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Reaginic Antibodies and Immunity to *Nippostrongylus brasiliensis* in the Rat

II. SOME PROPERTIES OF THE ANTIBODIES AND ANTIGENS

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Summary. 1. Passive transfer of immunity to *Nippostrongylus brasiliensis* with pooled antiserum from immune rats was neutralized *in vivo* by intravenous injection of small amounts of a saline extract of adult worms. This inhibition of protection was associated with systemic anaphylaxis and appeared to result from the neutralization of protective antibodies.

2. Serum from infected rats was fractionated by G-200 Sephadex gel-filtration. Reaginic antibodies were shown to be intermediate in molecular size between 7S and 19S globulins in sera from both singly and multiply infected animals. In immunoelectrophoresis they migrated with fast immunoglobulins but could not be related to either IgG or IgA rat immunoglobulins. The same serum fractions gave both homologous passive cutaneous anaphylaxis (PCA) and systemic anaphylaxis.

3. Blocking antibodies were found both in the 7S and 19S fractions after separation on G-200 Sephadex. These antibodies were found in sera from rats immunized with worm extracts as well as in sera from singly and multiply infected animals.

4. The saline extract of adult worms was fractionated on G-200 Sephadex. The isolated antigenic material (allergen) for both homologous PCA and systemic anaphylaxis seemed to be a protein with a molecular weight of approximately 12,000–17,000.

INTRODUCTION

Nippostrongylus brasiliensis in the rat is a convenient model system for study of the nature of immunity to helminth parasites. It has been known for many years that immunity can be transferred passively with serum from immune animals (Sarles and Taliaferro, 1936). More recently, it has been shown that an immediate hypersensitivity state develops in rats as they acquire immunity to this parasite (Mulligan, Urquhart, Jennings and Neilsen, 1965) and that this hypersensitive state is associated with the presence of antibodies resembling human reagins (Ogilvie, 1964, 1967). Passive cutaneous anaphylaxis in rats (PCA) is used to measure these rat reagins. It is known that a non-specific anaphylactic reaction in the rat gut, induced by interaction of an antigen and antibody which are unrelated to the infection, has no effect on the elimination of worms from the gut (Barth, Jarrett and Urquhart, 1966; Ogilvie, 1967) but that it does enhance immunity conferred passively with antiserum (Barth *et al.*, 1966). In this paper we have investigated the effect of an anaphylactic reaction induced with an extract of worms on the course of a living infection of *N. brasiliensis* in passively immune rats.

Because of the possible relationship between reagins and protective antibodies, we have attempted to separate rat reagins from other immunoglobulins and to define some of their properties. Antibodies which blocked the homologous PCA test for reagins have also been studied. In addition, we have partially purified and studied the worm extract used as antigen in systemic anaphylaxis and in PCA.

MATERIALS AND METHODS

The Sprague Dawley albino strain of rat was used throughout this work. Details of the test for reagins by homologous PCA and also the methods for maintenance and recovery of *Nippostrongylus brasiliensis* and for counting eggs in rat faeces and adult worms in rat small intestines are in earlier reports (Ogilvie, 1965, 1967). For estimation of antigen (allergen) (as worm equivalents/ml) see Ogilvie (1967).

Antisera

Rabbit antisera: All antisera were prepared in rabbits (N.I.M.R. Sandylops strain) by immunization with antigen emulsified in an equal volume of Freund's complete adjuvant (Difco), fortified with 5 mg/ml of tubercle bacilli, and injected into the footpads and scruff of the neck. The rabbits were bled 4 weeks later. Rabbit antiserum against rat serum was prepared with rat anti-*N. brasiliensis* serum which contained a high level of reagins (reciprocal PCA titre of 640). Rabbit antiserum against rat immunoglobulins was prepared by injecting a carefully washed specific precipitate of diphtheria toxoid and rat antitoxin to diphtheria toxoid suspended in saline (Arnason, de Vaux St Cyr and Relyveld, 1964). This latter antiserum gave three lines when tested in immunoelectrophoresis against pooled whole rat serum. These lines were identical to those formed by an antiserum kindly given to us by Dr Barry Arnason and, following the classification of Arnason *et al.* (1964), they will be referred to as IgG, IgA and IgM.

Rat antisera: Antisera for passive immunization were prepared by giving rats three or four subcutaneous infections of *N. brasiliensis*; each infective dose contained approximately 3000–5000 larvae given over a period of 2 months. Rats were bled 5–7 days after the final infection. These sera also contained reagins and are referred to as 'highly immune'.

The preparation of antiserum in rats by immunization with a saline extract of adult worms was described by Ogilvie (1967).

Assay for blocking antibody

The method for measurement of antibody which blocked or neutralized the PCA reaction was described by Ogilvie (1967). Results are given as residual PCA titres. This crude quantitative method was used rather than neutralization of the Prausnitz–Kustner (P-K) test in rats which is unsatisfactory because the Evans blue leaks into the area around the injection site even when saline is given intradermally.

Fractionations

G-200 Sephadex: A column (150 × 3·4 cm) was packed with G-200 Sephadex (Pharmacia) and maintained at 4°. Volumes of 10 or 20 ml of material were applied to the column and eluted with 0·1 M NaCl containing 10 per cent borate buffer, pH 8·0 (Fireman, Vannier and Goodman, 1963). Flow rates were 30–40 ml/hr.

DEAE-cellulose: DEAE-cellulose (Whatman Chromedia DE 11), equilibrated with 0·0175 M phosphate buffer at pH 6·3 for 4 days, was packed into a column 40 × 2·2 cm,

and kept at 4°. Twenty millilitres of rat antiserum from highly immune donors were applied and eluted stepwise with three buffers, as shown in Fig. 5. The flow rate was 15 ml /hr.

CM-cellulose (Whatman Chromedia DE 11) was equilibrated with 0·005 M phosphate buffer at pH 6·4 for 4 days, packed into a column 20 × 1 cm and kept at 4°. Fractions 1, 2 and 3 eluted from DEAE-cellulose were re-chromatographed on CM-cellulose and eluted stepwise with either the three buffers given in Fig. 5, or the first two only.

Eluates from all fractionations were collected as 3- or 6-ml fractions and optical densities were measured at 280 mμ in a spectrophotometer (Optika). Eluates were pooled into larger fractions, as shown in the text, and concentrated by negative pressure dialysis or by osmosis with Carbowax (B.D.H.) at 4°. Protein concentrations were measured by the Biuret method (Campbell, Garvey, Cremer and Sussdorf, 1963).

Thin layer G-200 Sephadex: This method for the estimation of molecular weights of proteins was described by Morris (1964). The solvent was 0·5 M NaCl and after chromatography the plates were dried at 55–60° for 30 minutes before staining with 1 per cent amido black in methanol–water–glacial acetic acid in the proportions 50:40:10. The distance from the origin to the position of maximum absorption for each protein was measured with the use of a double-beam recording microdensitometer. Rat haemoglobin was used as a standard marker and molecular weights were calculated on the assumption that rat haemoglobin behaved as if it had a molecular weight of 33,000 (Morris, 1964).

Agar electrophoresis

A solution of 1 per cent agar (Ionagar No. 2 Oxoid) in 0·05 M barbitone buffer, pH 8·4, was made into a block measuring 100 × 145 × 2 mm. Nine wells were cut in the midline of the longer axis and filled with a total volume of 0·5 ml of antiserum. Troughs were cut at each end for immunoelectrophoresis. After electrophoresis under petroleum ether at 27 mA and 60 V for 5 hours the agar was cut into 1 cm wide strips. Protein was extracted from the agar strips with saline by alternate freezing and thawing, followed by centrifugation. Extracts were concentrated to 0·5 ml by negative pressure dialysis.

The agar and buffer for immunoelectrophoresis (Grabar, 1959) were the same as above. Agar plates were 80 × 50 × 2 mm and serum fractions were separated by electrophoresis in agar for 80 minutes at 40 mA and 110 V under petroleum ether.

RESULTS

NEUTRALIZATION OF PROTECTIVE ANTIBODIES BY WORM EXTRACT

Three groups, each of seven rats, were infected with approximately 1000 larvae/rat. The rats in two groups were each given 4 ml of highly immune serum intraperitoneally on the day before infection and a further 3 ml of serum intraperitoneally 5 days after infection. The antiserum was prepared as described in 'Methods' and had a reciprocal PCA titre of 640. On the 6th day after infection and again on the 7th, all the rats in one group treated with serum were given an intravenous injection of worm extract which had been kept for 1 month at −20°. Each dose of this extract contained material extracted from 500 adult worms (500 worm equivalents). Egg counts were made on a faeces sample from each of the three groups from days 6 to 9 after infection, and on the 9th day all the rats were killed and the number of worms present in the gut was counted (Fig. 1). Control rats given normal serum or worm extract alone were included in this experiment but the

results are not given because the course of infections in these groups was the same as in controls.

After the injection of worm extract into rats previously treated with antiserum, the rats showed signs of moderate or severe systemic anaphylaxis on day 6 but slight or no signs of anaphylaxis after the second injection on day 7. The control group of rats which were given worm extract only were not shocked. Throughout the experiment, egg production by rats given serum alone was markedly less than egg production by controls. Before worm extract was injected on day 6, egg counts were similar for both groups of rats given serum (350 and 0 eggs/g) whereas controls passed 3000 eggs/g.

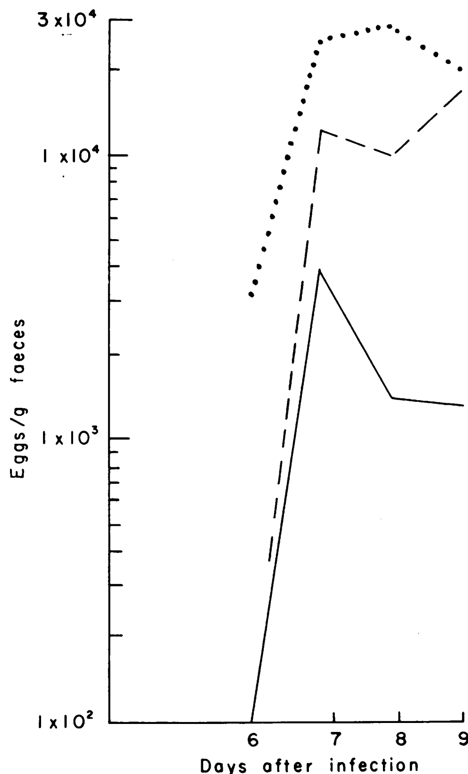

Fig. 1. Neutralization of passive immunity by injection of worm extract; shown by group egg counts from days 6 to 9 and mean worm populations recovered on day 9, after infection with 1000 larvae. · · · ·, Controls (worm counts, 559 ± 43); - - -, serum + antigen (488 ± 61·5); ——, serum (197 ± 83).

However, on day 7 and until the experiment ended on day 9, the number of eggs found in the faeces of rats given both serum and worm extract was much greater than the number passed by rats given serum alone. When the worms in the intestine were counted on day 9, more worms were found in rats given serum and worm extracts (488±61·5) than in rats given serum alone (197+83) (P = 0·001–0·01) but there were slightly more worms in controls (559±43) than in rats given serum and worm extract (P = 0·02–0·05). This experiment has been repeated with similar results.

It is concluded that adult worm extract contains an antigen which, when injected intravenously into rats passively protected with antiserum, will neutralize some of the antibodies concerned in protective immunity.

FRACTIONATION AND PROPERTIES OF RAT REAGINS AND OTHER ANTIBODIES TO *N. brasiliensis*

G-200 Sephadex gel-filtration

Ten millilitres of highly immune serum with a reciprocal PCA titre of 1280 were fractionated on G-200 Sephadex (Fig. 2b). The eluate was collected as 6-ml fractions, pooled to 30-ml volumes and each pool was concentrated to 5 ml. Reagins, measured by the PCA test, were found in the region between the first and third peaks with maximum titres in the fractions from the second peak and the ascending side of the third peak. The recovery of reaginic antibody from this procedure was approximately 90 per cent. Thus rat reagins seem intermediary in size between 19S and 7S globulins.

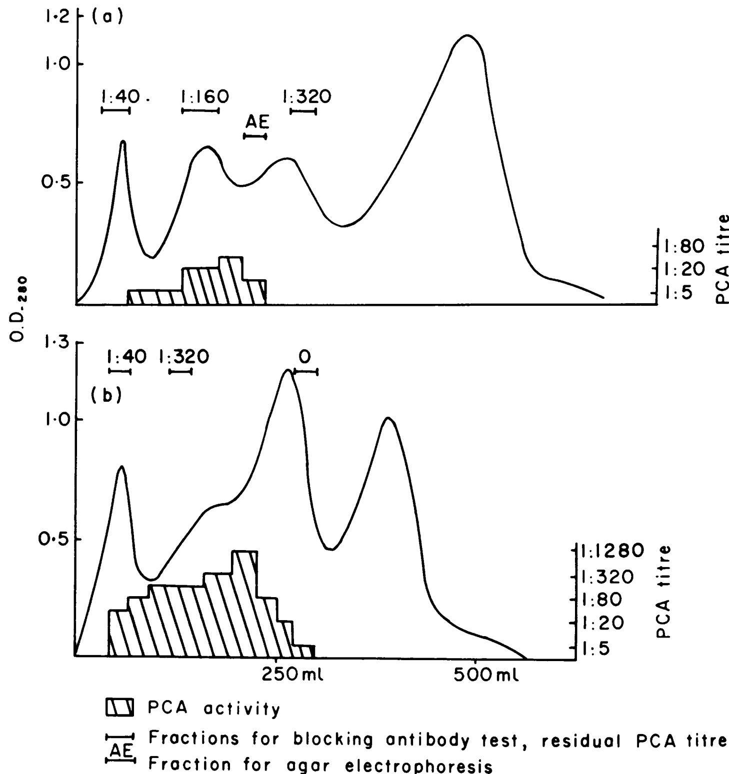

FIG. 2. Gel-filtration of antiserum on G-200 Sephadex to separate antibodies responsible for PCA and blocking activity. Serum taken: (a) after a single infection, and (b) after a series of infections.

A pool of antiserum taken from rats 4 weeks after a single infection with 3000 larvae was also fractionated on G-200 Sephadex (Fig. 2a). Reagins, although present in the antiserum in small amounts (reciprocal PCA titre of 40), were eluted from the same region as the reagins in sera from highly immune rats.

Systemic anaphylaxis was also induced with fractions from G-200 Sephadex (Fig. 3). Highly immune rat serum, reciprocal PCA titre of 320, was fractionated and 1·0 ml of each pooled and concentrated fraction was injected into four rats intraperitoneally. Twenty-four hours later a saline extract of 500 adult worms was given intravenously. Anaphylactic symptoms such as respiratory difficulties and lethargy, associated with haemorrhage of the small intestine, became apparent after 10–15 minutes in some rats.

The number of rats which showed symptoms of anaphylaxis compared with the number of rats injected is given in the relevant zones of Fig. 3. Anaphylaxis was evident only in rats given fractions from the region of the second and the ascending side of the third peak,

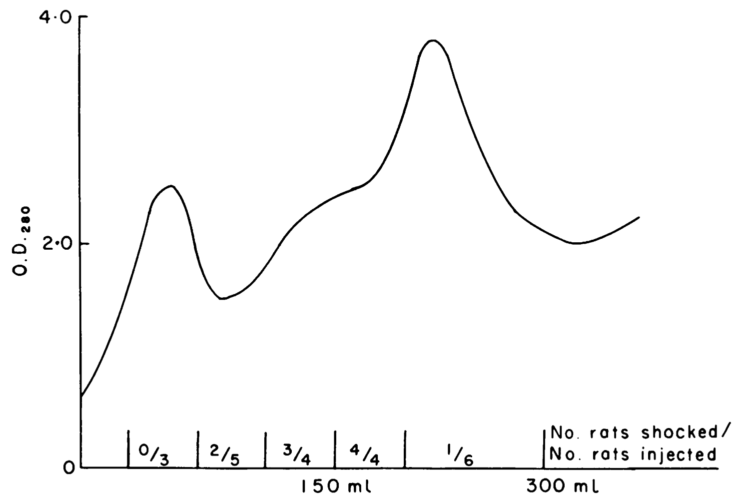

FIG. 3. Activity of antiserum fractions for systemic anaphylaxis obtained by gel-filtration on G-200 Sephadex.

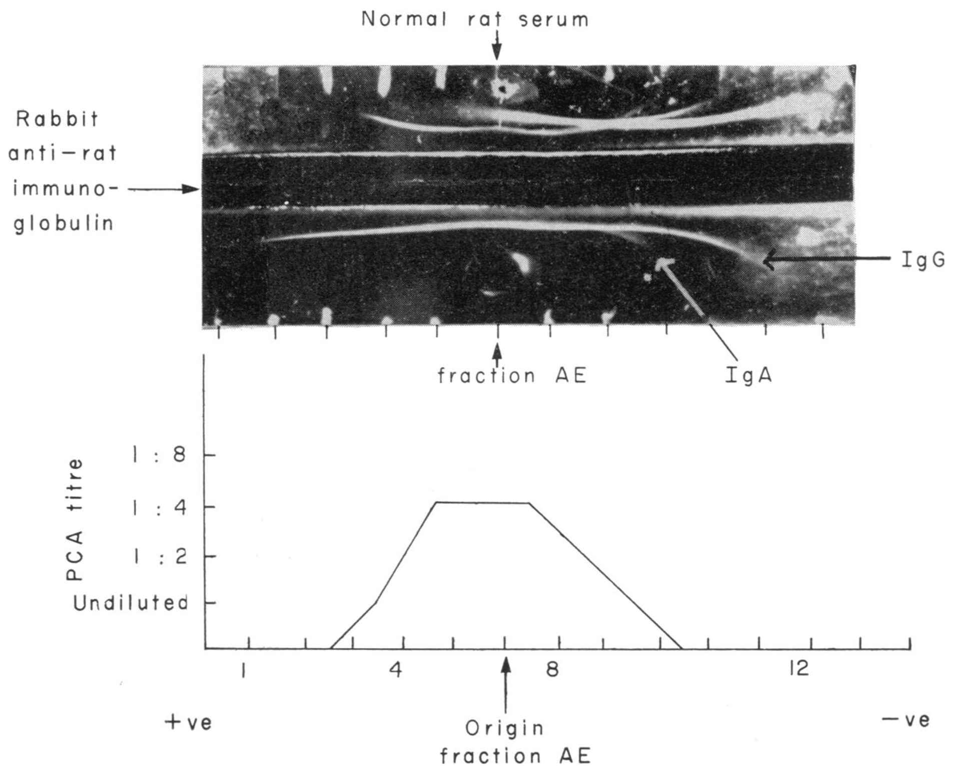

FIG. 4. Electrophoretic mobility in agar of PCA antibodies in fraction AE from G-200 Sephadex (see Fig. 2a); immunoglobulins in fraction AE shown by immunoelectrophoresis.

where reagins were also found. It was impossible to measure the degree of shock in each individual rat as the susceptibility of rats to anaphylaxis is too variable, but maximum passive systemic anaphylaxis was induced with the eluate from the region which also gave maximum amounts of reaginic antibody.

Blocking antibodies, which neutralized the antigen for PCA, were found in eluate of the first and third peaks corresponding to 19S and 7S globulins. The residual PCA titres, which indicate the relative amount of blocking antibody present, are given in Fig. 2 above

Fig. 5. Chromatography of antiserum on DEAE-cellulose. Fractions from hatched areas were not active in PCA test and were discarded. Re-chromatography of fractions 1, 2 and 3 from DEAE- on CM-cellulose.

the region where the eluate was measured for blocking antibody. After a single infection, blocking antibody was found chiefly in the 19S region (Fig. 2a) whereas in the antiserum from rats which had had several infections blocking antibody was predominantly in the 7S region (Fig. 2b).

Similar levels of blocking antibody of the 7S type were also found in sera from rats immunized with a saline extract of 3600 worms in Freund's complete adjuvant.

Electrophoresis in agar

The electrophoretic mobility of reaginic antibody in agar was compared with the mobilities of rat immunoglobulins. A sample of eluate (AE) taken from the region with high reaginic activity (Fig. 2a) was separated by electrophoresis in agar and the PCA titres of eluates from slices of agar were measured (Fig. 4). Immunoelectrophoresis of the sample together with a sample of normal rat serum is also shown in Fig. 4. Maximum reaginic activity was found toward the centre of the strong and very extended IgG band

It coincided also with the second band in immunoelectrophoresis, which according to Arnason *et al.* (1964), might be the IgA band. There was no trace of IgM in fraction AE.

Ion-exchange chromatography

Serum from highly immune rats was fractionated on DEAE-cellulose and fractions of the eluate were subsequently re-chromatographed on CM-cellulose (Fig. 5). The method is essentially the same as that described by Raynaud, Iscaki and Mangalo (1965) for the separation of IgG and IgA horse antitoxin. Eluates in the zone of each peak were pooled and concentrated before reaginic activity was measured (Table 1) and immunoelectrophoresis was carried out. The immunoelectrophoretic behaviour of each sample of eluate

TABLE 1

DISTRIBUTION OF REAGINS IN EACH FRACTION AFTER CHROMATOGRAPHY OF RAT ANTISERUM TO *N. brasiliensis* ON DEAE-CELLULOSE AND THEN FRACTIONS 1, 2 AND 3 ON CM-CELLULOSE

		Volume (ml)	Protein concentration (mg/ml)	PCA titre
Original antiserum		20		1:640
DEAE-cellulose	Fraction 1	5	71	1:320
	Fraction 2	5	6	1:160
	Fraction 3	5	48·6	1:320
CM-cellulose	Fraction 1a	5	24·5	1:160
	Fraction 1b	4		1:5
	Fraction 1c	5		0
	Fraction 2a	5	2·3	1:40
	Fraction 2b	8		Undiluted
	Fraction 3a	2	24·05	1:40
	Fraction 3b	4		0

was examined with rabbit antiserum to rat immunoglobulins and rabbit antiserum to rat serum (Fig. 6). Fraction 1 eluted from DEAE-cellulose with the first buffer, 0·0175 M PO_4HK_2/KH_2 at pH 6·3, contained all the electrophoretically slow, together with some fast, immunoglobulins as well as a high proportion of the total recovered reagins. The remaining fast moving immunoglobulins and the remaining reagins were eluted with 0·05 M PO_4HK_2/KH_2 at pH 4·3 (fractions 2 and 3 in Fig. 5). There were no detectable reagins in any other portions of the eluate. The presence of a proportion of reagins in fraction 3, with fast immunoglobulin suggests that at least some of the rat reaginic antibody might be associated with the IgA type.

Further chromatography of fractions 1, 2 and 3 on CM-cellulose was done with PO_4 HK_2/KH_2 buffer 0·005 M, pH 6·4; 0·02 M, pH 6·4 and 0·06 M, pH 6·3, by stepwise elution. The bulk of the protein and of the reaginic activity was eluted in the first peak of each run (Fig. 5 and Table 1). After chromatography of fractions 2 and 3 somewhat faster immunoglobulin was found in the first peaks together with the reagins. However, the second peaks also contained fast moving immunoglobulin and little or no reaginic activity.

Chromatography of antiserum on DEAE-cellulose succeeded in separating a fast immunoglobulin fraction together with high reaginic activity (fraction 3, Table 1 and Fig. 6). Further chromatography of either 1, 2 or 3 fractions on CM-cellulose however did not markedly improve this separation.

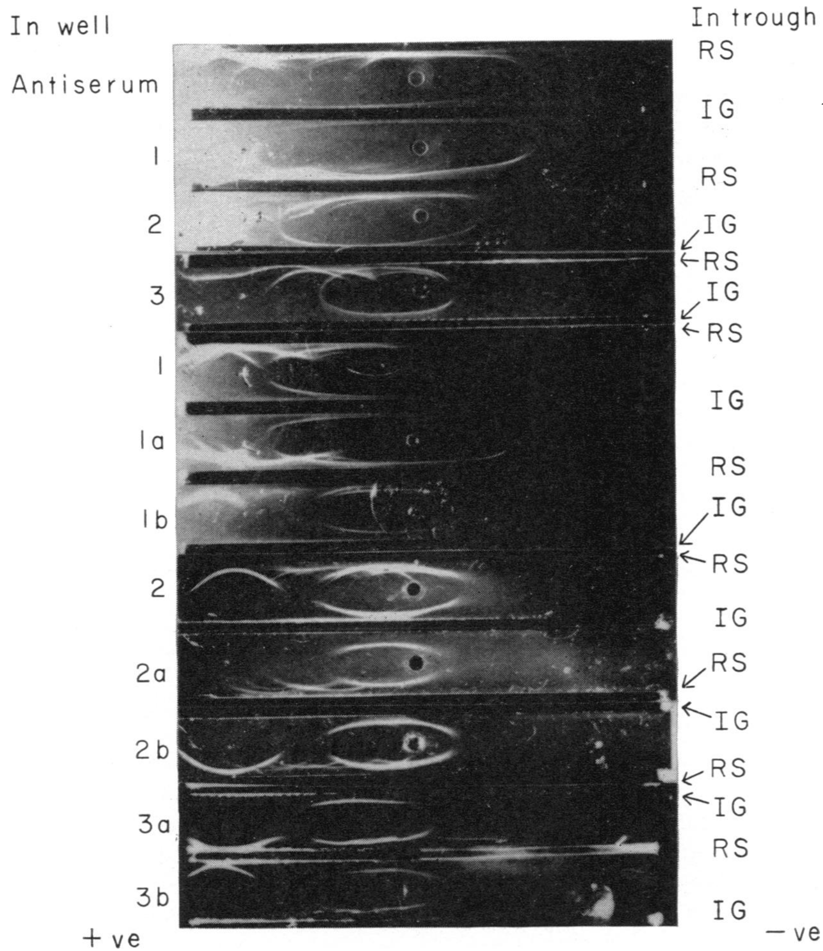

FIG. 6. Immunoelectrophoresis of fractions from DEAE-cellulose (fractions 1, 2 and 3) and CM-cellulose (fractions 1a, 1b, 2a, 2b, 3a and 3b). See Fig. 5. RS = Rabbit anti-rat serum; IG = Rabbit anti-rat immunoglobulin.

FRACTIONATION AND PROPERTIES OF *N. brasiliensis* ANTIGEN SPECIFIC FOR RAT REAGINS

Adult worms were collected, washed and the numbers estimated by volume (Ogilvie, 1967). Twenty thousand worms or more were crushed in a tissue grinder with a minimum volume, usually 5 ml, of saline. After centrifugation at 2000 rev/min for 10 minutes, the volume of supernatant was adjusted to contain the extract from 2000 to 4000 worms/ml, and therefore concentrations of extracts are expressed as worm equivalents/ml. Protein estimations on several extracts gave a range of values between 5·5 and 7·5 mg protein for 1000 worm equivalents.

This saline extract, as it was the antigen used to elicit PCA reactions, was fractionated on a column of G-200 Sephadex. The equivalent of 110×10^3 worms in 20 ml was eluted and the optical density of fractions was measured at 280 mμ for protein and at 540 mμ for *Nippostrongylus* haemoglobin (Fig. 7). In subsequent fractionations of worm extracts on G-200 Sephadex the large amount of heavy material, shown by the first peak, was removed by ultracentrifugation for 45 minutes at 50,000 rev/min before the extract was placed on the column; the material in the second and third peaks remained in the supernatant. Haemoglobin was eluted in the second protein peak (Fig. 7).

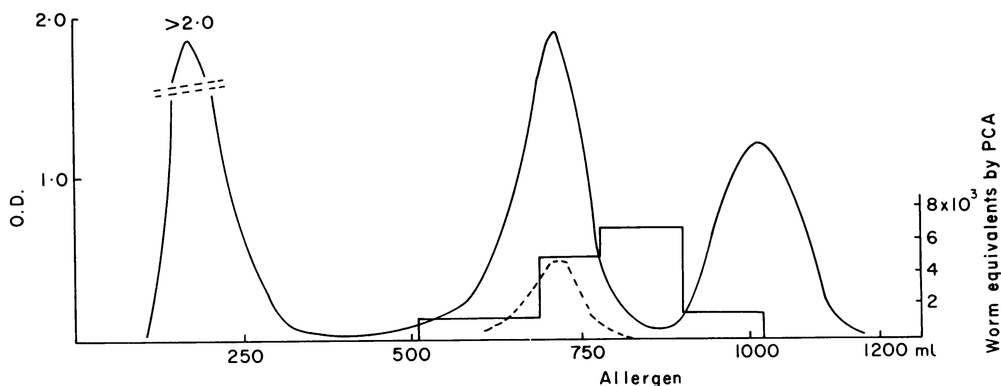

FIG. 7. Gel-filtration of worm extract on G-200 Sephadex; optical density measurements at 280 (——) and 540 mμ (- - -) (for *Nippostrongylus* haemoglobin). Histogram shows region of antigenic activity in PCA test measured as worm equivalents. Fraction with maximum PCA activity used as allergen.

After concentration of pooled eluates their PCA activity was measured and is shown in Fig. 7. The activity was estimated in relation to that of a saline extract of worms before fractionation (Ogilvie, 1967). Although there was some antigenic activity in the second peak, the major portion of PCA active antigen was eluted just after the haemoglobin in eluate containing a small amount of protein. The third peak, which contained the material of lowest molecular weight, had only a trace of activity.

The low protein eluate with maximum PCA activity is subsequently called the allergen (Fig. 7). It was contaminated with a trace of haemoglobin and after concentration to 2 mg protein/ml gave five bands which stained for protein after electrophoresis in starch gel. It was therefore difficult at this stage to relate activity of the allergen in PCA to protein concentration, so concentration continues to be expressed as worm equivalents/ml.

The allergen could also induce systemic anaphylaxis in rats. Each rat was sensitized by intraperitoneal injection of 5 ml of highly immune serum. Twenty-four hours later the rats were injected intravenously with allergen containing 50 or 250 worm equivalents. Fifty worm equivalents (estimated by PCA) induced only mild shock in both of two rats but 250 worm equivalents induced severe anaphylaxis in all of three rats. The other fractions of eluate which were inactive in the PCA test were also unable to induce systemic anaphylaxis although large volumes were used. This similarity between the antigenic specificities of antibodies which induce PCA and of those which induce systemic anaphylaxis underlines the relationship of these antibodies as demonstrated in the previous section.

Blocking antibody, as expected, also had a similar antigenic specificity. Antiserum from rats immunized with worm extract and containing blocking antibody was mixed with

allergen. A volume of 0·5 ml of antiserum completely inhibited the PCA activity of 100 worm equivalents of allergen. The crude saline extracts of worms contain a number of antigens that form precipitates with rat antisera in agar gel (unpublished). These antigens were also present in various Sephadex fractions but could not be related to the allergen.

Properties of the allergen

Preliminary study of the chemical nature of the allergen included the following tests and 300 worm equivalents of allergen were used for each test. Residual activity was measured by the PCA test; each of two skin-sensitized rats was given 150 worm equivalents intravenously.

All PCA activity was lost after: (1) Heating for 15 minutes at 100°; (2) 15 minutes exposure to 1 per cent periodic acid in the dark at room temperature, followed by neutralization with 0·1 N NaOH and then dialysis; (3) exposure to 8 M urea at 37° for 18 hours; and (4) 2 hours exposure to N trichloracetic acid (TCA) at 0°, followed by

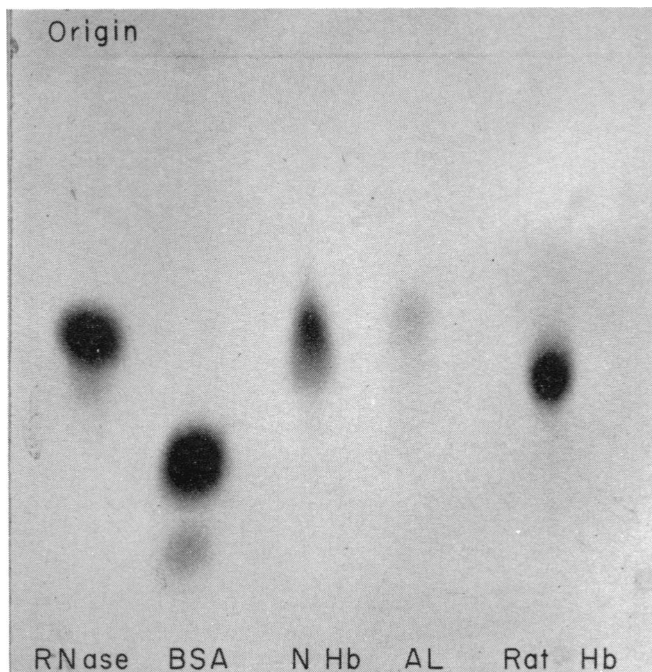

FIG. 8. Estimation of molecular weight of allergen (AL), by chromatography on thin-layer G-200 Sephadex, compared wtih ribonuclease (RNase), bovine serum albumin (BSA), *Nippostrongylus* haemoglobin (N Hb) and rat haemoglobin (Rat Hb).

centrifugation at 1500 rev/min for 20 minutes. The supernatant was dialysed at 4° for 18 hours before testing for PCA activity.

There was no loss of antigenicity for the PCA test after storage of the saline extract of worms at 18° for several hours or at 4° for 10 weeks (Ogilvie, 1967). The loss of antigenic activity by heat, periodic acid, TCA and even by 8 M urea suggests that the major portion of the allergen is protein.

The molecular size of the allergen was estimated by thin-layer chromatography with

G-200 Sephadex but was first concentrated to 3·7 mg protein/ml. Four proteins of known molecular weight were used for reference; solutions of 20 mg/ml of ribonuclease (RNase), bovine serum albumin (BSA) and pepsin, and rat haemoglobin (Rat Hb). Rat haemoglobin was the standard protein; the molecular weights of the other proteins were calculated from the ratio of the distance migrated by rat haemoglobin to the distance migrated by the unknown protein. Two sets of chromatograms were made, six in the first set and nine in the second set, and Fig. 8 shows one of these. The distance from the origin to the position of the maximum of each protein band was measured in all chromatograms and the molecular weight of each protein was calculated. The calculated molecular weights and the standard deviations from these estimates are given in Table 2. By this method the molecular weight of allergen is similar to that of RNase, within the range of 12,000–17,000.

TABLE 2

ESTIMATION OF MOLECULAR WEIGHT OF ALLERGEN BY CHROMATOGRAPHY ON THIN-LAYER G-200 SEPHADEX

Mean	Mean values ($\times 10^3$) for molecular weights with standard deviations			
	Allergen	RNase	BSA	Pepsin
Six estimates	12·8±0·9	16·9±3	Not done	36·4±3
Nine estimates	16·7±4	15·1±3	53·9±17	Not done

DISCUSSION

In this paper we have shown that the protective effect of antiserum to *N. brasiliensis* was neutralized *in vivo* by intravenous injection of an extract of worms. Following the injection, egg production by the worms rose immediately to high levels and remained high for 3 days. The worm population recovered on the 3rd day from rats given antiserum and worm extract, was found to be significantly greater than that recovered from rats given antiserum alone. The injection of antigenic worm extract induced severe systemic anaphylaxis as well as inhibition of protection.

It has already been shown that the pharmacological mediators and resulting tissue damage of systemic anaphylaxis induced by an unrelated antigen–antibody system do not affect the worms at any stage in their parasitic life cycle (Barth *et al.*, 1966; Ogilvie, 1967). But Barth *et al.* have shown that anaphylactic shock induced by an unrelated antigen–antibody system will enhance passive immunity conferred with antiserum, producing a significant expulsion of worms compared to rats which were only passively immunized.

These authors have explained their results on the assumption that protective antibodies do not cross the intact gut mucosa; they suggest that the integrity of the gut mucosa is destroyed either by the mechanical action of the worms themselves or by an anaphylactic reaction, and protective antibodies are thus able to reach the parasites. If anaphylactic antibodies have this simple role in worm elimination, then in our experiments specific anaphylaxis should have induced an immediate enhancement of protection. The reverse of this occurred. In fact, inhibition of protection persisted to the end of the experiment, although according to Barth *et al.* it might have been expected that leakage of protective antibodies would finally occur because of mechanical damage by the persisting worm population. Our results therefore can only be explained according to this hypothesis if we

assume that protective antibodies, as well as anaphylactic antibodies, were neutralized when specific worm extract was injected intravenously into passively immunized rats. If this is the case, the antigenic determinants for protective antibodies must be present in worm extract, although the worm extract cannot stimulate active immunity (Ogilvie, 1967).

There are, however, alternative explanations for the results obtained with systemic anaphylaxis in passive immunity and definite conclusions cannot yet be made on the role of anaphylactic antibodies in protective immunity.

There is another puzzling aspect of our experiment on inhibition of passive immunity. Why should a single injection of a small amount of worm extract reverse passive immunity when the living adult worms, already present in the passively immune rats, do not? This suggests that the antigen released by the living worms is not sufficient to neutralize the protective effect of the antiserum.

Separation of antiserum on G-200 Sephadex showed that rat reagins have a molecular size between 19S and 7S globulins and that this does not alter whether reagins are formed after an initial infection or are formed in highly immune rats after several infections. The size of rat reagins is similar to that of human reagins to pollen allergens (Goodfriend, Perelmutter and Rose, 1965; Ishizaka, Ishizaka and Lee, 1966).

Systemic anaphylaxis was induced with antibodies of the same size range as those which induce homologous PCA and both reactions had similar antigenic specificities. This strongly suggests that both reactions are initiated by the same antibody.

Blocking antibody on the other hand was found in 19S and 7S globulin fractions from G-200 Sephadex. After a single infection blocking antibody was mainly 19S, whereas after several infections it was predominantly 7S. As expected, blocking antibody was specific for the partially purified allergen.

Rat reagins were no more effectively separated from IgA and IgG by ion-exchange chromatography than by G-200 Sephadex. One fraction contained fast immunoglobulin and reagins but no slow moving immunoglobulin (fraction 2a, Table 1 and Fig. 6); but another fast moving immunoglobulin fraction (2b) contained no reagins. Therefore reagins could not be identified with any particular fast moving immunoglobulin in our experiments although they had similar electrophoretic mobilities. (It was not possible to distinguish IgA from IgG in the fast immunoglobulin component revealed by immuno-electrophoresis as our antiserum was multivalent, detecting IgM, IgA and IgG.)

The electrophoretic mobility of reagins to *N. brasiliensis* is similar to that of the rat anaphylactic antibody described by Binaghi, Benacerraf, Bloch and Kourilsky (1964). But two kinds of rat anaphylactic antibodies have now been described by Stechschulte, Bloch and Austen (1966); and Nussenzweig and Binaghi (1965) conclude that the rat can form at least five types of antigenically distinct immunoglobulins to DNP hapten, one or more of which may have anaphylactic properties. Now that analysis of human reaginic antibody such as that found in ragweed sensitive patients has shown that a distinct immunoglobulin, E, may represent human reagin (Ishizaka, Ishizaka and Hornbrook, 1966), it is possible that rat reagins also represent a separate class of rat immunoglobulin.

The antigen responsible for formation of reagins in *N. brasiliensis* infections was present in extracts of adult worms but was not investigated for immunogenicity as it is difficult to stimulate reagins with non-living worm preparations (Ogilvie, 1967). Antigenicity was studied from the ability to combine with reagins in the PCA test and to induce systemic anaphylaxis. Antigenic activity for these two reactions was also present in material released by the living worms when they were incubated *in vitro* (unpublished results).

Partially purified antigen (allergen), was readily fractionated from saline extracts of worms by G-200 Sephadex. By G-200 Sephadex thin-layer chromatography the molecular weight of allergen was calculated to be between 12,000 and 17,000. Various tests indicated that the antigenically active portions of the allergens are protein. In its characteristics of molecular weight and protein nature, this allergen resembles allergens from other sources, for example, rye grass pollen (Marsh, Milner and Johnson, 1966). Moreover, allergens which have been extracted recently from other helminths have a molecular weight of 10,000–20,000 (Jones and Smithers, unpublished; Hogarth Scott, unpublished). A highly concentrated preparation of allergen separated into five protein components after electrophoresis in starch gel (Beaven and Jones, unpublished). Therefore more than one antigen for reagins formed to living infections could be present in the allergen.

The neutralization of passive immunity with whole worm extract reported in this paper provides a system for the investigation of the antibodies and antigens concerned in protective immunity to *N. brasiliensis*. The close association of reagins with active immunity in infected rats and the involvement of reagins in the neutralization of passive immunity, indicates a protective function for these antibodies. However, there is evidence which suggests that they are not the only class of antibody involved in protection (Ogilvie, 1967; Ogilvie and Jones, 1967). At this stage, it is impossible to define concisely the role of reagins or to define the nature of any other protective antibodies in immunity to this parasite.

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