following Freund's adjuvant administration).
- Partridge, R. E. H., and Duthie, J. J. R. (1963). *Brit. med. J.*, **1**, 89 (Incidence of macrocytic anaemia in rheumatoid arthritis).
- Pazdur, J., Szpilman, H., and Stachurska, J. (1965). *Reum. pol.*, **3**, 1 (Blood clotting and fibrinolytic system in rheumatic patients. I. Number of plates and disturbances in their adhesion *in vivo*).

- Pearson, C. M. (1956). *Proc. Soc. exp. Biol. (N.Y.)*, **91**, 95 (Development of arthritis, periarthritis and periostitis in rats given adjuvants).
- , Waksman, B. H., and Sharp, J. T. (1961). *J. exp. Med.*, **113**, 485 (Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. V. Changes affecting the skin and mucous membranes. Comparison of the experimental process with human disease).
- and Wood, F. D. (1959). *Arthr. and Rheum.*, **2**, 440 (Studies of polyarthritis and other lesions induced in rats by injection of mycobacterial adjuvant. I. General clinical and pathologic characteristic and some modifying factors).
- Ryzewska, A., and Ryzewski, J. (1965). *Reum. pol.*, **3**, 209 (Experimental models of polyarthritis in rats).
- Shloesser, L. L., Kipp, M. A., and Wenzel, F. J. (1965). *J. Lab. clin. Med.*, **66**, 107 (Thrombocytosis in iron-deficiency anemia).

Altérations hématologiques chez le rat après des injections d'adjuvant de Freund. I

RÉSUMÉ

Après des injections intradermiques répétées d'adjuvant de Freund dans la patte du rat on trouva dans le sang périphérique une anémie hypochromique et macrocytique prononcée, une réticulocytose et une leucocytose augmentée. Les frottis de la moelle osseuse révélèrent une hyperplasie marquée et un pourcentage augmenté des érythroblastes dont la maturation se trouvait inhibée. Le chiffre total des globules blancs chez les rats expérimentaux fut augmenté, mais le rapport lymphocyte/granulocyte diminué. Ce tableau sanguin a certains traits commun avec l'anémie humaine qui accompagne les états inflammatoires chroniques.

Alteraciones hematológicas en la rata después de inyecciones de adyuvante de Freund. I

SUMARIO

Después de inyecciones intradérmicas repetidas de adyuvante de Freund en la pata de la rata, se observaron en la sangre periférica una anemia hipocrómica y macrocítica pronunciada, una reticulosis y una leucocitosis aumentada. El frotis de la médula ósea reveló una hiperplasia destacada y una proporción aumentada de eritroblastos cuya maduración se vió inhibida. Se observó una aumentación de la cifra total de glóbulos blancos pero una disminución de la proporción linfocito/granulocito en estas ratas experimentales. Este cuadro sanguíneo muestra ciertos rasgos comunes con la anemia humana que suele acompañar los estados inflamatorios crónicos.