Study of Humoral Factors Regulating the Production of Leukocytes. I. Demonstration of a "Neutropoietin" in the Plasma after Administration of Triamcinolone to Rats.

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Introduction

THE DISCOVERY of erythropoietin, a humoral substance regulating production of erythrocytes,[1-3] has raised the question whether humoral factors for the control of other blood elements exist. It is well known that following the administration of ACTH and cortico-steroids, lymphopenia and granulocytosis occur.[4-8] The mechanism responsible for this phenomenon is not clearly understood. It is possible that the hormone alters or "triggers" some chemical or physiological agent which by stimulating the elaboration or release of a humoral factor causes increased proliferation of granulocytes. The present study was made in the attempt to demonstrate the existence of such a factor in the plasma of experimental animals following the administration of triamcinolone, a potent synthetic glucocorticoid.

Method

The administration of triamcinolone and collection of plasma

Triamcinolone† (9-alpha-fluoro-16-alpha hydroxyprednisolone) was prepared in water in a concentration such that 1 cc. supplied 0.02–0.03 mg. per Gm. body weight. Male white Wistar rats of 200–300 Gm. weight were selected and designated as Group I. Daily administration of triamcinolone solution by gastric catheter was continued until the neutrophil counts were substantially and consistently elevated, usually a period of 10 to 14 days. Hematocrit and hemoglobin determinations were done on tail blood once before and once a week during this period, and also immediately before exsanguination, the white cell count and differential count 2 to 3 times before and three times a week subsequently. The tail blood for these determinations was always taken immediately before administration of triamcinolone or plasma. Twenty to 48 hours after the last dose the rats were exsanguinated by cardiac puncture under ether anesthesia. The heparinized blood was centrifuged and the plasma removed with sterile technique. The plasma so collected from each rat was pooled and stored in the freezer ready for the experiment. Plasma to serve as controls was collected in like manner from normal rats. Each batch of plasma was cultured to exclude the possibility of bacterial or fungal infection.

Administration of plasma

Male white Wistar rats were screened to exclude those with great fluctuation in white cell counts and divided into 2 groups: Eight rats designated Group II, received 10 cc. of plasma daily from normal rats by intraperitoneal injections for 10 days and another 8 rats,

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†Kindly supplied for this study as Kenacort by Squibb Co.

Group III, were given intraperitoneally for 10 days 10 cc. of plasma from rats given triamcinolone. Peripheral blood studies were carried out as described in preceding section. Direct eosinophil counts were done on 5 of each group.

Studies of the bone marrow

The total number of nucleated cells per mg. of bone marrow was estimated on some rats in each group after exsanguination using the following modification of Burke's original method[9]: The marrow of each femur was scraped out with a wood applicator, smears were made from each femur for differential counts and then the marrow was introduced into individual weighed siliconized test tubes which were then immediately reweighed to determine the weight of the marrow. Two cc. of normal rat serum was added to each marrow sample, the test tubes were agitated for 2 minutes, and the cells were counted and the concentration of cells per cu. mm. calculated as for a white count. The total nucleated cells per mg. of marrow was thus calculated. The percentages of different types of cells counted on the differential smear were used to calculate the absolute number of each type of cells per mg. of bone marrow. The figures from the 2 femurs of each rat were averaged.

Staining method for bone marrow smears

Although Wright's stain was ordinarily employed for peripheral blood smears, it was sometimes unsatisfactory for bone marrow due to the thickness of the smears and the large aggregations and sheets of cells. For these smears therefore a "double stain" method was developed incorporating the advantages of Giemsa stain for nuclear details and Wright's stain for the cytoplasm.

Direct eosinophil counts were performed in peripheral blood according to the method of Randolph.[10] All white cell counts were done in duplicate and averaged. For differential counts at least 400 cells were counted in peripheral blood smears and 2,000 cells in bone marrow smears.

RESULTS

Table I shows the average values and standard deviation (S.D.) of counts of the total white cells, neutrophils (band and segmented), and lymphocytes of each group before and after the last administration of various materials. Figures 1, 2, and 3 are the normalized graphs showing the changes in neutrophils (band and segmented) and lymphocytes of Groups I, II, and III during period of administration of triamcinolone, plasma from normal rats and plasma from rats given triamcinolone respectively. There is no significant change of

Table 1.—*Average Value in Thousands of Cells per cu. mm. of Neutrophils (Band and Segmented) and Lymphocytes in the Peripheral Blood of Each Groups Before and After Administration of Various Materials*

Administered:		Total White Cells	Neutrophils Band	Neutrophils Segmented	Lymphocytes
Triamcinolone by Gastric Catheter (8 rats)	Before	18.6 ± 5.4	0.65 ± 0.23	2.77 ± 1.05	14.2 ± 4.5
	After	17.3 ± 6.4	1.86 ± 0.92	11.21 ± 5.65	3.2 ± 1.6
Plasma from Normal Rats (8 rats)	Before	14.9 ± 4.1	0.43 ± 0.21	2.16 ± 0.69	11.6 ± 3.8
	After	12.8 ± 3.1	0.26 ± 0.13	1.42 ± 0.44	10.2 ± 2.4
Plasma from Rats given Triamcinolone (8 rats)	Before	13.6 ± 2.3	0.26 ± 0.12	1.84 ± 0.98	10.9 ± 3.3
	After	20.0 ± 4.0	0.98 ± 0.39	5.25 ± 0.93	12.9 ± 3.8

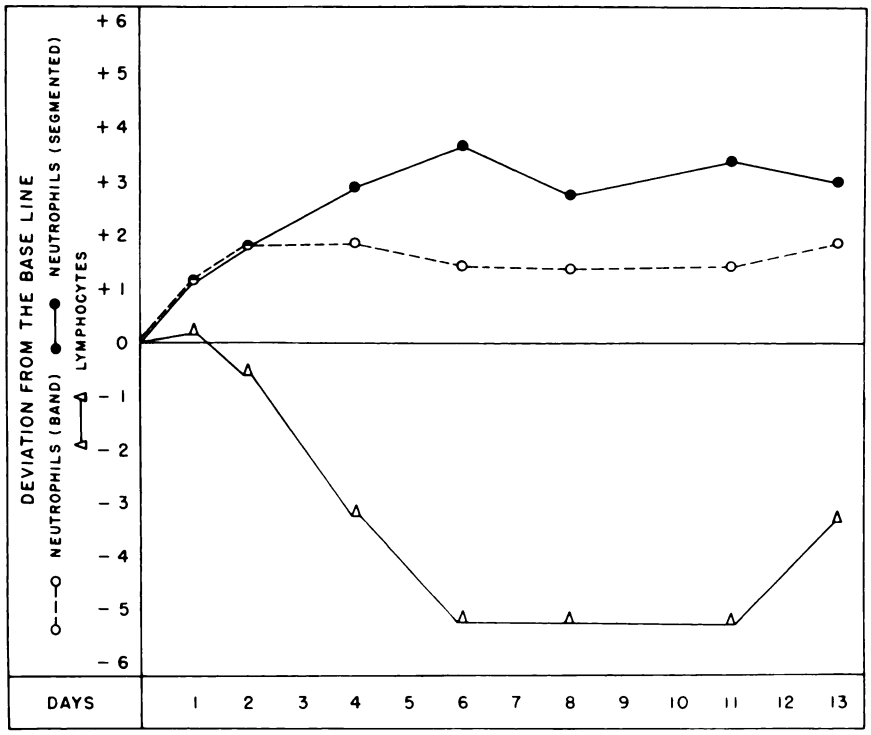

Fig. 1.—Normalized graph of changes in neutrophils (band and segmented) and lymphocytes of Group I rats during period of administration of triamcinolone.

monocytes in any group. Eosinophils were usually decreased during the experimental period in Group I but not in Groups II and III. The fall in lymphocytes in Group I accounts for failure of the total white count to rise in this group.

The neutrophil response occurred promptly after the first administration of triamcinolone and was usually followed by a drop or leveling off with a delay of 3 to 4 days before the rise resumed or leveled off with no further significant rise. Changes usually reached a plateau after 10 to 14 days of daily administration of triamcinolone as shown in figure 1, whereas in Group III no evidence of neutrophil response was noted but rather there was a 3 to 4 day delay after the first injection before a rise occurred and reached a plateau after 10 days.

Both table 1 and figure 2 reveal no evidence of an increase in neutrophils or other white cell elements in Group II rats.

Table 2 shows the average value of the nucleated cells in the bone marrow of the 3 groups of rats. The myeloid-erythroid ratios (M:E) were definitely increased in Groups I and III, while Group II had normal M:E ratio. The erythroid series of these three groups showed no significant differences. There is no evidence of decreased erythropoietic activity in these marrows, as hemoglobin and hematocrit remained relatively constant. Eosinophils appeared increased in the Group III rats receiving plasma from rats given triamcinolone, and decreased in the Group I rats receiving triamcinolone in comparison with

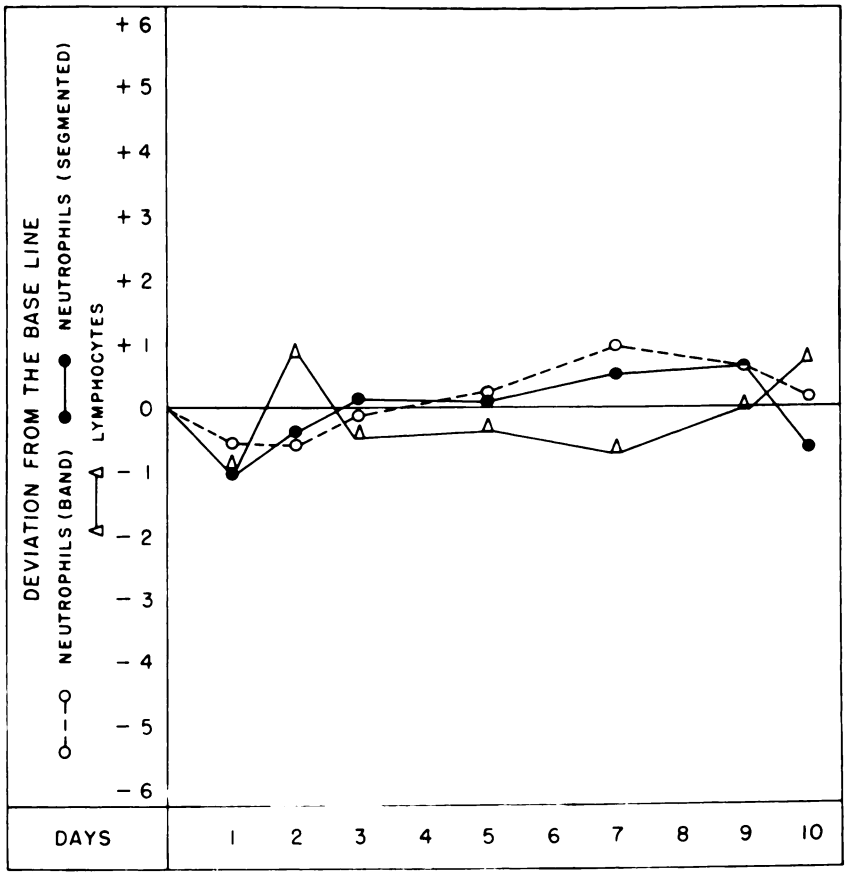

Fig. 2.—Normalized graph of changes in neutrophils (band and segmented) and lymphocytes of Group II rats during period of administration of plasma from normal rats.

the Group II rats given normal plasma. However, the total number of these cells is so low that the accuracy of the method is limited and values are subject to question.

Although the half life of steroids in the blood stream is known to be extremely short,[11,12] there is the possibility of accumulated residual steroid present in the plasma of the rats repeatedly given triamcinolone. For this reason eosinophil counts were done on 10 rats of Groups II and III at 0, 2, 4, 6, 12 and 24 hours after the first plasma injection and also after small and large oral doses of triamcinolone and intraperitoneal injections of triamcinolone suspended in saline or in normal rat plasma. Table 3 summarizes the results. Triamcinolone, no matter how administered, caused a complete suppression of eosinophils in 6 hours. The plasma, whether from normal rats or those given triamcinolone, caused no lowering of eosinophil counts.

While it is true that the rats given large doses of triamcinolone lost weight and were highly susceptible to infection, cultures of the plasma given to the rats were found to be negative for bacterial and fungal organisms, and in

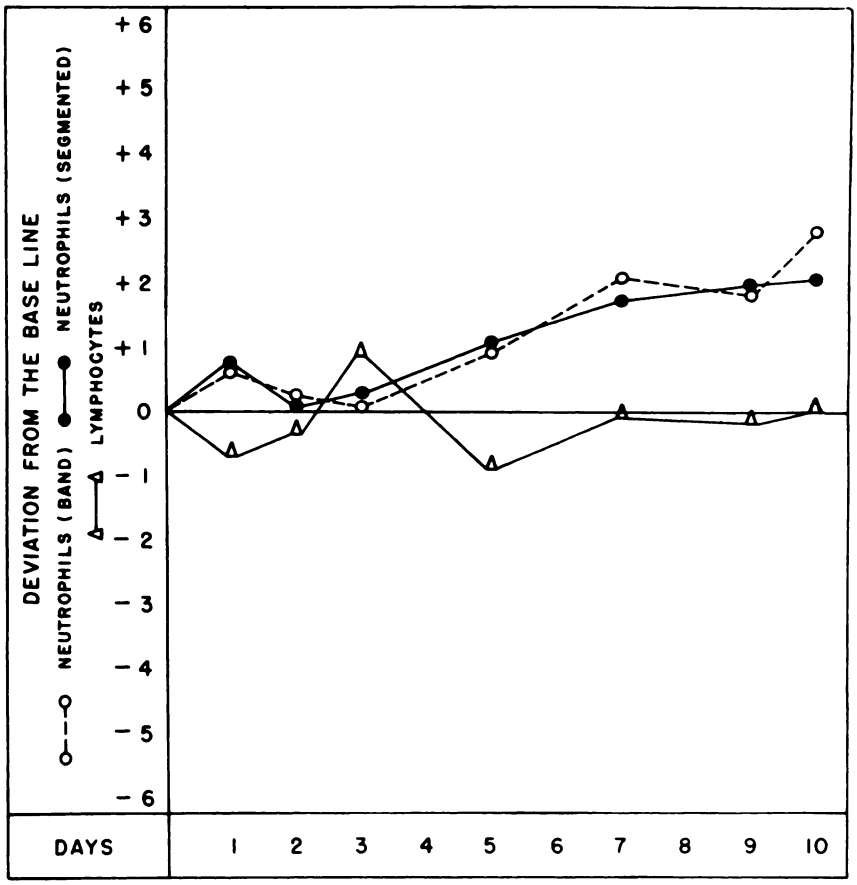

Fig. 3.—Normalized graph of changes in neutrophils (band and segmented) and lymphocytes of Group III rats during period of administration of plasma from rats given triamcinolone.

contrast to those rats given triamcinolone, those rats receiving the plasma from rats given triamcinolone were healthy and had no weight loss.

Discussion

The existence of a humoral mechanism governing red cell production is well established.[13,14] It may be postulated that other formed blood elements are likewise affected by humoral factors arising under the influence of some other specific derangements of physiological processes. However, since such specific derangements are not known at present and because methods for the study of the kinetics of white cell production are not sufficiently refined, demonstration of a humoral factor has been difficult. Menkin[15,16] has suggested that inflammatory exudates contain a factor for leukocytosis as well as one for leukopenia. Steinberg and Martin[17] claimed to have induced leukocytosis in rabbits with a factor in human plasma. They also stated that epinephrine alters the concentration of a factor in the plasma reported to cause the expulsion of granulocytes from the marrow into the circulation. Unfortunately the design of their

Table 2.—*Nucleated Cell Counts in the Bone Marrow of Rats After Administration of (1) Triamcinolone (2) Plasma from Normal Rats (3) Plasma from Rats Given Triamcinolone*

			Triamcinolone by Gastric Catheter (4 rats)	Plasma from Normal Rats (I.P.) (5 rats)	Plasma from Rats Given Triamcinolone (I.P.) (6 rats)
Total Nucleated Cells			1830.8 ± 212.9	1093.0 ± 414.1	1494.9 ± 297.9
Myeloid Series	Neutrophils	Segmented	282.6 ± 58.9	117.9 ± 28.7	213.0 ± 68.2
		Band	264.0 ± 29.3	122.8 ± 46.4	216.0 ± 60.2
		Metamyelocytes	257.9 ± 30.9	92.9 ± 31.9	202.4 ± 82.4
		Myelocytes & Myeloblasts	299.2 ± 100.4	93.2 ± 36.4	195.2 ± 56.3
	Eosinophils		26.5 ± 4.9	46.1 ± 13.2	70.1 ± 24.9
	Basophils		0.9 ± 0.85	3.5 ± 2.6	2.5 ± 2.2
Lymphocytes			63.9 ± 28.3	105.6 ± 54.1	122.0 ± 36.0
Monocytes			39.2 ± 20.9	28.7 ± 16.7	23.0 ± 11.7
Plasma Cells & Megakaryocytes			28.4 ± 7.9	15.4 ± 5.8	15.1 ± 1.6
Erythroid Series	Normoblasts		418.7 ± 59.3	344.6 ± 118.5	295.4 ± 77.0
	Erythroblasts		132.1 ± 88.6	110.3 ± 63.6	124.4 ± 41.1
	Proerythroblasts		17.6 ± 6.2	12.7 ± 6.0	15.8 ± 7.0
Total Myeloid Series			1131.0 ± 207.3	475.7 ± 167.6	899.2 ± 207.0
Total Erythroid Series			568.3 ± 57.7	467.6 ± 184.7	435.6 ± 116.8
M:E Ratio			2.0:1	1.0:1	2.1:1

The figures are expressed in thousand of cells per mg. of bone marrow.

Table 3.—*Direct Eosinophil Counts of the Peripheral Blood of Rats Following Administration of Various Materials*

Material Administered	Rat No.	Direct Eosinophil Count (per cu.mm.)				
		0 hr.	2 hr.	6 hr.	12 hr.	24 hr.
Triamcinolone (8 mg. by gastric catheter)	254	350	272	0	0	3
	255	450	75	0	0	22
Triamcinolone (1 mg. by gastric catheter)	101	147	75	3	–	3
	102	238	94	9	–	50
Triamcinolone (0.5 mg. in 10 cc. saline I.P.)	348	122	22	3	44	203
	349	103	12	0	34	119
Triamcinolone (0.5 mg. in 10 cc. normal plasma I.P.)	346	316	62	9	90	184
	347	106	9	3	72	128
Plasma from Rats Given Triamcinolone (10 cc. I.P.)	328	50	53	47	38	59
	329	56	112	112	53	69
	337	62	50	81	106	97
	340	165	209	134	97	131
	345	75	90	78	50	38
Plasma from Normal Rats (10 cc. I.P.)	320	81	119	125	75	112
	335	97	116	94	116	122
	341	128	103	97	72	103
	342	269	231	184	162	162
	344	128	137	128	56	100

experiments casts doubt on the validity of their results. Craddock et al.[18] demonstrated that the loss of leukocytes in itself will stimulate production. No good evidence of a humoral factor for leukocytosis existed until recently with the demonstration by Gordon[19,20] of a circulating leukocytosis-promoting factor appearing in rats after removal of peripheral white cells by peritoneal lavage (leukocytaphereses). Leukocyte release occurred in (1) rats given subcutaneous injections of plasma from leukocytapheresed rats; (2) rats whose hind legs were perfused with such plasma; (3) the parabionts of rats subjected to leukocytaphereses.

There is considerable documentation for the occurrence of neutrophilia, lymphopenia and eosinopenia and sometimes an increase of erythrocytes following severe exercise[21] or the administration of ACTH and some steroid hormones.[22] Quittner et al.[23] reported a significant increase in myeloid:erythroid (M:E) ratio in bone marrow of mice after a large dose of cortisone. Yoffey et al.[24] reported that 7 days of daily administration of corticosteroids to intact guinea pigs induced an increase in the cellularity of the marrow involving both neutrophilic and erythroid elements.

The data obtained in this investigation suggest that a humoral factor for the production of neutrophils appears following the administration of triamcinolone to rats. Thus, the administration of triamcinolone resulted in an early neutrophil response followed in several days by a decrease (or no further rise) for 3 to 4 days before the increase resumed. Following the administration of plasma from rats given triamcinolone, a period of 3 to 4 days elapsed before a rise in neutrophils occurred. A possible explanation of this takes into account

the following findings reported by others. Thus, Gordon demonstrated a circulating leukocytosis-promoting factor appearing in the rat after removal of peripheral white cells. Craddock et al.[25] reported an increased leukocyte production in the dog 3 to 4 days after repeated leukocytaphereses; in the human a period of 5 days was required for maturation of granulocytes in the marrow following the use of DNA tagging technique. Hamilton[26] pointed out in 1954 that the adrenal medullary factor (epinephrine) may act on the release of neutrophils from tissue sites into the blood. It may therefore be postulated that triamcinolone causes an immediate early release of stored neutrophils into the circulation, promptly raising the white count. The depletion of leukocytes from tissue storage sites brings about elaboration of the humoral neutropoietic factor which stimulates production in the marrow. There is, however, a period of 3 to 5 days required for the maturing of these neutrophils. Their emission into the peripheral blood stream is apparent in the continuing rise in neutrophils in the blood. The pattern and time of occurrence of leukocyte response with the plasma obtained by leukocytaphereses in Gordon's experiments are not identical with those of the Group III rats receiving plasma from rats given triamcinolone in this experiment. These differences may be attributed to the design of the two experiments. Furthermore, the result obtained in Group III rats, i.e., neutrophilia without lymphopenia and eosinopenia indicates that the increase in neutrophil production was not caused by the effects of stress or of so called non-specific factors; nor by infections, since the cultures of the plasma injected were negative for bacterial or fungal organisms and the injected rats were quite healthy.

Summary

Male Wistar rats were divided into 3 groups:

Group I. Rats given the triamcinolone daily by gastric catheter all developed neutrophilia accompanied by lymphopenia.

Group II. Rats given daily intraperitoneal injections of plasma from normal rats manifested no significant alteration in the peripheral blood elements.

Group III. Rats given daily intraperitoneal injections of plasma from rats given triamcinolone invariably developed neutrophilia without lymphopenia.

Studies of the bone marrow of these groups at the end of the experiments revealed increased myeloid:erythroid ratios in Groups I and III but not II.

It is therefore believed that this experiment suggests the existence of a neutrophilia-promoting factor in the plasma following the administration of triamcinolone.

Summario in Interlingua

Mascule rattos Wistar esseva dividite in 3 gruppos:

Gruppo I. Rattos tractate diurnemente con le corticosteroide triamcinolona per catheter gastric disveloppava sin exception neutrophilia accompaniate de lymphopenia.

Gruppo II. Rattos tractate diurnemente con injectiones intrape ritonee de plasma ab rattos normal manifestava nulle significative alteration in le ele-

mentos de sanguine peripheric.

Gruppo III. Rattos tractate diurnemente con injectiones intraperitonee de plasma ab rattos tractate con triamcinolona disveloppava sin exception neutrophilia non accompaniate de lymphopenia.

Studios del medulla ossee in iste gruppos al fin del experimentos revelava augmentos del proportion myeloido-erythroide in gruppo I e gruppo III sed non in gruppo II.

Es opinate per consequente que iste experimento suggere que il existe in le plasma post le administration de triamcinolona un factor neutrophilio-promovente.

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