Immunology, 1969, **17**, 777.

Eosinophil Granule Lysis *in vitro* Induced by Soluble Antigen–Antibody Complexes

G. T. ARCHER, MARGARET NELSON AND JILL JOHNSTON

New South Wales Red Cross Blood Transfusion Service, Sydney, Australia

(*Received* 31*st March* 1969)

Summary. A simple test system is described, for the demonstration of antigen–antibody reactions capable of causing eosinophil granule lysis *in vitro*. The antigen preparations used were extracts of the nematode *Amplicaecum robertsi* and body fluid of *Ascaris suum*. Antisera were obtained from rats infested with *Amplicaecum*. Eosinophils were obtained from the peritoneal cavity of normal rats. Centrifugation of the cells to form a cell button was an essential step in the procedure. Lysis of eosinophils occurred with antiserum obtained from the animals between the 12th and 32nd days of infestation with *Amplicaecum*, and was accompanied by vacuole formation in macrophages and mast cell disruption. The reaction was most pronounced during the 3rd week. Serum from adrenalectomized infested animals caused the most marked changes in eosinophils. Serum from cortisone-treated infested animals failed to cause eosinophil changes.

Attempts at purification of the antigen in *Ascaris* body fluid resulted in two fractions with marked activity in the test system. The same two fractions were found to form precipitin lines on agarose gel diffusion against rat antiserum.

It is postulated that antigen–antibody complexes soluble in low concentration were responsible for the changes observed in the eosinophils, macrophages and mast cells. One or more labile factors in the serum were found to be necessary for eosinophil granule lysis. The evidence, though incomplete, would favour the suggestion that both labile antibody and complement were necessary.

INTRODUCTION

Phagocytosis by eosinophils of insoluble antigen–antibody complexes is known to be followed by lysis of those eosinophil granules near the phagocytic vacuole (Archer, 1969). Eosinophil granule lysis has been observed in preparations containing fresh serum, antigen and antibody, and in red cell antigen–antibody systems studied, granule lysis occurred at the same time as red cell lysis (Archer and Hirsch, 1963). It has, therefore, been suggested that complement factors may be responsible for eosinophil granule lysis. This paper reports the finding of rapid degranulation of eosinophils in the presence of soluble antigen–antibody complexes. Labile serum factors were found to be necessary for granule lysis, and complement was fixed during the reaction.

MATERIALS AND METHODS

Antigens

The antigens were obtained from the nematode parasites *Amplicaecum robertsi* (Sprent, 1963) and *Ascaris suum*. Adult worms of *Amplicaecum* were obtained from the stomach of the

carpet python and homogenized with a small volume of physiological saline in a teflon tissue grinder. Insoluble material was deposited by centrifugation. The clear supernatant had a protein concentration of approximately 5 mg/ml. *Ascaris* body fluid was obtained from adult worms by cutting off the posterior tip of the worms and expressing the fluid. Insoluble material was removed by centrifugation and the clear supernatant was dialysed against 0·005 M phosphate buffer at pH 8·0. Four millilitres of the dialysate was applied to a DEAE-cellulose column 16 cm high × 1·6 cm wide, equilibrated with the phosphate buffer. Gradient elution was performed using 100 ml of each of the following phosphate buffer solutions: 0·01 M at pH 8·0; 0·05 M at pH 8·0; 0·1 M at pH 8·0; 0·15 M at pH 6·0; 0·15 M at pH 6·0 in 2 M sodium chloride; 0·15 M at pH 6·0 in 4 M sodium chloride. Two hundred drop fractions (approximately 6 ml) were collected. The major protein fraction was concentrated in a rotary evaporator after dialysis against distilled water. The residue was dissolved in saline and applied to a Sephadex G-200 column, 32 × 1·6 cm, equilibrated with saline. Fifty-drop fractions (1·5 ml) were collected.

Agarose block electrophoresis of nematode protein

Agarose 1 per cent in veronal buffer at pH 8·6 and ionic strength 0·04 was used in blocks measuring 27 × 10 × 1 cm. Veronal buffer at pH 8·6 and ionic strength 0·08 was used in the electrode chambers. The protein sample was injected together with a warm solution of 2 per cent agarose into a slit approximately 1 cm wide cut towards the cathode end of the block. Electrophoresis was performed at 7° for 18 hours at 50 V and 50 mA. Strips 1 cm wide were cut across the block. Each strip was homogenized in approximately 25 ml physiological saline and filtered. The filtrates were placed in Visking casing and dialysed against 10 per cent Carbowax in water for 20 hours. The sacs were then dialysed against saline for approximately 3 hours, the contents made up to 10 ml with saline, and centrifuged. The supernatant solutions were analysed for protein and for activity in promoting eosinophil degranulation *in vitro*.

Protein was estimated by the Lowry modification of the Folin–Ciocalten method, using crystalline lysozyme as a protein standard (Lowry, Rosebrough, Farr and Randall, 1951).

Antisera

Young adult male rats of the Long Evans strain were each injected with 2000 larvae of *Amplicaecum robertsi* by the intramuscular route (Archer and McGovern, 1968). Blood samples were collected from the jugular vein by venepuncture, the overlying hair being first removed with a depilatory cream. Between 10 and 20 days before infestation one group of six rats was submitted to bilateral adrenalectomy; another group of six (sham operated controls) had a laparatomy performed and an incision made into the retroperitoneal tissue surrounding each adrenal gland. After infestation with *Amplicaecum*, a third group of six rats was given 1 mg hydrocortisone acetate ('Cortef', kindly made available by Upjohn Pty Ltd, Australia), injected into the right hind limb, and the dose was repeated every 2nd day for 20 days.

Antigen–antibody precipitate

The method of gel diffusion described by Ouchterlony (1962) was used to demonstrate precipitating antibody to worm antigens. Micro plates were made up with 1 per cent agarose in saline. Immunoelectrophoresis was performed, using 1 per cent agarose in

veronal buffer at pH 8·6, ionic strength 0·04. A layer 1-mm thick was poured on to microscope slides. Electrophoresis was performed for 90 minutes at 100 V and 20 mA.

Peritoneal cavity cells

Cells were obtained from normal rats by peritoneal lavage (Bosworth and Archer, 1961). In one experiment the cells were separated into mast cell rich, eosinophil rich and mononuclear cell rich preparations, but otherwise no attempt was made to separate the different cell types. The cells were washed in physiological saline using a polystyrene test tube and 10 per cent cell suspensions in saline were prepared.

In vitro *phagocytic system*

The reaction mixture consisted of four drops of rat serum, one drop of *Amplicaecum* extract or of an *Ascaris* body fluid fraction, and one drop of peritoneal cavity cell suspension (see Fig. 1). The polystyrene test tube containing the mixture was centrifuged at 500 ***g*** for 3 minutes to deposit the cells, then incubated at 37° for 15 minutes. The supernatant serum was removed and cell smears were prepared as for blood films. The smears were stained with Wright's haematological stain and examined with the light microscope.

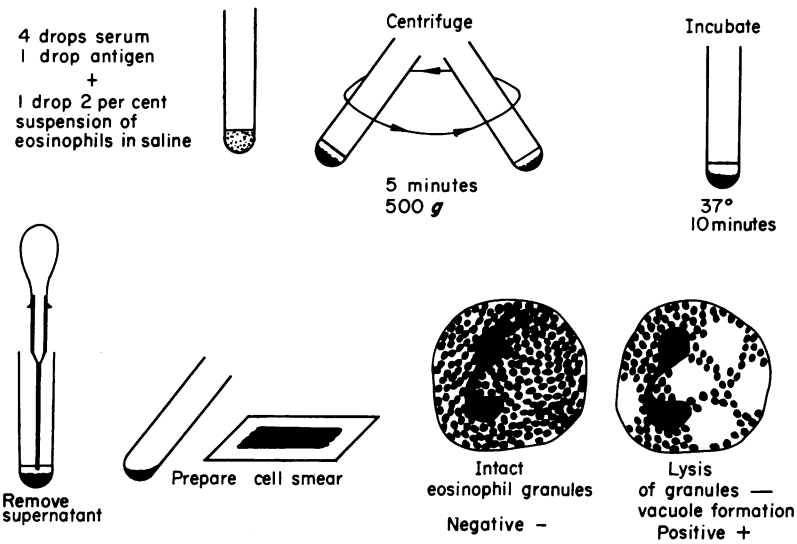

FIG. 1. Method used to detect the antibody in rat serum which is capable of causing eosinophil granule lysis.

Phase-contrast studies

Cell suspensions for phase-contrast studies and cinemicrophotography were prepared as follows: a platinum loop was dipped into the antisera under test and four loops full were placed at one end of a microscope slide. One loopful of *Amplicaecum* extract or *Ascaris* body fluid and one loopful of cell suspension were added and mixed with the antiserum. One loopful of the mixture was placed in the centre of the slide and a cover-slip applied. The slide was examined with a Leitz Ortholux microscope using a xenon light source and phase-contrast optics.

Complement fixation

Infested rats were hyperimmunized against human red cells by the intramuscular injection of 1 ml packed washed red cells. Fresh serum collected from these animals 1–2 weeks after the injection was found to cause haemolysis of human red cells. One volume of *Ascaris* body fluid was added to 4 volumes of serum and incubated at 37° for 30 minutes prior to the addition of human red cells. The positive control consisted of antigen solution added to the serum after the incubation step and immediately prior to the addition of the cells. Haemolysis in the positive control but not in the test preparation after incubation at 37° for 10 minutes was taken as evidence that complement had been fixed during incubation.

Effect of heat on immune serum

Sera from infested rats immunized against human red cells were incubated for 15 minutes over the range 45°–55°. After incubation the sera were tested for haemolytic activity against human red cells and ability to cause lysis of rat eosinophil granules. Equal volumes of fresh normal rat serum were added to the heated sera and the mixtures were submitted to the same tests.

RESULTS

EXAMINATION OF CELL PREPARATIONS

In all preparations which contained *Amplicaecum* extract or *Ascaris* body fluid the eosinophils showed marked vacuolation and granule lysis. Fig. 2 shows a number of eosinophils

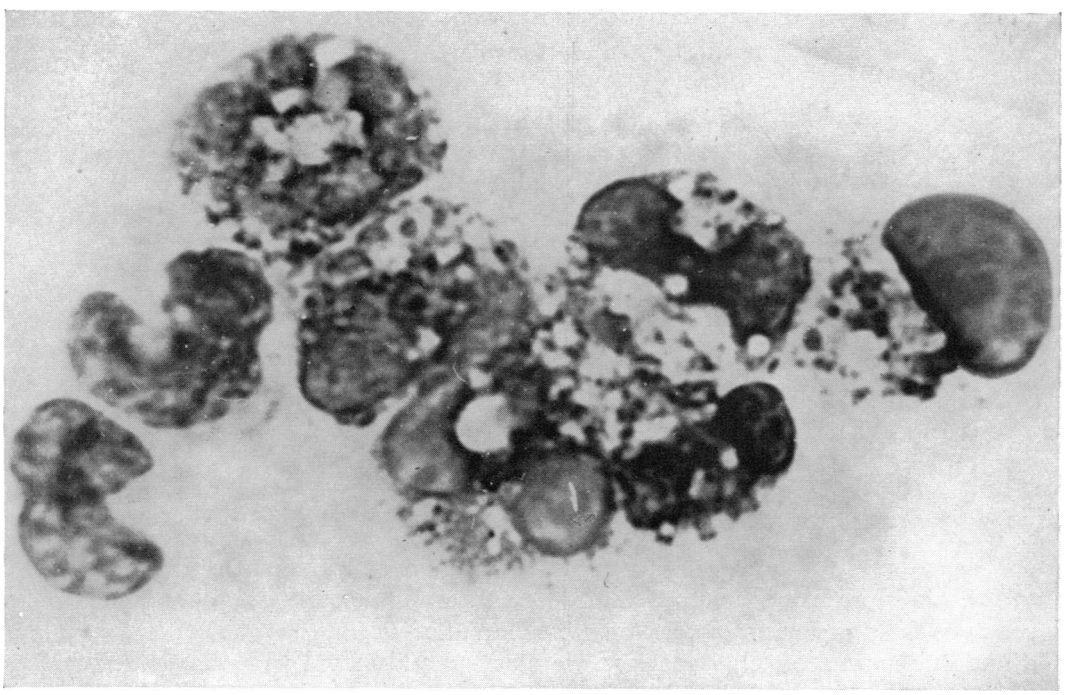

FIG. 2. Stained film showing a clump of six eosinophils, with two macrophages at the left of the photomicrograph. The vacuoles in the cytoplasm of the eosinophils appear white, the coalesced granular material as dark spots. Wright's stain, × 350.

TABLE 1

EFFECT OF SERUM FROM *Amplicaecum* INFESTED RATS ON EOSINOPHILS AND MACROPHAGES IN THE PRESENCE OF ANTIGEN

Day of infestation	Eosinophil granule lysis	Macrophage vacuolation
0	−	−
2	−	−
4	−	−
8	−	−
12	+	+
16	++	++
20	+++	+++
24	+++	+++
28	++	++
32	+	+

in which many of the granules have disappeared, leaving clear rounded areas in the cytoplasm. Other areas have taken up eosin stain and represent concentrations of coalesced eosinophil granule protein. Marked vacuolation was also observed in the mononuclear cells. The most pronounced changes were in macrophage-type cells, with indented nuclei and pale blue cytoplasm. The vacuoles usually appeared white and clear but in some preparations the ingested material seemed to have formed a precipitate in the cytoplasm.

Sera from rats infested with *Amplicaecum* varied in their ability to cause eosinophil and macrophage changes, as shown in Table 1.

It will be noted that sera collected up to 8 days after infestation were negative in the test system and that those collected between 12 and 32 days were positive, the most pronounced changes being observed with sera collected 3 weeks after infestation.

Table 2 shows the effect of adrenalectomy and of hydrocortisone on the ability of serum from *Amplicaecum* infested rats to cause changes in the eosinophils. Adrenalectomy increased the activity of the serum, whereas second daily injections of 1 mg hydrocortisone completely suppressed activity. Sera collected from sham-operated controls infested with *Amplicaecum* and from non-operated infested animals gave similar results. The mast cells present in the test system showed degranulation and swelling in those preparations where eosinophil and macrophage vacuolation was most marked. Mast cell degranulation was most conspicuous when the serum used was obtained from adrenalectomized animals.

TABLE 2

EFFECT OF ADRENALECTOMY AND HYDROCORTISONE TREATMENT ON THE PRODUCTION OF EOSINOPHIL LYTIC ANTIBODY

Rat serum used collected at the 24th day of infestation with *Amplicaecum*	Eosinophil granule lysis
(a) Non-operated controls	+
(b) Sham-operated controls	+
(c) Adrenalectomized animals	++
(d) Hydrocortisone-treated animals (1 mg every 2 days)	−

EFFECT OF HEAT ON IMMUNE SERUM

Heating immune serum at temperature up to 50° for 15 minutes did not prevent haemolysis of human red cells or lysis of rat eosinophil granules. Heating at 51° lowered the rate of both reactions. Heating at 52° destroyed activity. The addition of an equal volume of fresh normal serum to serum heated at 52° restored its ability to haemolyse human red cells, though the rate of haemolysis was less than with unheated serum. Lytic activity against rat eosinophil granules was not restored.

FIXATION OF COMPLEMENT

The haemolytic activity of immune serum disappeared when the serum was incubated at 37° for 30 minutes with *Ascaris* body fluid.

PHASE-CONTRAST STUDIES OF VIABLE CELL PREPARATIONS

Eosinophil granule lysis and vacuolation were seen to occur more rapidly than macrophage vacuolation. Eosinophil granules burst suddenly at one point in the cell, leaving a vacuole in the cytoplasm. In the macrophage, vacuoles appeared to be secondary to pinocytosis. Small pinocytic vesicles formed at the periphery of the cytoplasm, gradually increased in size, and frequently moved towards the perinuclear area.

Mast cell disruption and bursting of mast cell granules was frequently observed at the same time as eosinophil granule lysis, but it was difficult to interpret the results because non-specific mast cell disruption was observed when cover slips were applied firmly to cell preparations on microscope slides.

FRACTIONATION OF *Ascaris* BODY FLUID

Protein profiles of fractions of *Ascaris* body fluid separated by DEAE-cellulose column chromatography are shown in Fig. 3. The pooled Fraction 4–8 was found to be inactive,

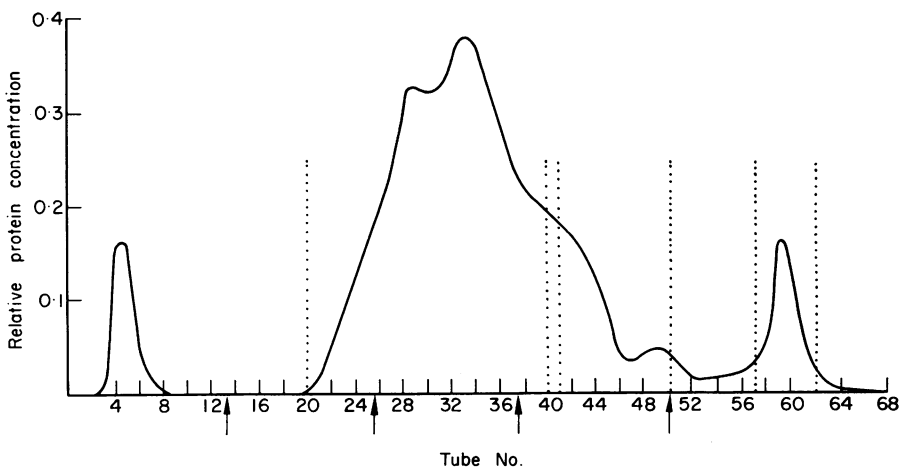

FIG. 3. Protein profiles of *Ascaris* body fluid after fractionation on DEAE-cellulose. Changes of buffer in the reservoir are indicated by the arrows. Fractions between the dotted lines (20–40, 41–50, 57–62) were submitted to further fractionation (Figs. 4 and 5).

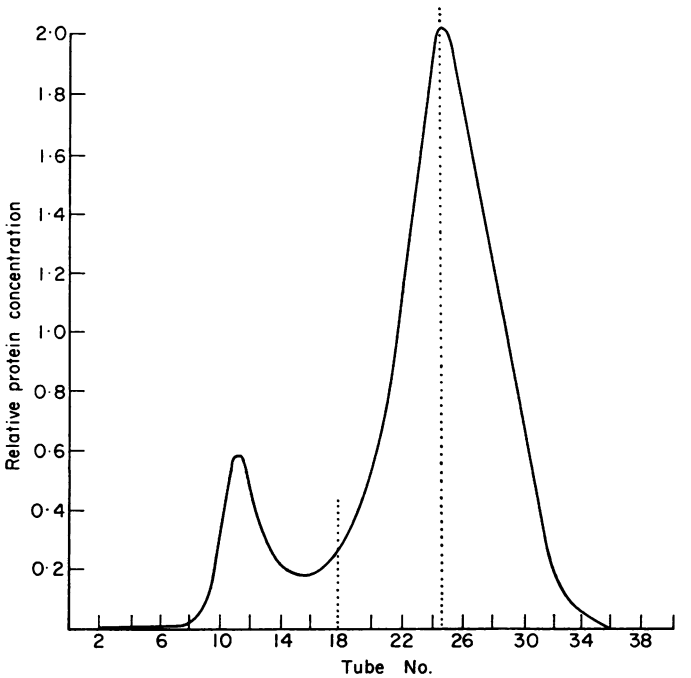

FIG. 4. Protein profiles of DEAE-cellulose Fraction 20–40 (see Fig. 3) after fractionation on a Sephadex G-200 column. For further fractionation tubes 18–25 were pooled and concentrated to give Fraction A, tubes 26–36 to give Fraction B (Fig. 5).

Fractions 20–40, 41–50 and 57–62 active, in causing eosinophil granule lysis in the test system. The major protein Fraction 20–40 was applied to a Sephadex G-200 column with the results shown in Fig. 4. Lytic activity occurred with the second protein peak only. This peak was divided into tubes 18–25 (Fraction A) and 26–36 (Fraction B). Fractions A and B, and the DEAE-cellulose Fractions 41–50 (Fraction C) and 57–62 (Fraction D) were subjected to electrophoresis on agarose blocks, with the results shown in Fig. 5. Each was found to contain a fraction which caused eosinophil granule lysis. The activity was marked in the respective sub-fractions of C and D and highest in the sub-fraction of D, which gave a strong positive reaction against eosinophils at a protein concentration of 1 μg/ml.

Precipitin patterns are shown diagramatically in Fig. 6. The agarose block fractions were run against two separate antisera, one obtained early in the infestation (5-day antiserum) and the other late (9-month antiserum). No precipitin lines were obtained with either serum against any of the fractions of A and B. Precipitin lines were obtained with both sera against the sub-fractions of C and D which were active in causing eosinophil granule lysis, a single line only with 5-day antiserum and multiple lines with 9-month antiserum.

DISCUSSION

In an earlier study (Archer, 1969) it was observed that eosinophils are attracted to antigen–antibody precipitate *in vitro*. Phagocytosis of the precipitate occurred and was associated with eosinophil granule lysis. The antigen used was a saline extract of the nematode *Amplicaecum robertsi* and the antisera were obtained from rats infested with the

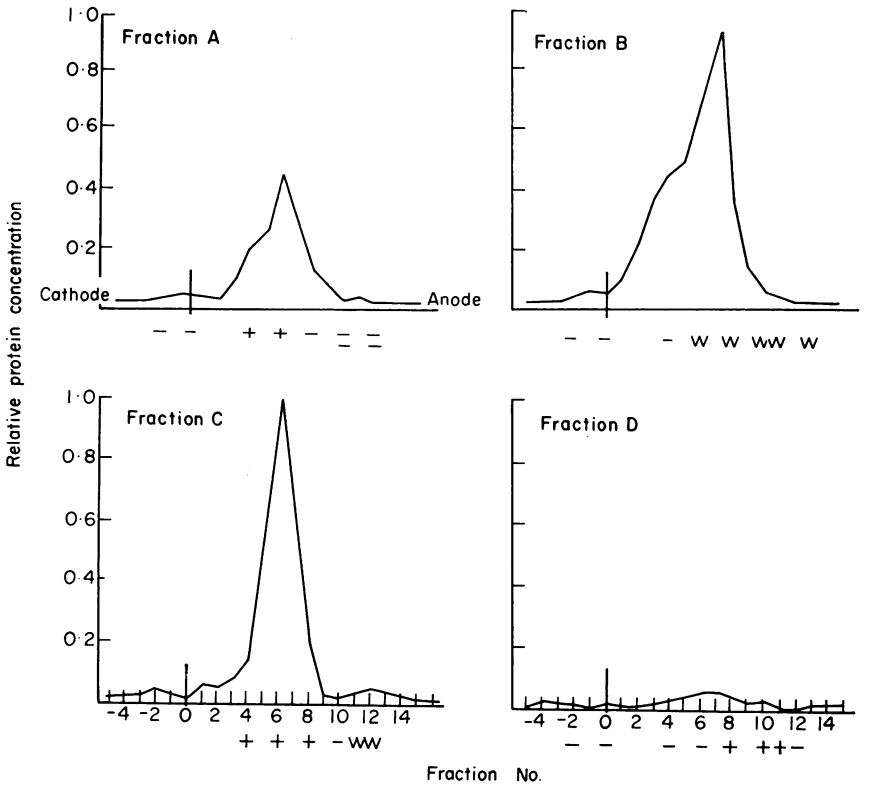

FIG. 5. Protein profiles after agarose block electrophoresis of Fractions A and B (see Fig. 4) and DEAE Fractions 41–50 (C) and 57–62 (D) (see Fig. 3). The ability of the various sub-fractions to cause eosinophil granule lysis is indicated as positive (+), weak positive (w) or negative (−).

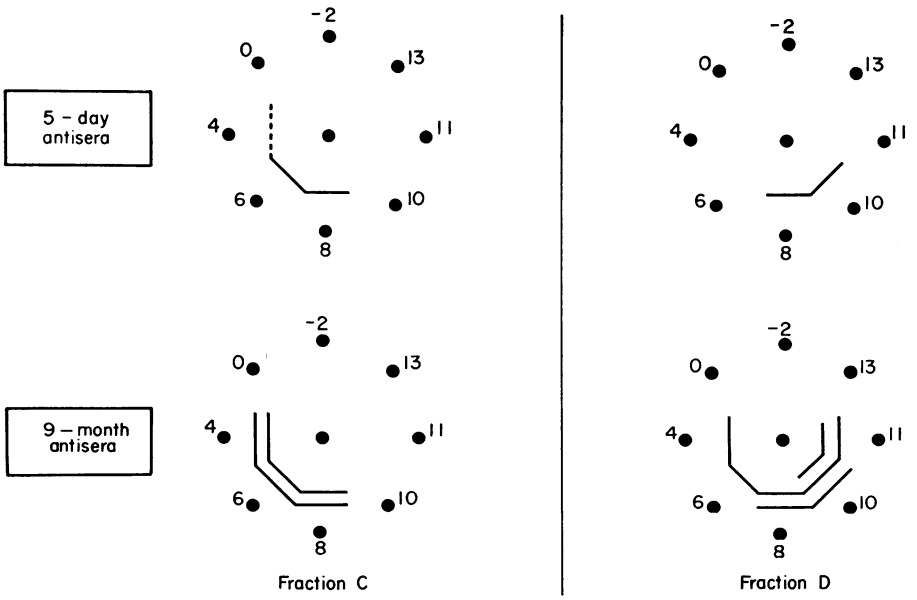

FIG. 6. Diagram of gel diffusion patterns obtained with sub-fractions of C and D (see Fig. 5). Antisera were placed in the centre well and the various electrophoresis sub-fractions, numbered according to distance (cm) of migration from the origin towards the anode, in the peripheral wells.

parasite. It does not seem likely that insoluble antigen–antibody precipitate is the cause of the massive pulmonary eosinophilia which occurs during the 3rd week of infestation, when the parasites have localized in the liver (Archer, 1968). The experiments now reported offer an alternative explanation for the pulmonary eosinophilia, namely, the passage through the circulation to the lungs of soluble antigen–antibody complexes formed at sites remote from the lungs. Eosinophilia is most marked in the loose connective tissue surrounding the pulmonary vessels and the bronchioles, and this would suggest that the soluble antigen–antibody complexes pass through the walls of the blood vessels in the lungs into the surrounding loose connective tissue.

The ability of preformed soluble antigen–antibody complexes to cause eosinophilia has been well demonstrated by Litt (1964), who has also shown that guinea-pig eosinophils are capable of ingesting fluorescent labelled antigen–antibody complexes. The results of the work reported here support Litt's finding.

Another mechanism which may be responsible for pulmonary eosinophilia has been suggested by Samter, Kofoed and Pieper (1953), who discovered a factor in sensitized guinea-pig lung which was capable of inducing peritoneal eosinophilia. They proposed that the active factor was produced following a reaction between the challenging antigen and cell-bound antibody in the lungs. In the present experiments attempts were made to attach antibody to macrophages, eosinophils and mast cells, without success. On the other hand, the addition of antigen to antisera followed by the addition of cells was uniformly successful in inducing eosinophil and macrophage vacuolation. Thus a cell-bound antibody *per se* appears to be excluded in so far as these *in vitro* experiments are concerned, but an interaction between antigen and antibody to produce a complex which binds to cells remains an attractive possibility.

Protein fractions of *Ascaris* body fluid and saline extracts of *Amplicaecum* were equally effective in the *in vitro* system. There is known to be a high degree of cross-reactivity between *Ascaris* and *Amplicaecum* antigens (Archer, 1969), and as *Ascaris suum* was much more readily available than *Amplicaecum robertsi*, *Ascaris* body fluid was used in attempts to isolate the antigens responsible for the changes in the eosinophils. Further purification is necessary, but it is apparent that at least two components of *Ascaris* body fluid are capable of causing marked eosinophil granule lysis in the presence of serum from rats infested with *Amplicaecum*. The fractions containing these two components gave precipitin lines in agarose gel against serum collected from rats five days after infestation with *Amplicaecum*, suggesting a close relationship between precipitin and eosinophil lytic antibodies. It is of interest that Kent (1963) found a close relationship between precipitin and skin sensitizing antibodies. The early appearance of precipitating antibody and the cross-reactivity between *Amplicaecum* and *Ascaris* (Archer, 1969) suggest that the antigens responsible may be commonly occurring antigens and that the animals were naturally immune prior to infestation with *Amplicaecum*. Infestation would then provide a secondary stimulus, with rapid appearance of significant amounts of antibody in the circulation.

An unexpected finding in the present work was the necessity to centrifuge the cells in the preparations prior to incubation. Eosinophil granule lysis occurred only when this step was included. No adequate explanation can be offered for this phenomenon. It was thought that the ingestion of soluble complexes might be most active when eosinophils and macrophages were in apposition. However, eosinophil-rich preparations with a few macrophages and no mast cells and normal peritoneal cavity cell suspensions alike showed vacuolation and granule lysis. The effect of centrifugation seems therefore to depend on the production

of a cell–serum interface rather than to an interaction between different cell types. Cochrane and Müller-Eberhard (1968) made the interesting observation that complement complexes are generated more readily on cell surfaces than in free solution. Complement was fixed when antigen was added to the antiserum in the present experiments. The observations of Cochrane and Müller-Eberhard (1968) may explain the necessity to centrifuge the cells in the test system. The cell layer formed during centrifugation may enhance the production of antigen–antibody–complement complexes at the cell surface, and these complexes may be responsible for eosinophil granule lysis. This necessity for centrifugation in the *in vitro* phagocytosis system is at variance with the results of direct observation using phase contrast, when no centrifugation was involved and eosinophil granule lysis occurred. A possible explanation, for which no proof is available, is that the cell surface is greatly increased in these thin cover slip slide preparations leading to increased production of antigen–antibody–complement complexes at the cell surface and in close proximity to the eosinophil granules.

The antibody responsible for the induction of the eosinophil and macrophage changes was shown to be heat labile, to appear in the serum early in infestation, to reach maximum levels several weeks after the commencement of infestation, and then to decrease in concentration. The characteristics of heat lability and appearance early in the infestation suggest that the antibody may be reaginic in type. Ishizaka and Ishizaka (1968) have shown reaginic antibodies to belong to a special class of immunoglobulins termed 'IgE'. Reaginic antibodies are known to be produced in parasite infestation (Jones and Ogilvie, 1967) and Johansson, Mellbin and Vahlquist (1968) have reported high levels of IgE antibodies in children infested with *Ascaris lumbricoides*. It is an attractive hypothesis, that reaginic (IgE) antibodies are responsible for eosinophil granule lysis. This would explain the heat-lability of the reaction. An alternative possibility would be the participation of a heat-stable antibody and complement. However, the preliminary experiments using heated serum suggest that normal serum cannot provide the heat-labile factor necessary for eosinophil granule lysis, and this may be evidence in favour of labile antibody rather than complement being involved. Further investigation is required.

Serum from infested rats treated with cortisone did not contain lytic activity against eosinophils. The absence of lytic activity is probably associated with the well-known effect of cortisone in suppressing antibody formation. It does not seem likely that the drug acts directly on the parasite, because the rate of growth and migration of the larvae to the liver was not impaired.

Vacuole formation in macrophages occurred under the same conditions as eosinophil vacuolation but more slowly. Another difference between eosinophils and macrophages was the appearance of precipitate in the vacuoles and over the surface of the macrophages in those preparations which showed the most marked cellular changes. It is not known whether this tendency for soluble antigen–antibody to precipitate out on the macrophage surface bears any relation to *in vivo* events, but it could be responsible at least in some situations for the binding of antibody to cell surfaces. A number of workers have suggested that reaginic antibodies bind to cell surfaces in the absence of antigen. On the other hand, Ishizaka and Ishizaka (1968) have shown that preformed antigen–IgE complexes are capable of inducing erythema weal skin reactions when injected into the skin of normal human volunteers.

Generalized mast cell disruption occurs in rats infested with *Amplicaecum* between the 1st and 2nd week of infestation (Archer and McGovern, 1968). Mota (1964, 1967) sug-

gested that special antibodies termed mast cell lytic antibodies may be responsible for mast cell disruption *in vivo*, and Becker and Austen (1966) coined the expression 'homocytotropic antibodies' to describe antibodies which become attached to mast cells early in an immune response. The present experiments suggest that the combination of antigen and antibody rather than antibody alone may attach to mast cells.

ACKNOWLEDGMENTS

The technical assistance of Mrs Angela Trube and Mr Nikolai Kalcenaus is gratefully acknowledged. Mr Terry Rothwell of the McMaster Laboratories, C.S.I.R.O., kindly arranged the supply of *Ascaris suum*.

This work was supported by the National Health and Medical Research Council and the Asthma Foundation of New South Wales.

REFERENCES

ARCHER, G. T. (1968). 'The function of the eosinophil.' *Biblio. haemat.*, No. 29, Part 1, p. 71 (Proc. 11th Congr. int. Soc. Blood Transf., Sydney 1966).

ARCHER, G. T. (1969). 'Mechanisms of eosinophilia and mast cell disruption in rats infested with the parasite *Amplicaecum robertsi*.' *Pathology*, **1**, 133.

ARCHER, G. T. and HIRSCH, J. G. (1963). 'Motion picture studies on degranulation of horse eosinophils during phagocytosis.' *J. exp. Med.*, **118**, 287.

ARCHER, G. T. and MCGOVERN, V. J. (1968). 'Mast cell changes in rats with eosinophilia.' *J. Path. Bact.*, **95**, 217.

BECKER, E. L. and AUSTEN, K. F. (1966). 'Mechanisms of immunological injury of rat peritoneal mast cells. I. The effect of phosphonate inhibitors on the homocytotropic antibody-mediated histamine release and the first component of rat complement.' *J. exp. Med.*, **124**, 379.

BOSWORTH, N. and ARCHER, G. T. (1961). 'The eosinophil content of the peritoneal cavity of the rat.' *Aust. J. exp. Biol. med. Sci.*, **39**, 165.

COCHRANE, C. G. and MÜLLER-EBERHARD, H. J. (1968). 'The derivation of two distinct anaphylatoxin activities from the third and fifth components of human complement.' *J. exp Med.*, **127**, 371.

ISHIZAKA, K. and ISHIZAKA, T. (1968). 'Induction of erythema-weal reactions by soluble antigen–γE antibody complexes in humans.' *J. Immunol.*, **101**, 68.

JOHANSSON, S. G., MELLBIN, T. and VAHLQUIST, B. (1968). 'Immunoglobulin levels in Ethiopian preschool children, with special reference to high concentrations of immunoglobulin E (IgND).' *Lancet*, **i**, 1118.

JONES V. E. and OGILVIE, B. M. (1967). 'Reaginic antibodies and immunity to *Nippostrongylus brasiliensis* in the rat. II. Some properties of the antibodies and antigens.' *Immunology*, **12**, 583.

KENT, N. H. (1963). 'Seminar on immunity to parasitic helminths. V. The antigens.' *Exp. Parasitol.*, **13**, 45.

LITT, M. (1964). 'Eosinophils and antigen-antibody reactions.' *Ann. N. Y. Acad. Sci.*, **116**, 964.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951). 'Protein measurement with the folin phenol reagent.' *J. biol. Chem.*, **193**, 265.

MOTA, I. (1964). 'The mechanism of anaphylaxis. I. Production and biological properties of 'mast cell sensitizing' antibody.' *Immunology*, **7**, 681.

MOTA, I. (1967). 'Biological characterisation of mouse early antibodies.' *Immunology*, **12**, 343.

OUCHTERLONY, O. (1962). 'Diffusion-in-gel methods for immunological analysis. II.' *Progr. Allergy*, **6**, 30.

SAMTER, M., KOFOED, M. A. and PIEPER W. (1953). 'A factor in lungs of anaphylactically shocked guinea pigs which can induce eosinophilia in normal animals.' *Blood*, **8**, 1078.

SPRENT, J. F. A. (1963). 'The life history and development of *Amplicaecum robertsi*, an ascariboid nematode of the carpet python (*Morelia spilotes variegatus*). I. Morphology and functional significance of larval stages.' *Parasitology*, **53**, 7.