Exp. Therap., 1959, v127, 175.

6. Linneweh, F., Ehrlich, M., Klin. Wschr., 1960, v38, 904.

7. Culley, W.J., Chance, R. E., Mertz, E. T., Fed. Proc., 1965, v24, 470.

8. Linneweh, F., Ehrlich, M., Graul, E. H., Hundeshagen, H., Klin. Wschr., 1963, v41, 18.

9. Cockburn, F., Sherman, J. D., Ingall, D., Klein, R., Proc. Soc. Exp. Biol. and Med., 1965, v118, 238.

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Role of PMN-Leukocyte Lyosomes in Tissue Injury, Inflammation And Hypersensitivity. III. Passive Cutaneous Anaphylaxis Induced in The Rat with Homologous and Heterologous Hyperimmune Antibody.* (31307)

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The mechanism of passive cutaneous anaphylaxis (PCA) in the rat has long been a subject of controversy, particularly with regard to the possible roles of mediators such as histamine, serotonin and complement (C'). Thus Ovary(1) reported that an antihistamine, mepyramine, suppressed the reaction to heterologous precipitating antibody. Other investigators, however, showed that these reactions produced no mast cell damage(2). Furthermore, the response could not be inhibited by prior depletion of histamine and serotonin with compound 48/80 or by pretreatment with antihistamines and the serotonin antagonist BOL-148(3,4). Bier *et al* (5) and Osler and co-workers(6) reported a positive correlation between the intensity of the cutaneous reaction and the levels of circulating C'; yet Bloch *et al*(7) have shown that both non-C'-fixing guinea pig γ_1 and C'-fixing γ_2 antibody can sensitize the rat for PCA.

Polymorphonuclear (PMN) leukocytes had been observed in the lesions of PCA but were generally dismissed as unlikely to have a primary role in the reaction in either the guinea pig(8) or the rat(1). Brocklehurst and colleagues(3), however, had demonstrated that the amounts of heterologous antibody required to produce an Arthus reaction in the rat were not a great deal larger than those used to produce PCA and suggested that the two reactions might share a common pathogenetic mechanism. The Arthus reaction is mediated primarily by PMN-leukocytes, since the phenomenon cannot be elicited in leukopenic rabbits(9,10).

The recent description by Mota and others (11,12,13,14) of a heat-labile homologous, "mast cell-sensitizing" or "anaphylactic" antibody, capable of sensitizing the rat for PCA, has opened up new paths for investigation. In view of the marked differences between this antibody and hyperimmune antibody, as described by Binaghi *et al*(14), it seems possible that two more or less distinct mechanisms are involved in the production of the PCA lesion in the rat, the operative mechanism being dependent on the type of antibody used for sensitization. The present study investigates the PCA induced in the rat with hyperimmune antibody, either heterologous or homologous.

Materials and methods. Male Lewis rats (Microbiological Associates) were used throughout the study. Rats weighing 500-800 g were hyperimmunized with ferritin (Pentex, Kankakee, Ill.), using complete Freund's adjuvant. The ferritin had been dialyzed against several changes of phosphate buffered saline to remove cadmium. Four mg of antigen was given into the foot pads and intramuscularly in the gluteal region. The sera were collected at 4 weeks, pooled, and antibody nitrogen (AbN) was determined by the Kjeldahl method. Anti-ferritin was also

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TABLE I. Total White Count and Differential Counts in Normal Lewis Rats and in Rats Treated with Rabbit Anti-Rat Leukocyte Serum.

	Normal*	Leukopenic*
Total WBC	11,600	1,900
Neutrophils	16.0%	1.3%
Eosinophils	≤3.0%	<1.0%
Basophils	≤1.0%	<1.0%
Monocytes	6.5%	4.5%
Lymphocytes	78.0%	94.0%

* Based on the means of the normal and intensely leukopenic rats listed in Table II.

prepared in rabbits by injecting 2.5-3 kg rabbits with 25-30 mg ferritin. Most of the ferritin was injected with complete Freund's adjuvant into the foot pads, intramuscularly and subcutaneously. About 5 mg ferritin was given without adjuvant intravenously. The rabbits were bled 6 and 8 weeks after the injection of antigen and the AbN determined as in rats. The anti-ferritin level in rats was 0.06-0.15 mg N/ml and in rabbits 0.8-2 mg N/ml of serum.

Rabbit anti-rat leukocyte serum was prepared by injecting rat peritoneal leukocytes with complete Freund's adjuvant intramuscularly into 2 kg albino rabbits. A booster was given at 2 weeks and the rabbits were bled at 4 weeks. The serum was sterilized by Seitz filtration, biological activity and dose being determined *in vivo* by injecting various doses intraperitoneally and/or intravenously into rats weighing between 150-200 g. Total white cell counts (WBC) and differentials were done at intervals up to 24 hours after injection. Some individual variation was observed, but in general a good depletion could be obtained by injecting 1 ml of the anti-leukocyte serum intraperitoneally, followed 20 hours later with 0.5 ml intravenously (Table I). Penicillin and streptomycin (0.2 ml Strep-Combiotic, Pfizer) were given intramuscularly at time of the first injection. Animals used for PCA were challenged at least 3 hours after the second injection of anti-leukocyte serum since C′ determinations had shown that C′, after an initial sharp drop, returned to normal levels by 3 hours post-injection.

Rats (150-200 g) were injected intracutaneously with 0.1 ml rabbit anti-ferritin (60 and 6 μg N) and rat anti-ferritin (6 μg N) at three sites on the shaved dorsal surface. Two control sites on each animal received 0.1 ml of normal rat serum and normal rabbit serum, respectively. One and one-half to 2 hours after sensitization, animals were challenged intravenously with ferritin (50 mg/100 g body weight) and 0.5 ml of 0.5% Evans blue in saline. One hour after challenge the animals were killed, the skin was reflected and 2 diameters of each lesion were measured on the inner skin surface. Intensity of the bluing response was recorded on an arbitrary scale of 0 to 4+.

In one experiment, normal rats, leukopenic rats and rats treated with 3 mg/kg mepyramine maleate (Neo-Antergan, Poulenc) and 2 mg/kg BOL-148 (Sandoz) 30 minutes before challenge were tested as described. Blood for a final white cell count was taken from all "leukopenic" rats immediately before challenge.

In another experiment, sensitized normal animals were challenged at intervals of 15 minutes, 1, 2, 3, 4, 5, 6, and 24 hours after sensitization, in order to determine the length of the latent period and the persistence of the response.

Tissue was excised from the lesions of normal and leukopenic rats at various times (2 minutes-1 hour) after challenge, fixed in Zenker's fixative or a mixture of osmium tetroxide and Tyrode solution, embedded in methacrylate or epoxy resin, respectively, and sectioned for examination by light and electron microscopy.

C′-depletion was attempted as described by Ward and Cochrane(15), using carrageenin or aggregated human gamma globulin or by intravenous injection of washed antigen-antibody precipitates(6).

White counts, differentials, and C′ levels were followed at intervals from 5 minutes to 24 hours after injections.

Results. Attempts to lower the C′ level of rats without lowering the leukocyte count were unsuccessful and were abandoned.

Animals challenged as early as 15 minutes after sensitization exhibited strong reactions at all test sites. Sharply circumscribed, 4+ reactions were obtained as long as 5 hours

TABLE II. PCA Reactions in the Rat to Homologous and Heterologous Anti-Ferritin.

Group	WBC ($\times 10^3$/mm^3)	Rabbit antiserum 60 μg N Diameter	Rabbit antiserum 60 μg N Intensity	Rabbit antiserum 6 μg N Diameter	Rabbit antiserum 6 μg N Intensity	Rat antiserum 6 μg N Diameter	Rat antiserum 6 μg N Intensity
Normals	11.5	14.5	+++	12.5	+++	11.5	+++
	10.0	13.5	+++	14.0	+++	10.0	++
	9.5	17.0	+++	12.0	+++	9.5	++
	10.0	16.5	++++	12.5	+++	10.0	++
	10.3	15.5	++++	15.0	++++	10.0	+++
	12.0	17.0	++++	13.5	+++	12.0	+++
	14.5	16.0	+++	13.5	+++	14.5	++
Mepyramine & BOL-148	13.5	17.0	++++	13.5	+++	10.5	+
	13.2	16.5	++++	13.0	+++	9.0	++
	13.0	15.5	+++	13.0	++	11.0	+
	12.5	14.5	+++	12.5	++	12.5	++
	13.8	16.5	++++	13.0	++++	12.5	+++
Partially leukopenic	3.3	<5	+	<5	+/—	<5	+/—
	3.0	<5	+	<5	+	<5	+
	4.3	<5	+	<5		<5	+
	6.5	6.5	+	<5		<5	+/—
Leukopenic	1.4	5	+/—	0	0	0	0
	2.4	0	0	0	0	0	0
	1.1	0	0	0	0	0	0
	2.6	0	0	0	0	0	0
	2.3	0	0	0	0	0	0
	2.4	0	0	0	0	0	0
	2.2	0	0	0	0	0	0
	1.2	<5	+/—	<5	+/—	<5	+/—

Intervals between intracutaneous injection of serum and challenge with antigen and Evans blue was 1.5-2 hr.

Diameter of the lesions is expressed in mm; intensity on an arbitrary scale 0-4+.

Control sites injected with normal rabbit and normal rat serum showed no reactions.

after sensitization, but challenge at 6 hours produced only 2 to 3+, rather diffuse reactions. At 24 hours all sites were negative. Control sites showed no bluing, and there was only a slight difference between reactions with rabbit antibody and those produced with the same concentration of rat antibody.

The results of suppression experiments are shown in Table II. Marked suppression of the bluing response was observed in both severely leukopenic and partially leukopenic rats but not in rats pretreated with antihistamine and antiserotonin (BOL-148). The effect of the anti-leukocyte serum on the differential count is shown in Table I. There was marked suppression of neutrophils and a slight effect on monocytes. The effect on eosinophils and basophils could not be assessed because of the low values found in normal rats. The antiserum had no effect on red cells and platelets. Using neat rat serum and 1:10 dilution in saline, a center to center distance of 1 cm, a diffusion time of up to 48 hours at room temperature and volumes of 0.1 ml, no reaction between the anti-leukocyte serum and rat serum could be detected by double diffusion.

In normal rats emigration of leukocytes was observed in biopsies taken 2 minutes after challenge with antigen. At 15 minutes after challenge there was marked leukocytic infiltration in the walls of vessels and in the surrounding connective tissue. No such changes were noted in the leukopenic rats. A few leukocytes were present in the tissue before challenge with antigen. Mast cell damage was observed only in late (1 hour) lesions, where mast cells in the vicinity of massive accumulations of PMN-leukocytes appeared to have released their granules into the surrounding tissues or showed signs of lysis. With the electron microscope it was seen that many of the PMN-leukocytes had phagocytosed material which could be identified as ferritin-anti-ferritin complexes. Many of these PMN-leukocytes appeared to be in the process of releasing their granules (Fig. 1).

Discussion. It has been stated(1) that a latent period is necessary between sensitization and challenge for the production of PCA. In our study the latent period, if it existed, appeared to be shorter than 15 minutes. It seems rather doubtful, therefore, that "fixation" of the antibody to tissues is necessary for the reaction induced in the rat

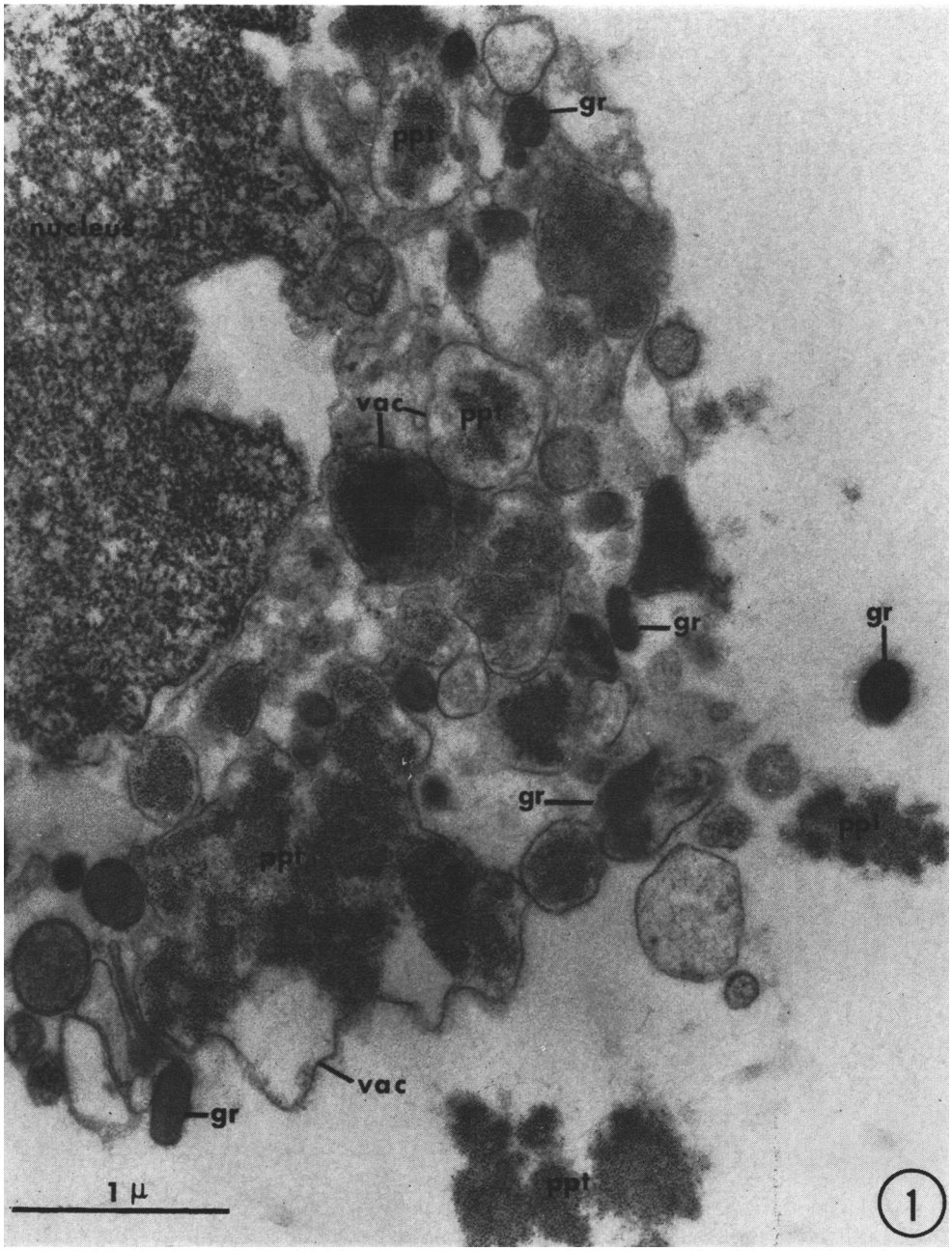

FIG. 1. Rat PMN-leukocyte from 5-minute PCA produced with 6 μg N of rabbit anti-ferritin. There are numerous precipitates (ppt) of ferritin-anti-ferritin in phagocytic vacuoles (vac) in the cytoplasm of the leukocyte. The cell shows signs of lysis with release of both granules (gr) and immune precipitates. × 30,000.

with hyperimmune antibody. Histological sections showed that degranulation of mast cells, as Mota reported(2), was minimal, except in late (1 hour) lesions. Furthermore, pretreatment of the animals with antihistamine and antiserotonin did not suppress the reaction. Leukopenia, on the other hand, produced virtually total suppression and even partial leukopenia was associated with a marked suppressive effect. Attempts to transfer cells passively were made in another experiment, but it was found that injection of peritoneal leukocytes causes only a very temporary increase of these cells in the circulation and attempts to try to produce PCA lesions in such animals were therefore abandoned.

It appears probable from our studies that the primary event in the PCA reaction induced in the rat with hyperimmune antibody is the combination of antigen with antibody to form micro-precipitates, which are then phagocytosed by PMN-leukocytes. Similar ultrastructural changes have been observed in PCA of the guinea pig with much smaller amounts of sensitizing antibody(16). It has also been shown that phagocytosing PMN-leukocytes release a substance (probably lysosomal contents) capable of increasing vascular permeability(17).

Boyden(18) and Ward *et al*(19) have shown that the binding of C′ to antigen-antibody precipitates *in vitro* releases a chemotactic factor. One could postulate that such a factor released *in vivo* attracts the PMN-leukocytes to phagocytose the micro-precipitates. Enhancement of the reaction by added C′(6) could then be explained as an increased release of the chemotactic substance, with a resulting increase in chemotaxis and in the speed and efficiency of phagocytosis. On the other hand, "decomplementation" may have an opposite effect, *i.e.*, there may be a decreased attraction of PMN-leukocytes to the immune precipitates formed in the skin due to a lack of serum C′ and the concomitant lack of the C′5-C′6 complex of Ward *et al*(19). In view of the fact that injection of anti-leukocyte serum caused a drop in serum C′ and that C′ levels could be estimated only in a pilot study and not in the animals used for PCA, partial "decomplementation" could not be excluded in our study. However, in another experiment suppression of PCA by leukopenia in the guinea pig was demonstrated when either anti-leukocyte serum or nitrogen mustard was used to elicit leukopenia(16). Nitrogen mustard does not cause a fall in complement, but unfortunately it could not be used in the rat for technical reasons.

The question of side effects from the anti-leukocyte serum cannot be answered fully. After the first injection of the anti-leukocyte serum, the rats were in mild to moderate shock, but they tolerated the second injection much better and were clinically normal when sensitized and challenged for PCA. Some evidence was obtained from another study that shock has no effect on the skin reaction. The injection of an anti-platelet antibody (adsorbed with PMN-leukocytes) causes a depletion of platelets and a degree of shock similar to that obtained with the anti-leukocyte serum, but has no effect on the bluing obtained when antiserum is injected intracutaneously simultaneously with intravenous antigen and Evans blue(20). The anti-leukocyte serum did not precipitate rat serum when tested by immuno-diffusion. In respect to the possible effect of leukopenia on tissue "fixation" of antibody, we have evidence that rat "anaphylactic" or "mast cell sensitizing" antibody(11,12,13,14) when injected into leukopenic rats was readily fixed(21,22). The PCA was not inhibited in such animals, but was readily suppressed by treatment with antihistamine and antiserotonin.

Summary. The PCA reaction induced in rats with hyperimmune antibody, whether homologous or heterologous, was suppressed in leukopenic and partially leukopenic animals but was not suppressed by pretreatment of the animals with antihistamine and antiserotonin. Mast cells did not appear to be primarily involved in the reaction. Ultrastructural observations showed that PMN-leukocytes in the lesions had phagocytosed micro-precipitates of antigen and antibody and subsequently degranulated. Based on these observations, it is suggested that PCA produced with hyperimmune antibody in the

rat is mediated by release of lysosomal material from PMN-leukocytes.

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1. Ovary, Z., Intern. Arch. Allergy, 1952, v3, 293.
2. Mota, I., Ann. N. Y. Acad. Sci., 1963, v103, 264.
3. Brocklehurst, W. E., Humphrey, J. H., Perry, W. L. M., J. Physiol., 1955, v129, 205.
4. Mota, I., Life Sci., 1963, v2, 465.
5. Bier, O. G., Siqueira, M., Osler, A. G., Int. Arch. Allergy, 1955, v7, 1.
6. Osler, A. G., Hawrisiak, M. M., Ovary, Z., Siqueira, M., Bier, O. G., J. Exp. Med., 1957, v106, 811.
7. Bloch, K., Kourilsky, F. M., Ovary, Z., Benacerraf, B., ibid., 1963, v117, 965.
8. Biozzi, G., Mené, G., Ovary, Z., Rev. Immunol., 1948, v12, 320.
9. Stetson, C. A., J. Exp. Med., 1951, v94, 347.
10. Humphrey, J. H., Brit. J. Exp. Path., 1955, v36, 268.
11. Mota, I., Immunology, 1964, v7, 681.
12. ———, ibid., 1964, v7, 700.
13. Binaghi, R. A., Benacerraf, B., J. Immunol., 1964, v92, 920.
14. Binaghi, R. A., Benacerraf, B., Bloch, K., Kourilsky, F. M., ibid., 1964, v92, 927.
15. Ward, P. A., Cochrane, C. G., J. Exp. Med., 1965, v121, 215.
16. Taichman, N. S., Movat, H. Z., Int. Arch. Allergy, in press.
17. Movat, H. Z., Uriuhara, T., Macmorine, D. L., Burke, J. S., Life, Sci., 1964, v3, 1025.
18. Boyden, S., J. Exp. Med., 1962, v115, 453.
19. Ward, P. A., Cochrane, C. G., Müller-Eberhard, H. J., ibid., 1965, v122, 327.
20. Uriuhara, T., Movat, H. Z., unpublished observations, 1965.
21. Lovett, C. A., Wardlaw, A. C., Movat, H. Z., Fed. Proc., 1966, v25, 681.
22. Movat, H. Z., Lovett, C. A., Taichman, N. S., Nature, in press.

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The Leukocyte Response to Intraperitoneal Ehrlich Ascites Tumor In Mice.* (31308)

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The defenses of the host against malignant invasion are at present ill-defined, but include reactive or immune counter measures by leukocytes. The qualitative and quantitative aspects of this relationship require further definition. The amount of tumor presented in an artificial situation such as transplantation does not necessarily reflect the natural circumstance, but host response can be evaluated thereby.

Ehrlich ascites tumor (EAT) has been shown to be a successful invader of many mouse strains; it appears to possess foreign antigenicity, since previously immunized mice will reject otherwise tumor-producing doses. Furthermore, McCarthy has shown that C_3H mice with intraperitoneally growing EAT will accept foreign skin grafts, which are retained until the death of the animal(1). Thus, the acceptance of the neoplasm resulted in an "acceptance" of the homografted skin, an outcome known to be dependent at least in part on lymphocytic functions.

The peritoneal cavity may be a rather specialized site for tumor cell growth. Not only are the structural, nutritional, and transport aspects somewhat unique, but the free and close contact between invading tumor cell and resisting leukocyte may allow an evaluation of factors, mechanical or biological, determining neoplastic invasion(2,3). Old *et al* have reported on their interest in the leukocyte/tumor cell ratio in the inva-

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