THE MECHANISM OF ARTHUS REACTIONS. II. THE ROLE OF POLYMORPHONUCLEAR LEUCOCYTES AND PLATELETS IN REVERSED PASSIVE REACTIONS IN THE GUINEA-PIG.

J. H. HUMPHREY.

From the National Institute for Medical Research, Mill Hill, London, N.W.7.

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In the preceding paper (Humphrey, 1955) evidence was given for the importance of neutrophil polymorph accumulation in the development of vascular necrosis and oedema at sites of reversed passive Arthus (R.P.A.) reactions in the rabbit. It was desirable to ascertain whether the sequence of events was broadly similar in some other species, and the guinea-pig was chosen, both because its response to acute anaphylaxis is different from that of the rabbit, and because an excellent quantitative study of passive Arthus reactions in this species had already been made by Benacerraf and Kabat (1950). Exact repetition of the experiments made in rabbits was not possible because nitrogen mustard (HN2) proved to be too toxic to guinea-pigs when administered in doses sufficient to remove neutrophil polymorphs effectively from the circulation. Furthermore, no guinea-pig sera were available containing sufficient precipitating antibody to give easily measurable macroscopic reactions in R.P.A. reactions, and heterologous (rabbit) antisera had to be used instead. The first difficulty was overcome by the use of potent specific rabbit antibodies against guinea-pig polymorphs, following the work of Bedson (1921), and these proved to be a very effective means of removing mature polymorphs not only from the circulating blood, but also from the bone marrow. It was also possible to obtain potent specific rabbit antisera against guinea-pig platelets. By means of these various reagents it has been found that in the guinea-pig, as in the rabbit, polymorphs are essential for the formation of gross oedema at Arthus reaction sites, but not for the rapid anaphylactic increase in capillary permeability. In the absence of platelets, the severity of such reactions is somewhat increased.

METHODS.

Animals.

Albino guinea-pigs were used, weighing 350–550 g., from the stock bred at the National Institute for Medical Research. They were fed on pelleted Diet No. 18 (Bruce and Parkes, 1946) supplemented with cabbage and hay. The bellies were clipped and depilated on the day before testing.

Production of R.P.A. reactions.

Antigen was injected intraperitoneally, dissolved in 1 ml. 0·9 per cent NaCl. After 1–1½ hr. two intracutaneous injections of 0·2 ml. suitably diluted rabbit antiserum were made symmetrically into the belly wall on either side of the midline. Oedema volume was estimated from direct measurements of the swelling visible when the animal was held supine. The amounts of antibody in each injection were 1–2·5 mg., and the amounts of antigen were approximately 50 × the neutralising equivalents of the antibody. The antigen-

antibody systems were pneumococcus Type III capsular polysaccharide, 5 × recrystallised ovalbumin, crystallised bovine serum albumin (Armour), and human gamma globulin together with their respective rabbit antisera, prepared as described in the preceding paper. Most of the experiments were made with pneumococcus polysaccharide and the corresponding antiserum, but results were similar with the other systems.

It was impossible to test each animal before and after depletion of polymorphs, because the duration of depletion exceeded the period required for sensitisation to the antigens and antisera injected in the first test. Control groups of matched guinea-pigs were therefore included in each experiment.

Preparation of antisera against neutrophil polymorphs and platelets.

Neutrophil polymorphs were obtained from exudates induced by intraperitoneal injection of 50 ml. 3 per cent solution of bacteriological peptone in 0·9 per cent NaCl. After 15 hr. a further 20 ml. was injected intraperitoneally, and 3 hr. later the exudate, containing 90 per cent or more of polymorphs was collected, with addition of 1/5 vol. 3·8 per cent sodium citrate. The cells were washed 3 × with buffered Ringer solution containing 0·1 per cent gelatine, and were used fresh, or after storage for up to 3 days at 0°.

Platelets were obtained by differential centrifugation of blood taken by cardiac puncture from heparinised guinea-pigs, into silicone-treated tubes containing 1/10 vol. of 1 per cent di-sodium salt of ethylenediamine tetra-acetic acid (" Versene "—Bersworth Chemical Co., Framingham, Mass., U.S.A.). They were washed twice with 0·9 per cent NaCl solution at 2°, and suspended in buffered gelatine Ringer.

Rabbits were given intravenous injections of approximately 10^8 polymorphs or 10^9 platelets at 3-day intervals for a course of 3 weeks. Sometimes a second course was given 6–8 weeks later. Serum samples were tested at intervals for ability to agglutinate *in vitro* (in silicone-treated tubes) the cells against which the rabbits had been immunised. The end-point was taken as that dilution of serum which, after standing overnight at 4°, caused just definite macroscopic agglutination of polymorphs (5 × 10^6 per ml.) or platelets (5 × 10^8 per ml.). Sera with titres of at least 500 against polymorphs or 1,000 against platelets were heated at 56° for ½ hr. and kept for subsequent absorption. Both types of antisera contained agglutinins against erythrocytes, which were absorbed by successive treatments with washed erythrocytes, until only a very fine agglutination occurred. They were then treated with ½ vol. of washed liver cells, ¼ vol. of washed kidney cells, ¼ vol. of washed spleen cells, and the anti-platelet sera were finally absorbed with polymorphs obtained from peritoneal exudate. After absorption the globulins were precipitated twice with Na_2SO_4 (final concentration 18 per cent w/v) at pH 7·8, dialysed to remove Na_2SO_4, and made up with 0·9 per cent NaCl to a volume equal to the original volume of serum. About half of the original antibodies against polymorphs or platelets remained after the treatment outlined above, and they were specific for the cell types concerned.

Haematological examinations.

These were made on blood taken from ear veins after smearing with soft paraffin, and causing vasodilatation with toluene. In examinations for the presence or absence of platelets, the veins were pricked through sodium citrate solution.

RESULTS.

Effect of nitrogen mustard on blood neutrophils.

Although HN2 administration did not prove to be satisfactory, it is worth recording that a dose of 2 mg./kg. intravenously caused a marked reduction of polymorphs, beginning on the 4th day after administration and maximum on the 6th day (average count 100/c.mm.), with relatively little reduction in lymphocytes and monocytes. Doses of 1 mg./kg. were much less effective, while doses of 3 mg./kg. caused death after 4 days. There was thus no margin of safety when HN2 was used, and furthermore, even when polymorph counts were at their lowest, they increased 10-fold or more in response to injections of antigen.

Effect of antibodies on neutrophil polymorphs.

The effect of intraperitoneal injection of 2·0 ml./kg. anti-neutrophil globulins is illustrated in Fig. 1. The reduction in neutrophil polymorphs was rapid, and persisted for five days, in contrast to the delayed onset and transient nature of the neutropenia due to HN2 administration. Other formed elements of the blood (erythrocytes, lymphocytes and platelets) were unaffected, or—in the case of lymphocytes—suffered at most a 2–4-fold reduction. The animals showed no obvious effects from the injections, except that for two days afterwards the skin of the belly, exposed by depilation, was mildly erythematous.

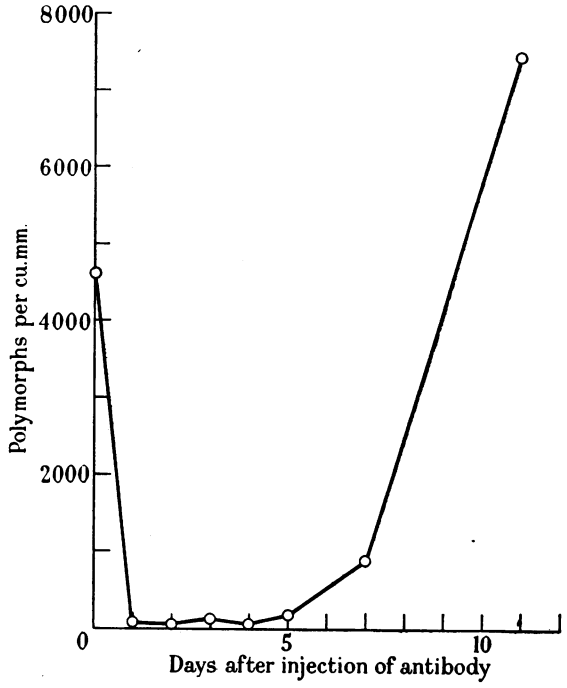


FIG. 1.—Effect of 2·0 ml./kg. anti-neutrophil globulins on neutrophil polymorph counts in peripheral blood of guinea-pig.

Of 46 guinea-pigs which received antibody, and whose differential blood count was done two days later, 22 had 0–1 per cent neutrophils, 16 had 2–4 per cent and 7 had 10 per cent or more. The neutropenia in the peripheral blood was accompanied by similar changes in the bone marrow, the percentage of mature neutrophils plus neutrophil myelocytes falling to between 9 and 20 per cent, instead of the normal 40 per cent or over. Guinea-pigs with neutropenia were very susceptible to bacterial infection, and it was found that strict sterile precautions were necessary where intravenous injections were made in the thigh, in order to avoid local cellulitis.

Effect of antibodies on platelets.

A single injection of 0·5 ml./kg. anti-platelet antibody globulins caused no immediate ill effects on the guinea-pig, but 24 hr. later the platelet counts had dropped to 20,000/c.mm. or lower. It was frequently impossible to detect any platelets in smears of peripheral blood, although leucocytes were unaffected. The animals had petechial haemorrhages in many parts of the body, particularly retroperitoneally and at sites of friction, and they became increasingly anaemic. Those which survived for 5 days recovered rapidly, and platelets returned to the circulation at this time.

Effect of neutropenia on oedema of R.P.A. reactions.

Reversed passive Arthus reactions were performed as described under "Methods" in guinea-pigs which had received 2·0 ml./kg. anti-neutrophil serum intraperitoneally two days previously, and in similar animals which had received corresponding amounts of normal rabbit globulins. An additional control was provided by animals of both series which were injected intracutaneously with rabbit antisera but did not receive antigen.

In the Table are recorded the measurements of gross oedema in treated and untreated animals. It is evident that in the absence of neutrophils oedema was minimal, as compared with the massive oedema which appeared in control animals. Despite this reduction in gross changes, however, neither the early increase in capillary permeability nor the later mononuclear cell invasion at the reaction sites was appreciably changed, as will be shown below.

TABLE.—*Mean Oedema Volumes at Sites of R.P.A. Reactions in Guinea-pigs with and without Neutrophil Polymorph Depletion.*

Treatment	Number of guinea-pigs.	Oedema vol. (ml.) after 7 hr.	24 hr.	48 hr.
Anti-polymorph antibody i.p.	9	0·4	0·5	*nil*
Normal globulin i.p.	11	1·4	3·0	2·6
Controls without antigen	16	0·3	0·13	*nil*

Each animal was injected at 2 sites.

Effect of neutropenia on changes in vascular permeability.

Reaction to histamine, 48/80 and leukotaxine.—Guinea-pigs were injected intraperitoneally with anti-neutrophil polymorph antibodies or with normal rabbit globulin. Two days later they were clipped and depilated, and were then injected intravenously with 0·6 ml./kg. 5 per cent Pontamine Sky Blue 6BX (G. Gurr). Immediately afterwards, 0·1 ml. quantities of 0·9 per cent NaCl containing graded doses of histamine (0·33–3 μg.), or the histamine liberator 48/80 (1–9 μg.) or leukotaxine (12–48 μg.) were injected intracutaneously, and the area and intensity of local blueing were noted. There were no differences between depleted and control animals.

Direct passive local anaphylaxis.—Guinea-pigs were sensitised passively by intraperitoneal injection of 1 mg./kg. rabbit antibody to pneumococcal capsular polysaccharide (Type III) at the same time as they received anti-neutrophil or

normal rabbit globulins. Two days later they were depilated, blued as above and straightway injected intracutaneously with 2 and 0·5 μg. of the polysaccharide in 0·1 ml. Blueing at the injection sites was rapid—being maximal in 10 min.—and was equal, both in area and intensity, in depleted and control animals.

Reversed passive local anaphylaxis.—In order to study the time course of the changes in capillary permeability, R.P.A. reactions were induced, as described above under "Methods", in 32 guinea-pigs of which half had been depleted of polymorphs in the usual way. Two doses of antibody, 1·0 and 0·04 mg., were used in separate experiments. Each animal received 4 intracutaneous injections of antibody, at different times relative to each other, and after a further interval Pontamine Blue was administered intravenously. In this way the distribution and intensity of capillary leakage was observed at intervals from a few minutes to 6 hr. after injecting antibody.

With the high doses of antibody blueing began around 30 min., and was maximal at 3½–5½ hr. During the later stages only the periphery of the lesions blued, the centres remaining pale, except for small central areas of haemorrhage. The pattern of blueing was similar to that described at sites of R.P.A. reactions in the rabbit (Humphrey, 1955), except that the reaction was more intense. There was no significant difference between the blueing reactions in polymorph-depleted and control animals.

With the low doses of antibody, the extent of blueing was much less, and there were no haemorrhages. Blueing began sooner, and was maximal at ½–1 hr. after injecting antibody. After 1½ hr. in normal guinea-pigs, the reaction site remained pale compared with the surrounding skin, whereas in polymorph-depleted animals increased capillary permeability was still evident up to 6 hr. It appeared that the closure of the vessels in the centre of these less intense lesions was in some way bound up with polymorph infiltration.

Effect of platelet depletion on R.P.A. reaction.

Six guinea-pigs depleted of platelets by injection of anti-platelet serum on the previous day, were subjected to R.P.A. reactions in the usual way. The reactions were compared with those in normal controls treated at the same time.

When judged by oedema formation (measured at 24 hr.) and haemorrhage, the reactions in the platelet-depleted animals were more severe than in the controls. Since the depleted animals had generalised petechial haemorrhages, strict comparison is impossible, but it was notable that much of the massive oedema at the reaction sites in these animals was not blood-stained—*i.e.*, it was true oedema fluid and not seepage from haemorrhages.

Histological changes at R.P.A. sites in normal and polymorph-depleted guinea-pigs.

Skin sites were excised 5, 24 and 48 hr. after the start of R.P.A. reactions in normal and polymorph-depleted guinea-pigs. Control sites, injected with normal rabbit serum or normal rabbit gamma globulin, were examined for comparison. They were stained with haematoxylin and eosin after fixation in corrosive sublimate and acetic acid.

The general picture—*i.e.*, oedema, vascular necrosis and thrombosis (with or without haemorrhage), perivascular infiltration by polymorphs and mononuclear cells—was similar in normal guinea-pigs to that in normal rabbits (see previous

paper), except in certain features. These were that already after 5 hr. the mononuclear cell accumulation was well marked; that oedema continued to increase and to persist for longer; and that polymorphs disappeared more slowly, being still present in very large numbers after 48 hr. In polymorph-depleted animals the histological picture was similar to that in normals as regards mononuclear cell accumulation, occurrence of platelet thrombosis, and oedema in the early stages; vascular necrosis, although present in all lesions to a variable degree, was less extensive than in normal animals, and at 24 and 48 hr. there was very much less oedema despite the marked mononuclear cell accumulation. These features are illustrated in Fig. 2–7.

DISCUSSION.

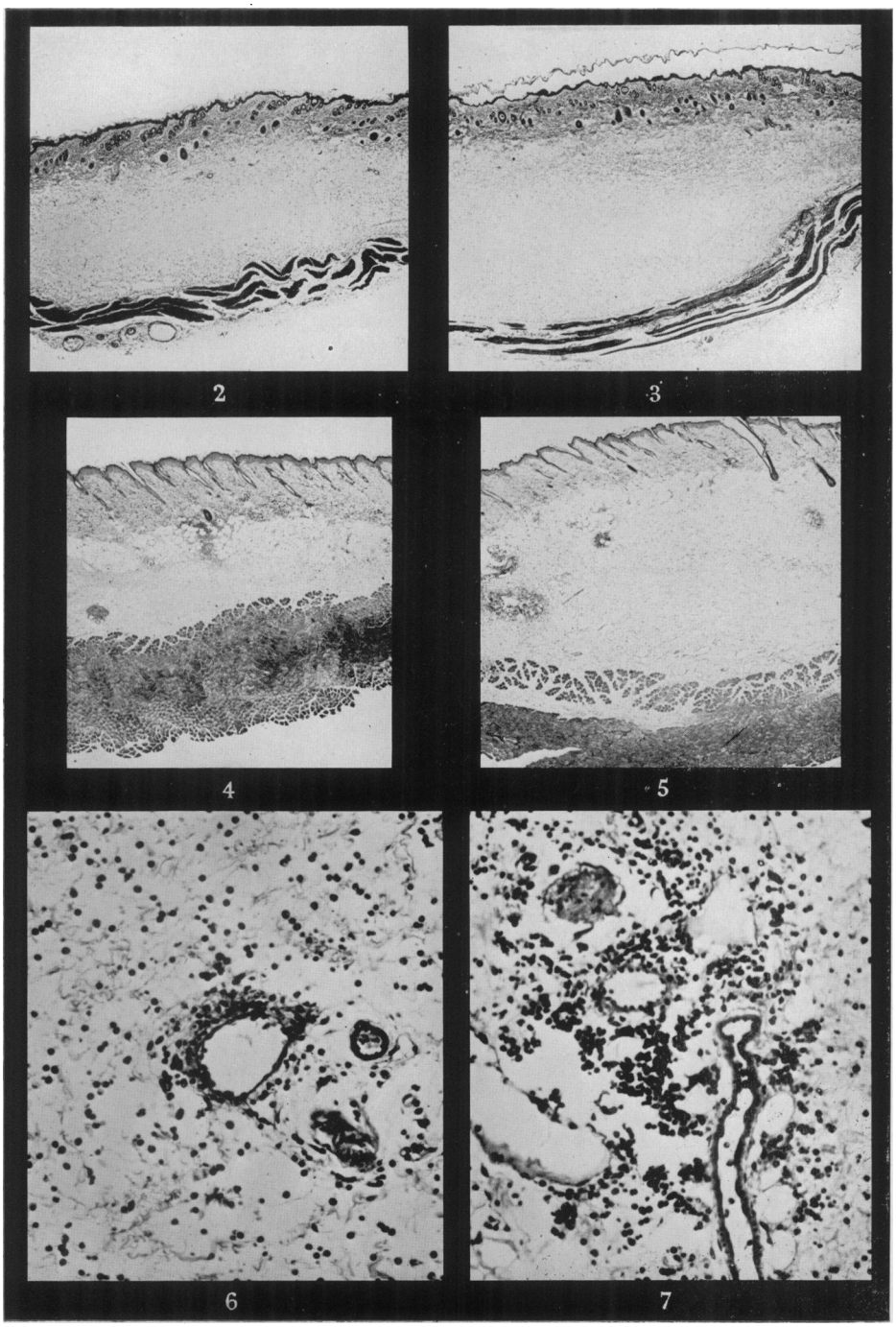
Fundamentally, the course of R.P.A. reactions in guinea-pigs and rabbits, and the effect of polymorph depletion on them, is similar. Since depletion was achieved by entirely different mechanisms, it seems that absence of polymorphs, rather than some other unknown factors, accounted for the marked diminution of oedema and the decrease in vascular damage observed in depleted animals. The conclusion reached in the previous paper, that polymorph accumulation was a major factor in exaggerating the damage to walls of capillary venules and, to a lesser extent, arterioles, and in blocking the lymphatics during the second stage of the reaction, is thereby substantiated. In the guinea-pig, in contrast with the rabbit, the increased vascular permeability of the earliest phase was very marked, and was uninfluenced by polymorph depletion. This may mean that guinea-pig skin vessels are relatively highly sensitive to the initial product(s) of the antigen antibody reaction, whereas in the rabbit the corresponding changes cause little more than increased endothelial stickiness. Differences of this nature could also explain the fact that whereas " passive cutaneous anaphylaxis " (Ovary and Bier, 1953) is readily elicited in guinea-pigs, no such reaction occurs in rabbits.

The more rapid accumulation of mononuclear cells at the reaction sites in guinea-pigs than in rabbits may be due to the use of heterologous antisera in the first species and of homologous antisera in the second. It could also be due to a species difference, and the experiments described do not allow of distinction between these possibilities.

Thrombosis being a prominent feature of severe or moderate Arthus reactions, it might be expected that prevention of thrombosis by platelet depletion would lessen the severity of the reaction—despite the fact that in rabbits large amounts of heparin were relatively ineffective. The severity was, however, actually increased, and platelets must, to some extent, play a protective rôle, either by causing thrombosis or by vasoconstriction (due to liberation of their contained 5-hydroxytryptamine), thereby limiting the area of inflammation.

EXPLANATION OF PLATES.

FIG. 2–7.—All show sites of reversed passive Arthus reactions in guinea-pig skin. Fig. 2: Polymorph-depleted; 5 hr. (× 18). Fig. 3: Normal guinea-pig; 5 hr. (× 18). Note greater oedema, and cellular invasion. Fig. 4: Polymorph-depleted; 2 days. (× 20). Perivascular cell collections are mononuclear cells. Fig. 5: Normal guinea-pig; 2 days. (× 18). Note persistent oedema. Perivascular cell collections are both mononuclear and polymorphonuclear. Fig. 6: Higher power of Fig. 2, showing early invasion by mononuclear cells in polymorph-depleted animal. (× 210). Fig. 7: Higher power of Fig. 5. (× 210).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SUMMARY.

Guinea-pigs were deprived of blood neutrophil polymorphonuclear leucocytes and/or platelets by means of specific antisera.

Reversed passive Arthus reactions were studied in polymorph-depleted guinea-pigs and compared with those in normal animals. In the absence of polymorphs, oedema was very much diminished, polymorph invasion was absent, and vascular damage was decreased. Other changes, notably acute early increase in vascular permeability, and a relatively early mononuclear cell invasion, were unaffected.

The severity of the reactions was increased in animals without platelets.

The findings are compared with those in rabbits.

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