THE BIOSYNTHESIS AND MEASUREMENT OF IMMUNOGLOBULIN E IN THE RAT

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SUMMARY

In Section 1, various methods employed for the quantification of IgE are discussed, and detailed accounts are given of the techniques used in this laboratory.

Total IgE levels in human sera have been estimated using the Phadebas® radioimmunosorbent test (RIST). This is a competitive radioimmunoassay in which IgE in a sample competes with radiolabelled IgE for sites on sephadex-anti-IgE particles. The usefulness and limitations of the test are discussed. A rat radioimmunosorbent test (RIST), and the Rowe modification of the Mancini single radial diffusion test are both described in detail. Both tests measure total IgE levels in rat sera. The rat RIST is a direct 'sandwich' radioimmunoassay in which IgE in a sample binds to anti rat IgE bound to activated paper discs. Finally addition of radiolabelled anti-rat IgE acts as a marker for the amount of sample IgE bound. RIST is more sensitive than the Rowe-Mancini test, detecting IgE levels as low as 10ng/ml. Technical aspects of the tests are discussed in detail.

Antigen specific rat IgE is measured by the radio allergo-sorbent (RAST) and passive cutaneous anaphylaxis (PCA) tests. The RAST is based on the same principle as the rat RIST, but antigen instead of anti-IgE is bound to activated paper discs. The RAST has been used in this laboratory to detect IgE directed against egg albumin and N. brasiliensis antigens, and of the two systems the egg albumin RAST is the more efficient, N. brasiliensis /

(Radio allergosorbent tests)
RASTS being hampered by lack of pure antigen preparations. The RAST was found to be only partially reproducible, but extremely sensitive. The PCA test is the test of choice in this laboratory for the quantification of antigen specific IgE, and is discussed in detail in this section.

Section III presents a study of the relationship in time between the elevation of total serum IgE, the parasite-specific IgE response, and the potentiated IgE response to unrelated antigen which occurs in rats following infection with the worm parasite N. brasiliensis.

During a first infection the potentiated IgE response (to egg albumin) and elevation of total IgE occur synchronously rising to a peak on days 12-14 after infection, with the fastest rate of increase occurring between days 8 and 10. N. brasiliensis-specific IgE rises to a peak some 2 - 3 weeks later when both total IgE and the potentiated response have largely declined.

A strain difference is shown in that Wistar rats produce far lower levels of total and parasite-specific IgE than Hooded Listers.

Events following reinfection differ in that total IgE rises more rapidly, very high levels being reached 6 days after reinfection together with a secondary specific IgE response to N. brasiliensis.

/ The total IgE level, however, rises by a far greater factor than parasite-specific IgE and declines rapidly while the parasite-specific response declines slowly over many weeks. The egg albumin response is not repotentiated.

It is proposed that the total IgE response and the potentiated IgE response which forms a small component of it results from the release of a non-specific IgE-stimulating factor produced by *N. brasiliensis*-specific T cells. In this scheme the same or similar cells are involved in the production of *N. brasiliensis*-specific IgE through a separate specific helper function.

In Section II experiments were described in which primary and booster IgE antibody responses were elicited in Hooded Lister rats by the intradermal injection or oral administration of very small quantities of egg albumin. Oral immunization was effected by giving antigen by stomach tube or in the drinking water.

The minimum primary dose of antigen found to be effective was I µg intradermally and 10 µg orally, administered together with an intraperitoneal injection of *B. pertussis* adjuvant. In rats immunized with these doses secondary responses could be evoked by giving even smaller quantities of antigen, thus 1 ng intradermally or 1 µg orally without adjuvant. Smaller challenge doses were not tried.

Large primary doses of antigen (> 100 μg) presented by these routes were, on the other hand, found to be inhibitory to the production of secondary IgE responses, this effect being similar to that observed in previously reported intraperitoneal immunization experiments. By contrast with previous experiments, however, tertiary responses could be obtained following immunization by these routes, and I believe this to be a reflection of the absorption of smaller and therefore less inhibitory quantities of antigen.

The results are discussed in relation to the control of IgE antibody production, current concepts of the control of antigen absorption through mucosal barriers, and possible implications for the genesis of naturally occurring IgE responses in man.

GENERAL INTRODUCTION

A REVIEW OF THE LITERATURE

IgE forms a fifth and distinct antibody class in man. It mediates type 1 (Immediate or anaphylactic) hypersensitivity reactions which are responsible for atopic disorders such as hay fever, allergic asthma, dermatitis and allergic eczema. In these conditions and in helminth parasite infections, raised levels of IgE are found.

The fundamental event in immediate hypersensitivity is the immunological release from target cells - identified as tissue mast cells and circulating basophils (Ishizaka, Tomioka and Ishizaka, 1970) - of granules containing pharmacologically active mediators. These include histamine, serotonin (5-hydroxytryptamine), and slow reacting substance of anaphylaxis (S.R.S.-A.). These compounds act on surrounding tissues to cause vasodilation and smooth muscle contraction, and it is these effects which give rise to the clinical symptoms of immediate hypersensitivity. Another factor, termed eosinophil chemotactic factor of anaphylaxis (E.C.F.-A.), attracts eosinophils to the site of anaphylaxis (Kay and Austen, 1971).

It is thought the mechanism of mediator release requires that specific antigen (allergen) combines with two adjacent IgE molecules bound to the target cell membrane via their Fc portions (Stanworth 1971). Antigen - antibody interaction could then cause a conformational change in the antibody molecules, activating a membrane bound enzyme system (probably

adenyl cyclase) which in turn triggers off a series of intracellular enzymatic events, ultimately resulting in the non-cytotoxic secretion of granules from the cell. Degranulation is dependent on the presence of divalent cations - such as calcium (Ca^{++}), which has been seen to enter the cell just prior to mediator release (Lichtenstein, 1972).

Discovery of IgE

The presence of a tissue sensitising humoral factor in the blood of allergic individuals was discovered by Prausnitz in 1921. The antibody nature of this factor had also been assumed and it had been given the names; reagin, homocytrotropic, or anaphylactic antibody. Later, Ishizaka and his colleagues isolated the reaginic activity from the serum of a ragweed sensitive patient and found that this belonged to a new, unique class of immunoglobulin. They designated the new antibody class Ig'E' because of its specific binding to ragweed allergen E. (Ishizaka, Ishizaka and Hornbrook, 1966,a,b). Simultaneously, Bennich and Johansson discovered a unique protein being produced in a patient with myelomatosis. This myeloma protein was found to be antigenically distinct from the other four immunoglobulin classes, and the new class was tentatively designated Ig'ND' after the initials of the patient (Johansson and Bennich, 1967,a,b). Comparison of Ig'E' and

Ig'ND' showed that the two molecules shared major antigenic determinants, and that they represented the same immunoglobulin class, which was designated "Immunoglobulin class E" (IgE) (Bennich, Ishizaka, Johansson, Rowe, Stanworth and Terry, 1968).

Properties of IgE

The IgE molecule consists of two heavy and two light polypeptide chains. The heavy chain, called the epsilon (E) chain carries the antigenic determinant which distinguishes the molecule from the other classes of immunoglobulin (Bennich and Johansson, 1968). To date, five human IgE myelomas have been discovered of which two have been extensively characterised - namely from patient P.S. (Ogawa, Kochwa, Smith, Ishizaka and McIntyre, 1969) and patient N.D. (Johansson and Bennich, 1967a). IgE has been identified with both Kappa or Iambda light chains. Most investigations of the properties of IgE have been performed with the myeloma form IgND (Bennich and Johansson, 1971). The larger molecular weight of the epsilon chain (72,000) as compared to the gamma chain of IgG (50,000) is thought to be due to the existance of an extra domain in the Fc portion of the molecule. This domain has been postulated to be responsible for the unique biological properties of IgE (Stanworth, Humphrey, Bennich and Johansson, 1967). The carbohydrate content of the molecule is unusually high for an immumoglobulin (11.7%) - the reason for this is not known. The total molecular

weight of the IgE molecule ($\sim$ 196,000) is greater than that of IgG because of the extra domain and the carbohydrate content. Recently, the amino acid sequence of a large portion of the IgND molecule has been elucidated (Bennich and Bahr-Lindstrom, 1974) and the regions of the epsilon chain responsible for the various biological properties of IgE have been located more precisely. For example, the C terminal region of the Fc portion of the molecule is hypothesised to be essential for the binding of IgE to target cells (Bennich and Dorrington, 1972). Further investigation on the cleavage of disulphide bonds in myelona PS (Takatsu, Ishizaka and Ishizaka, 1975) indicated that IgE loses its affinity for target cells after cleavage of five disulphide bonds in the $F(ab)_2$ portion of the molecule. A similar effect was observed when two intra heavy chain disulphide bonds were cleaved. The authors speculate that both events could cause a conformational change in the tissue binding Fc portion of the molecule, thus causing it to lose its tissue binding capacity.

As well as the above mentioned tissue binding capacity (a long lasting affinity for mast cells and basophils), IgE is also heat labile, its activity being destroyed by heating for one hour at 56°C. It is also susceptible to reduction by 0.1M, 2-mercaptoethanol. In these latter two respects it is identical to the properties of reagin as described by Stanworth (1963). Electrophoretic mobility of the globulin is in the fast γ_1 region (Ishizaka, Ishizaka and Hornbrook, 1966a, Bennich and Johansson, 1971). In vitro, aggregated IgE binds late complement components (C3 to C9)

via an alternate pathway of complement fixation (Osler, Oliveira, Shin, and Sandberg, 1969), the activity here being associated with the Fc portion of the molecule (Ishizaka, Soto and Ishizaka, 1972). However, IgE antibodies have not been demonstrated to fix complement by the classical pathway, and the importance of alternate complement fixation by aggregated IgE is not known. IgE does not cross the placenta, thereby eliminating the possibility of passive sensitisation of the foetus by the mother.

IgE forming plasma cells have been found predominantly in the tonsils, respiratory and gastrointestinal mucosae and regional lymph nodes (Ishizaka and Ishizaka, 1971).

Turnover studies on IgE using the myeloma protein Ig'ND' in normal individuals demonstrated that the antibody has a half life ($t_{\frac{1}{2}}$) in the circulation of 2 to 3 days, compared with 25 days for IgG (Waldeman, 1969). Thus IgE appears to be the most rapidly catabolosed protein amongst the immunoglobulin classes. The mean IgE synthetic rate has been calculated to be 2.3. μg/kg/day in normal subjects (Ishizaka, 1973), which gives IgE the slowest synthetic rate of all the immunoglobulin classes. Waldeman (1969) also found that radiolabelled Ig'ND', injected into local skin sites, disappeared from these sites in two distinct phases; the first phase was a rapid disappearance of IgE closely paralleling that of IgG. This was followed by a slow rate of disappearance of IgE, where 1 to 5% of the original protein injected was retained at local skin sites, disappearing slowly with a half life of between

8.5 and 14 days. The initial phase is thought to represent diffusion of protein away from skin sites, but whereas IgG continued to disappear at its initial rate, IgE is retained - presumably because of its capacity to fix to mast cells.

IgE Levels in Health and Disease:

IgE can be detected in cord serum at an average value of 36 to 3[illegible] ng/ml as measured by the radioimmunosorbent test (R.I.S.T.) (Johansson and Bennich, 1967b). This level increases slowly with age (Berg and Johansson, 1969) and does not significantly vary between the sexes. Many workers have attempted to establish the IgE serum level in healthy adults, the average value lying in the range 10 to 1,000 Units per ml. However, the major drawback to these results is that the sera under test were taken from individuals chosen on the basis that they showed no clinical allergic or parasite infected condition. This group could however contain individuals tolerating a low threshold allergy of which they are not aware, or individuals from families showing a history of allergies, while they themselves are not affected clinically. A recent report (Nye, Merrett, Landon and White, 1975) has described the isolation of a group of subjects with no known family history of allergy and no known allergy. The mean IgE level in the circulation for this group was found to be 36 Units/ml (as measured by a highly sensitive double antibody radioimmunoassay.). This is significantly lower than values obtained by previous workers. This work indicates

that the IgE level in the serum of non-allergic individuals lies within the range 1 to 178 Units/ml and values of over 200 Units/ml can be considered as indicative of an atopic or pathological state. Raised IgE levels can be detected in atopic diseases such as allergic asthma, hay fever and atopic eczema (Bennich and Johansson, 1971). Significantly high levels have also been detected in the serum of patients infected with any one of a variety of helminth parasites, for example: Ascaris lumbricoides (Johansson, Melbin and Valquist, 1966); Toxocara canis (Hogarth - Scott, Johansson and Bennich, 1969); Capillaria philipinensis (Rosenberg, Whalen, Bennich and Johansson, 1971) and ancylostomes (Bennich and Johansson, 1971).

Clinical Importance of IgE and Inhibition of Allergic Reactions

It seems reasonable to assume that IgE does not exist merely to cause allergies. A possible benificial role of this immunoglobulin class could lie in the expulsion of certain helminth parasites from the gastrointestinal tracts of their hosts. Support for this lies in work done on Nippostrongylus brasiliensis infected rats (Barth, Jarrett and Urquhart, 1966), where an IgE mediated local anaphylaxis in the gut causes increased macromolecular leakage. It has been postulated in this work that this facilitates an influx of anti-worm precipitating antibodies, which then act directly on the worms which leads to their immune expulsion.

The clinical importance of IgE lies in the fact that a large

number of people suffer from atopic disorders. At present the routine detection for the cause of allergy is by the prick test. In this test, a small drop of various allergen extracts is placed at sites on the forearm of the suspected allergic individual and the skin is pricked through these drops allowing small amounts of extract access to the underlying skin tissues. A wheal and flare response in positive reactions appears after a few minutes and is maximal at around 20 minutes. Although the individual may give positive skin reactions to a variety of allergens, clinical allergy may only be demonstrated to one (Brostoff, 1973). Provocation tests are also used in the diagnosis of allergy, where allergen is presented to an allergic patient by the route which it would naturally take. For example, presentation of allergens intranasally to patients whose target organ for anaphylaxis is the respiratory tract, and orally to those who exhibit a food allergy.

There are several ways of treating allergic conditions, none of which appear to be permanent. Desensitisation involves giving an allergic patient increasing doses of the relevant allergen in an attempt to elevate the IgG antibody component of the immune response. These IgG antibodies compete with IgE, and in many cases successfully block IgE from combining with allergen and causing anaphylactic reactions. These γG antibodies have been referred to as 'blocking' antibodies. IgG combination with allergen may not be exclusively responsible for the success of hyposensitisation, as the treatment has been hypothesised to decrease the IgE

response to the relevant allergen by increasing the T suppressor cell population (Taylor, 1974).

Various drugs which affect the allergic response are used in the treatment of allergic diseases. Antihistamines act by inhibiting histamine release from target cells. Disodium cromoglycate is effective and is thought to act by preventing the influx of calcium ions always observed before degranulation (Orr, 1973). Drugs that increase the intracellular level of cyclic A.M.P. will also reduce the amount of histamine released from mast cells. Isoprenaline and theophylline function in this way. Steroids have been demonstrated to depress immediate hypersensitivity reactions although their mode of action is unknown (Brostoff, 1973).

IgE production in other species:

Homocytrotropic antibodies similar to human reaginic antibodies have been detected in various animal species. In rats, immunisation with antigen and B.pertussis killed organisms as adjuvant was found to give rise to the production of reagin-like antibodies (Mota, 1964a). Subsequently, antibody capable of sensitising homologous skin was found in the mouse (Mota, 1967); dog (Rockey and Schwartzman, 1967); rabbit (Zvaifler and Becker, 1966); monkey (Weiszer, Patterson and Fruzansky, 1968); guinea pig (Levine, Chang and Vaz, 1971) and in cattle (Hammer, Kickhofen and Schmid, 1971). The physical, chemical and biological properties of these

homocytotropic antibodies are similar to those of human IgE. Monkey and rat IgE have been shown to react under certain conditions with antihuman IgE (Ishizaka, Ishizaka and Tada, 1969; Kanyerezi, Jaton and Bloch, 1971). Thus the IgE produced in these three different species share similar antigenic and structural components.

Control of IgE production:

Working with various strains of mice, Levine and Vaz (1970) have shown two different types of genetic control of IgE production. One of these controls immune responses per se and lies at single autosomal locus closely linked to the H-2 system. By this control mechanism, responsiveness of a given mouse strain was found to be antigen specific (i.e. a good responder to one antigen while a poor responder to another) and when good and poor responders to any given antigen were grouped, responsiveness was found to correlate with the H2 genotype. Small amounts of antigen were required to bring out strain differences. The gene derived controlling factor is thought to act in T cells (Mitchell, Grumet and McDevitt, 1972). A second control system was found to be specific for reaginic responses in mice and differed from the above control in that there was neither dose effect, nor antigen specificity and the genetic control was exerted by more than one locus, not linked to the H2 system (Levine, 1973).

In family studies on ragweed hay fever, Levine (1973) has postulated the presence, in the human system, of a genetic locus closely linked to the HL-A system which controls both reaginic and γG responses

to this allergen. This system also shows antigenic specificity. However, a recent study (Marsh, Bias and Ishisaka, 1974) indicated that a gene regulating basal serum IgE levels in most allergic families appeared to mask the effect of the above hypothesised HL-A haptotype associated immune response genes for a specific IgE response. These workers also observed that inheritance of high IgE levels in these family studies, followed a Mendelian recessive pattern.

Work done by Tada and his colleagues (see Indroduction to section II - IgE production in the rat) on the control of IgE production in the rat, indicated two control systems acting on reaginic antibody production, Firstly, they observed an antigen specific γG feedback inhibition of IgE production, and secondly a cellular control mechanism involving the suppressive effect on reagin production by thymus derived (T) cells. These cells appear to act after an initial helper T cell function causing B cells to produce reagin. The authors suggest that defects in the above control mechanisms could theoretically result in uncontrolled IgE production to cause an allergic state in man (Tada, Okumura, and Taniguchi, 1973b). Whether the same control mechanisms operate in man remains to be elucidated. The fact that hyposensitisation schedules are successful in at least some individuals is an indication that similar mechanisms may be involved.

Conclusion:

The role of IgE in allergic reactions has been partially defined. Its benificial role, however, remains enigmatic. Further work is required to elucidate this and to provide the basis of information from which to devise prophylactic or curative schems for the alleviation of allergic disease.

Work in animal systems is already begining to resolve some basic aspects of immediate hypersensitivity.

In this thesis, various aspects of IgE production in the rat have been studied which will hopefully lead to a better understanding of the conditions for the production of this immunoglobulin class.

MATERIALS AND METHODS

Experimental Animals:

Adult female Hooded Lister rats (Animal Suppliers (London) Limited) weighing 150 to 200 grams were used in the following experiments unless otherwise stated. A small breeding unit of inbred Hooded Lister rats (Originally from the National Institute for Medical Research, London) were kept at the Veterinary Hospital. In some experiments, Wistar strain (CFHB) rats were used, and these have been maintained at the Veterinary hospital as a closed random bred colony.

Animal Maintenance:

The rats were kept in plastic cages with wire tops and floors, suspended above sawdust-containing trays. The cages were stacked in racks and were washed regularly.

The temperature in the animal house was constant at 22°C and a change of air took place 12 to 15 time an hour.

The rats were fed a pelleted diet (Primrose diet 41) and this was used exclusively.

Adjuvant:

In order to raise IgE in rats immunised with protein antigens, the use of a suitable adjuvant is necessary. This was first shown by Mota (1964a), who demonstrated that killed Bordetella pertussis organisms were extremely effective. A saline suspension of

Bordetella pertussis organisms (Wellcome Biological/reagents) was therefore used as adjuvant in the following experiments. As standard proceedure 10^{10} B.pertussis organisms in 0.5 ml. saline were given intraperitoneally. Adjuvant was only given with the initial sensitising dose of antigen and not on subsequent antigen challenge.

Antigen:

The antigen used in the following experiments to induce reagin synthesis was egg albumin (EA) (Sigma grade V). This antigen was chosen because of its ability to induce good reagin responses in rats (Mota 1964a). The EA was initially made up into stock solutions at a concentration of 10 mg/ml and then immediately diluted to the required concentration using 0.85% saline.

The preparation of N.brasiliensis antigen is described later.

Administration of Antigen:

In the experiments which follow, antigen was administered by one of the three routes - (a) Intraperitoneally, (I.P.)

(b) Intradermally (I.D.)

or (c) Orally

For intraperitoneal injection, antigen was dissolved and diluted so that the correct dose was contained in 0.1 ml saline. This was injected together with the adjuvant. Intradermal and oral administration of antigen is described in Materials and Methods

in section II.

Anaesthetic:

Rats were anaesthetised in a large jar containing cotton wool moistened with trichloroethylene.

Collection and titration of reaginic sera:

Adult rats were bled from the tail vein by cutting off the tip of the tail and manually 'milking' the tail to deliver the blood into a test tube. 1 to 2 ml of blood was collected in this way. Baby rats were bled by cardiac puncture, 0.5 to 1 ml of blood being collected. Heavily anaesthetised rats were bled out by opening up the thoracic cavity and collecting as much blood as possible by cardiac puncture. The blood was allowed to clot in the test tube and serum was collected after centrifugation. The sera obtained from individual rats were stored separately at -22°C until required.

The IgE content of individual sera was estimated using the passive cutaneous anaphylaxis (PCA) test (Ovary, 1958; 1964) (see Section I).

Parasite:

The parasite used throughout these experiments was the nematode Nippostrongylus brasiliensis. This is maintained in the Wellcome Laboratories for Experimental Parasitology by repeated subinoculation

in Hooded Lister and occasionally Wistar strain rats.

Culture:

The cultrue of N.brasiliensis larvae was as described by Jennings and his colleagues (Jennings, Mulligan and Urquhart, 1963) based on the method of Bakarat (1951). Faeces were collected from rats with a 7 to 10 day old infection by placing paper beneath the cages. The faecal pellets were mixed to a paste with a little water in a mortar and a portion of the paste was spread on to the centre of circles of Whatman's no.1 filter paper, diameter 7 cm., the outer edges of the filter papers being kept clear of faeces. The filter papers were then moistened and placed on circles of plastic foam of lesser diameter, saturated with water. These were place in disposable plastic petri dishes (Oxoid Ltd.) which were stacked with lids on, in a humid incubator at 27°C.

After 4-5 days larvae which had migrated outward could be seen as a fringe around the edges of the filter paper. The larvae were harvested between 5 and 10 days after setting up the culture. This was achieved by flooding the petri dish with water at 37°C and allowing a short time to elapse (2 to 5 minutes) for the larvae to swim away from the filter paper leaving a fringe of empty sheaths still attached.

The filter paper and plastic foam were then discarded. The water containing the larvae was filtered under suction in a large Buchner

funnel through strong filter paper (Greens Hydro 904, 18.5 cm. diameter). The filter paper was then placed inverted on an Endecott sieve (mesh 400) in a Baerman apparatus filled with water at 37°C. The larvae swam down through the sieve and after a while collected at the bottom of the funnel from which they were subsequently run off. Larvae prepared and collected in this fashion were thus separated from any faecal residue.

Larval Count:

Larvae were counted as follows: The larval suspension was diluted to 100 or 200 ml., depending on concentration, using warm saline. The larvae present in 0.025 ml. aliquots of this were counted under a dissecting microscope until a total of at least 400 larvae had been counted. Care was taken to ensure that the larvae were properly and evenly suspended before dispensing aliquots for counting. The number of larvae present in the original suspension was then calculated, and this was diluted or concentrated so that the required number of larvae for infection purposes was contained in 1 ml. of the suspension.

Infection of Rats:

The rats were infected by subcutaneous inoculation of the larvae in 1 ml. saline in the groin region using a 20 guage, 1 inch needle

and a 1 ml. disposable plastic syringe. The usual larval dose was 4,000 per rat.

Faecal egg count - The McMaster Technique:

A 3g sample of faeces from infected rats was homogenised in 42 ml. water to give a total volume of 45 ml. This was passed through a sieve (Mesh 50) and 15 ml. of the filtrate centrifuged at 2,000 r.p.m. for 2 minutes. The supernatant was discarded while the pellet containing the eggs was re-suspended in 15 ml. of saturated sodium chloride solution (in which the eggs float). The suspension was agitated by inversion before samples were withdrawn using a pasteur pipette. The two counting chambers of a McMaster slide were filled with the suspension. Both chambers, each representing 0.15 ml., were examined under the microscope for eggs. The mean value from both chambers, when multiplied by 100, gave the number of eggs present in 15 ml. of homogenate, and thus 1 gram. of faeces.

Recovery of worms from the intestine of infected rats:

Anaesthetised rats were killed by cervical dislocation. The small intestine was removed and opened longitudinally with blunt scissors and placed in a gauze bag suspended in a 250 ml. beaker filled with warm saline. This was incubated at 37°C for between 40 minutes and 1 hour during which time the worms migrated through

the gauze and collected at the bottom of the beaker. Most of the fluid was decanted from the beaker, and the worms were then counted in petri dishes under a dissecting microscope. If large numbers of worms were present, they were diluted up to 200 ml. in water and several 4 ml aliquots of the well agitated suspension were counted. The total number of worms obtained from each rat could then be calculated. Recovery of worms was optimal around D.7 and 8 of an infection when the maximum number of adult worms could be found in the gut.

Preparation of N.brasiliensis whole worm antigen:

Adult worms were obtained from rats 7 to 8 days after a larval infection. The worms were washed with saline and finally suspended in saline at a concentration of 1,000 worms per ml. The worms were then homogenised in the cold at high speed using a Silverson homogeniser. Samples of homogenate were withdrawn at various intervals and examined microscopically for the state of disruption of the worms. When this was judged to be sufficient (after about 3 minutes of homogenisation) the homogenate was centrifuged at 3,000 r.p.m. at 4°C for 30 minutes to remove particulate matter. The supernatant was collected and stored at -20°C until required.

The Caprylic Acid Technique for the Isolation of the Gamma Globulin Fraction of Serum:

The isolation of pure IgG from the sera of various animal species

using caprylic (n-octanoic) acid has been described by Steinbuch and Audran (1969).

The purity and yield of IgG from any given serum sample source was found to be dependent on the amount of caprylic acid used; the pH of the reaction mixture, and the molarity of the diluting buffer used. The optimum conditions found by these workers were the ones used below.

Proceedure:

1. To 1 volume of serum was added 2 volumes of 0.06 M Acetate buffer pH 4.0. This gives a serum-buffer pH of 4.8.

2. The calculated amount of caprylic acid (n-ostanoic acid, reagent grade -B.D.H. Ltd.) was added dropwise to the solution in a large glass centrifuge container, with shaking. Agitation was continued for between 15 and 20 minutes.

The following amounts of caprylic acid were recommended for the serum sources indicated:-

Goat .. .. 4.0 g/100 ml. serum (Steinbuch, Audran and Pejaudier, 1970)

Rabbit . .. 4.8 g/100 ml. serum (Steinbuch, Audran and Pejaudier - personal communication)

3. The solution was centrifuged at 3,000 r.p.m. for 10 minutes at room temperature. At this stage, the supernatant has been estimated to contain 90% pure IgG (Steinbuch and Audran, 1969).

4. The supernatant was removed and adjusted to pH 5.7 with dilute NaOH.

5. The salt concentration of the solution was lowered by 24 hour dialysis against 0.015 M Acetate buffer pH 5.7 at room temperature with 3 changes of buffer. Any IgA present in the sample would be removed by the above procedure (Steinbuch and Audran, 1969).

6. Remaining contaminants (depending on the serum source, but largely ceruloplasmin, α 1-acid glycoprotein, and possibly prealbumin) (Steinbuch and Audran, 1969) were removed by batch adsorption of the solution with D.E.A.E. cellulose (Whatman DE 52 medium) as follows:-

DE 52 was equilibrated by stirring 6 g of humid pressed medium per gram of protein in the sample into 0.1 M Acetate buffer pH 5.7. The particles were allowed to settle and the buffer decanted. The process was repeated. The next few washes were performed using 0.015 M Acetate buffer pH 5.7 until the effluent buffer pH was 5.7. The particles were allowed to settle after the final wash and buffer removed, leaving the medium in a paste-like consistency.

7. To the medium was added the impure gamma globulin solution with gentle stirring. Adsorption was allowed to proceed - with occasional stirring - for two hours at room temperature.

8. If necessary, a small amount of 0.015 M. Acetate buffer pH 5.7 was added to the mixture and the particles removed by centrifugation at 3,000 x g for 5 minutes. The supernatant was collected and the IgG solution concentrated by surrounding a piece of dialysis tubing containing the solution with carbowax (polyethylene glycol, B.D.H. Ltd.). When the volume of the IgG solution in the tubing was one half the starting serum volume, the sample was removed,

and concentration estimated using a spectrophotometer. The purity of the fraction was checked by agar slide electrophotesis using 0.05 M barbitone buffer pH 8.4 in a Shandon electrophoresis tank powered by a Vokam power pack.

Antiserum used was as follows:-

For Goat IgG	.. ..	Rabbit anti-goat whole serum and
		Rabbit anti-goat 7S globulin.
For Rabbit IgG	. ..	Goat anti-rabbit whole serum and
		Goat anti-rabbit 7S globulin.

The pure IgG preparation was stored at -20°C until required. Before use, it was dialysed against the relevant buffer - in this case 0.1 M P.B.S. (for use in the Rowe-Mancini technique - see section 1).

SECTION 1

DETECTION AND MEASUREMENT OF TOTAL IgE AND ANTIBODY IgE

INTRODUCTION

The methods available for the detection of reaginic antibodies can be grouped into two broad categories: first, those which utilise the ability of reagins to sensitise homologous tissue as assessed by the occurrence of immediate hypersensitivity reactions on subsequent encounter with specific allergen; and secondly those which use specific anti-E chain antisera to detect the presence of IgE.

Passive Sensitisation of Tissues in Vivo:

In the first category, the pioneering test was the so called Prausnitz-Kustner (P.K.) test (Prausnitz and Kustner, 1921) which is based on the discovery, by Prausnitz, that intradermal injection of the serum from an allergic donor (Kustner) could sensitise the skin so that an immediate wheal and flare reaction occurred on challenge (after a latent period) in the same skin sites, with the relevant allergen. This test subsequently became a widely used research assay for reagins in the human system, and provided the reference against which later tests for reagin were compared. Briefly, the test as used more recently (Stanworth and Kuhns, 1965) involved the intradermal injection of 0.1 ml. dilutions of test serum into the back or forearms of a non-allergic recipient. A latent period of 24 to 72 hours was then allowed to elapse before challenge with the relevant allergen. This was achieved by pricking in a small amount of concentrated allergen extract into the same skin sites into which antibody was previously injected. After 30 minutes,

the wheal area was measured and recorded for each serum dilution. A linear relationship was obtained by plotting the logarithm of the serum dilution against mean wheal area. The test is extremely sensitive but is no longer widely used because of the risk of transferring serum hepatitis.

A modification of the P.K. test, has, however, provided the most practicable method for detecting circulating levels of allergen specific reaginic antibody in experimental animals. This is the passive cutaneous anaphylaxis (PCA) test developed by Ovary (1958; 1964). It differs from the P.K. test in that allergen challenge is given intravenously along with a high molecular weight dye, e.g. Evans blue. The presence of reaginic antibody in the samples under test is detectable by the leakage of dye at the site of local cutaneous anaphylaxis, causing a blue spot. The PCA reaction will be discussed in detail below.

Passive Sensitisation of Tissue in Vitro

Passive sensitisation of tissue can also be achieved in vitro, and there are many assays for reaginic antibody activity in this category. For example, passive sensitisation of contractile tissue (e.g. guinea pig ileum); chopped tissue (e.g. human or monkey lung), and peripheral leucocytes has been achieved (see Stanworth, 1973). In these systems, serum under test is incubated with tissue or cell preparations and relevant allergen. An immediate hypersensitivity reaction will take place if reaginic antibody was present in the test serum. The amount of

histamine released, as measured biologically (see Stanworth, 1973) (e.g. guinea pig ileum in Schultz-Dale bath) or spectrofluorimetrically (Shore, Bukhacter and Cohn 1959) is proportional to the amount of reagin in the test sample. These assays, while useful for the better understanding of immediate hypersensitivity reactions, are hampered by a lack of reproducibility, a requirement for fresh tissue or cells, and the fact that non specific release of histamine unavoidably occurs. Furthermore, it is also possible that IgE is not the only factor in serum causing specific histamine release (Stanworth 1973).

The rat mast cell degranulation technique (R.M.C.T.) (Perelmutter, Liakopoulou and Phillis, 1973) has been claimed to be a successful assay to detect human reagins. The technique involves the incubation of allergic serum, allergen, normal rat serum and a suspension of rat peritoneal mast cells for 3 minutes, followed by centrifugation and the microscopic examination of cells for degranulation. The supernatant is analysed for histamine content using the Schultz-Dale system. Results of the test were expressed as the minimum concentration of allergen giving a response. Of all the in vitro passive sensitisation techniques for allergen specific reagin detection, this is perhaps the most practicable - but its application for the detection of human reagins remains unconfirmed by workers in some other laboratories who have tried to reproduce the technique.

Tests Involving the Use of Specific Antisera to IgE

The second category of techniques for the detection of IgE antibodies involves the use of specific antiserum to IgE. The production of such antiserum has been made possible by the discovery of myeloma IgE proteins in the human (Johansson and Bennich, 1967a) and rat (Bazin, Beckers, Deckers and Moriame, 1973). The tests in this category

may be used to detect both total circulating IgE (or the IgE content of other body fluids), and allergen specific IgE.

Measurement of Total IgE:

The radioimmunosorbent test (RIST) developed by Wide and Porath, (1966) and modified for IgE detection by Johansson, Bennich and Wide (1969) has been widely used for detection of total IgE. This is a competitive radioimmunoassay in which standard IgE or IgE present in samples compete with labelled myeloma IgE for sites on an insolubilised anti-IgE matrix (usually Sephadex bound anti-IgE). The test is very sensitive and reproducible. The normal range of IgE concentration detected by RIST is between 2 and 8,000 Units/ml as used by the Swedish workers, and 10-4,000 Units/ml range for Phadebas^{R} RIST. This test is described in detail below.

Another test of great sensitivity is a double antibody liquid phase radioimmunoassay (Morgan and Lazerow, 1963; Gleich, Averbeck, and Swedlund, 1971; Polmar, Waldeman and Terry, 1973) which is especially valuable for detecting low levels of IgE. The test involves unknown or standard IgE samples competing with labelled myeloma IgE for anti-IgE antibodies (e.g. rabbit and anti-human IgE). After incubation, the free and bound fractions are separated by adding goat anti-rabbit antibodies along with normal rabbit serum. After centrifugation and decantation of the supernatants, the precipitates are assessed for radioactive content on a gamma counter. The amount of IgE in an unknown sample is inversely proportional to the amount of radioactive myeloma bound. The sensitivity of the test can be increased (detection as low as 0.124 Units IgE/ml) by incubating standard and unknown samples with rabbit anti-human IgE for a period before the addition of labelled myeloma

IgE (Nye, Merrett, Landon and White, 1975). Immunological reactions due to idiotypic antibody specificities can be prevented by raising antiserum to one myeloma and adding labelled IgE of another myeloma. Optimal dilutions of rabbit anti-IgE and goat anti rabbit antibodies have to be worked out prior to use in the test (Polmar, Waldemann, and Terry, 1973).

'Sandwich' radioimmunoassays have also been developed for IgE detection in body fluids. One such test involves coupling anti-IgE antibodies to paper discs, incubating with test samples and standard IgE dilutions, followed by washing and incubation with ^{125}I labelled anti-IgE antibodies, then finally washing and measuring the radioactivity in the tubes on a gamma counter. In this system (Ceska and Lundkvist, 1972) the concentration of IgE in the sample is proportional to the amount of radioactive anti-IgE bound. The Rowe modification of the Mancini single radial immunodiffusion technique has been used for serum IgE detection where highly purified anti-IgE has been difficult to obtain (Rowe, 1969). The test involves the dilution of an anti-IgE antiserum preparation in agar into which wells are punched. Standard or unknown IgE sample dilutions are added to these wells and diffusion takes place. The invisible precipition ring formed after incubation can be visualised by further incubating the agar plates with radioactively labelled antiserum to the anti-IgE, and exposure to X-Ray sensitive film of the washed and dried plates. The test will be described in more detail later. The sensitivity of immunodiffusion techniques is not as great as with the Sandwich or competitive radioimmunoassays.

Various modifications of the above methods for the detection of IgE exist. For example, instead of the use of radioactivity as a marker, fluorescein (Centifanto and Kaufman, 1971) or alkaline phosphatase (Hoffman, 1973) have been used. Anti-IgE antibodies or IgE myelomas can be bound to a variety of insolubilising materials e.g. cellulose particles, activated paper discs, bromo-acetyl cellulose (Mann, Granger and Fahey, 1969) or even the insides of plastic tubes (Hoffman, 1973). A comparison of some of these methods for IgE detection has shown that the most sensitive technique is the double antibody liquid phase radioimmunoassay, however, this test operates only within a small range of IgE levels and takes longer to complete. The RIST is good for the clinically important levels of IgE concentration ($>$ 200 Units/ml) and is a quick and practicable test to perform (Polmar, Waldeman and Terry, 1973). Agar diffusion methods are relatively insensitive and tedious to perform as compared with the other methods mentioned above, - an average assay taking approximately 9 days to complete.

The detection of allergen specific IgE:

The two main and most widely used tests in this category are the Radioallergosorbent test (RAST) (Wide, Bennich, Johannson, 1967) and the Red cell linked antigen-antiglobulin reaction (R.C.L.A.A.R.) (Steele and Coombs, 1964; Coombs, Hunter, Jonas, Bennich, Johansson and Panzani, 1968). The RAST is based on the antiglobulin principle of the R.C.L.A.A.R., and involves the coupling

of allergen to paper discs and after incubation with test sera, identification of any IgE in the sera specific for the relevant allergen by further incubation with highly specific radiolabelled anti-IgE antibodies. The test is subsequently to be described in more detail.

The R.C.L.A.A.R. provides a sensitive in vitro test for allergen specific IgE based on an agglutination principle. Allergen is coupled to rabbit anti-red cell antibodies by photo-oxidation. This conjugate is then coupled to red cells. Following incubation with dilutions of test sera, agglutination can be achieved (in the samples where IgE was present in the test sera) by subsequent incubation with specific anti-IgE antibodies. The test is sensitive and has been compared in this respect with PCA testing of human sera in baboons (Stanworth, 1973). A major drawback is the fact that the coupling proceedure is effective for a few allergens only, damaging some other allergen preparations (Bennich and Johansson, 1971). The RAST compares very well with both P.K. and provocation tests in the human system. Both tests can be adapted to measure different classes of allergen specific antibodies present in test sera by using antibodies to the different classes in the second incubation stage. With respect to this, it should be pointed out that in IgE detection, any allergen specific IgE class antibodies will bind to the allergen coated red cells or paper discs in com-

petition with IgE, and therefore influence test results. Another major drawback to both test is that most allergens have not been properly standardised with respect to each other, and differences exist in the quality of a given allergen extract obtained from different laboratories or commercial sources (Stanworth, 1973). Such drawbacks make these tests only semi-quantitative and results are not comparable regarding IgE levels to different allergens, and results obtained between different laboratories.

In all the in vitro tests described above, with the exception of agar diffusion methods, highly purified anti-IgE antibodies are essential. In the assays for total IgE results were originally expressed in ng/ml, but in 1970 the W.H.O. introduced a research standard reference serum obtained from 91 adult West African donors all with high IgE levels (Rowe, Tackett, Bennich, Ishisaka, Johansson and Anderson, 1970). The sera were placed into ampoules in freeze dried form, the mean weight of the content of each ampoule was 92.84 mg. To this was assigned an arbitrary unitage of IgE such that each ampoule contained an average 10,000 Units of activity of IgE. Thus 1 Unit of IgE is defined as the activity present in 0.009284 mg. of the freeze dried powder in this reference preparation - known as 68/341. On reconstitution with 1 ml of water it has been calculated that 1 ml of the reference will contain 9346 Units of IgE. (This is because <u>total</u> volume will exceed 1 ml.). All recent measurements of IgE concentration in body fluids are expressed in Units/ml. Recent investigations indicate that 1 Unit is approximately equal to 2.4 ng (Bazaral and Hamburger, 1972).

There follows in this section a detailled description of a number of the techniques introduced above which have been extensively used in this laboratory. Some of these have been in common use in other laboratories, but others, particularly the RIST and RAST as applied to rat IgE detection have been developed here with the collaboration of Dr. H. Bazin of the University Catholique of Louvain, Brussells, and Dr. H. Bennich and his colleqgue Dr. T. Karlsson of Aarhus University, Denmark, and Uppsala University, Sweden.

DETECTION OF TOTAL CIRCULATING IGE IN MAN

Radio Immunosorbent Test (RIST):

The radio-immunosorbent test was developed in Sweden, shortly after the discovery of the IgE myeloma 'ND' (Wide and Porath, 1966; Johansson, Bennich and Wide, 1968). The test is suitable for the detection of IgE in serum and other body fluids especially pathological levels of 200 Units/ml and upwards (see General Introduction).

A recent study, described in more detail in the General Introduction, has furnished a 'normal' concentration range for circulating IgE (Nye, Merett, Landon and Whyte, 1975) and from this work it can be assumed that values of over 200 Units/ml are representative of an atopic or pathological state.

Principle of the Test:

The radioimmunosorbent test used throughout for these studies was the Pharmacia Phadebas^{R} IgE test. Anti-IgE antibodies are covalently bound to Sephadex^{R} particles as the solid phase. The concentration of IgE in an unknown sample is evaluated by its capacity to compete with a fixed amount of radioactively labelled IgE for the binding sites on the Sephadex anti-IgE complex. This competitive capacity is then compared with that of standard IgE preparations of known concentrations.

When performing the test, the samples are mixed with a given

amount of radioactively labelled IgE (IgE-I^{125}) and with a given amount of the immunosorbent (the anti-IgE antibodies coupled to Sephadex particles). This mixture is then incubated overnight during which time the labelled IgE added and the unknown amount of IgE present in the sample compete for the binding sites on the immunosorbent complex. Bound IgE is then separated from free IgE by centrifugation. The unbound IgE remains in the supernatant and is easily removed.

After washing the sedimented particles, the radioactivity bound to the Sephadex particles is measured and the IgE concentration in each sample is read off from the standard curve prepared from standard IgE preparations of known concentration. The radioactive uptake on the immunosorbent varies inversely with the quantity of unlabelled IgE present in the sample (see figure 1).

Materials and Methods:

Reagents used in the Test:

Buffer substance - This is supplied in Lyophilised form and is reconstituted by dissolving in 100 ml of distilled water, providing a buffer solution of pH 7.4.

Sephadex - anti-IgE complex and Tween solutions - The Sephadex - anti-IgE immunosorbent particles are supplied in powder form, and the Tween solution in concentrated liquid form (5 ml.). These are mixed together with 55 ml of buffer solution in stages to give a final volume of 60 ml. A magnetic stirrer is used to keep the particles in suspension.

IgE Standard Solution - Purified myeloma Ig'ND' calibrated against the British Research Standard for the human serum Immunoglobulin E, 66/341 is supplied lyophilised and is reconstituted by dissolving in 2.0 ml distilled water. The final solution contains 400 Units of activity of IgE per ml.

IgE - I^{125} Solution - This also is supplied lyophilised and is reconstituted by the addition of 5.5 ml distilled water. 150 ng at an activity of 3.7 μc, (at the date of manufacture) is supplied with each kit.

Each Phadebas IgE kit contains sufficient reagents for 50 single IgE estimations. This allows for duplicate determination in 16 patients or single determination in 32 patients as well as duplicate assays of the standard dilutions for the construction of standard curve.

The test is carried out in plastic centrifuge tubes (Lukham Limited, LP3) with round bottoms. The test is divided into two parts - the preparation of standard dilutions for the concentration of a standard curve and the preparation of the unknown serum samples with the subsequent calculation of IgE concentrations in these from the standard curve.

Preparation of the Standard Dilutions:

To prepare the standard curve, the IgE standard stock solution (400 Units /ml concentration) is diluted with buffer solution to obtain suitable concentrations in the range 1-400 Units IgE/ml. e.g.: 400,200, 100, 25, 2, 2.5 and 1 Units IgE/ml. These dilutions can be made using a combination of doubling and individual dilutions from the standard stock solution.

A 'zero' sample is included which contains only buffer and no unlabelled (sample or standard) IgE. In this sample binding of labelled IgE will be maximal as there is no competition for binding sites on the Sephadex anti-IgE particles. All the standard assays are performed in duplicate for greater accuracy. The lower limits of the test can be extended by further dilutions of the standard beyond 1 Unit IgE/ml.

Preparation of Unknown Samples:

The serum samples are conventionally diluted 10 times with buffer, because the high protein content of neat serum would adversely influence the results. In this laboratory, serum samples are diluted 20 times which extends the upper limits of the test range, which is 20-5,000 Units IgE/ml. This is found to be more convenient for the measurement of serum samples with pathologically high IgE levels such as those obtained from allergic patients.

Test Proceedure:

All pipetting was done using automatic pipettes and disposable plastic tips.

1. 0.1 ml of each standard dilution to be used in the construction of the standard curve was pipetted into plastic centrifuge tubes. Each standard was run in duplicate.

2. 0.1 ml of buffer only was pipetted into duplicate tubes. These tubes are the 'zero's' described above and therefore receive no unlabelled IgE.

3. 0.1 ml of each diluted unknown sample was pipetted into plastic centrifuge tubes.

4. 0.1 ml of the radioactively labelled IgE solution ($IgE\text{-}I^{125}$) was added to each of the tubes in steps 1, 2 and 3 above.

5. 0.1 ml of $IgE\text{-}I^{125}$ solution was pipetted into each of the two empty tubes which were immediately stoppered and put aside. These

tubes only contained IgE-I^{125} and were not incubated or washed. They were used to determine the total radioactivity added and were counted along with the other samples at the end of the test (step 10).

6. 1.0 ml of the sephadex - anti-IgE complex suspension was added to each tube in steps 1, 2 and 3 above. The suspension was stirred continuously whilst being dispersed in order to keep the particles in suspension and to ensure equal amounts were added to each tube.

7. The tubes were now stoppered and incubated overnight at room temperature, with constant vertical rotation in order to keep the particles in suspension.

8. After the overnight incubation, the tubes were centrifuged at 2,000 x g for 2 minutes to remove any droplets from the stoppers which were then removed. The supernatant was carefully aspirated to about 5 mm. from the bottom of each tube ensuring that the particles were not removed.

9. The suspension in each tube was then washed three times in 0.85% saline to remove any unbound IgE (labelled or unlabelled). For the washing, 2 ml of 0.85% saline wash added to each tube to resuspend the particles. The tubes were then re-centrifuged at 2,000 x g for 2 minutes after which the supernatant was removed as before. This was repeated 2 times more.

10. The radioactivity in each tube was then determined using a gamma counter. At this stage two empty tubes were included in the test to determine the background count of radiation. A routine

counting time of two minutes per tube was employed and proved adequate.

Calculation of Results:

The count rates in counts per minute obtained for the standard dilutions were plotted against the logarithm of IgE concentration in Units of IgE per ml. on linear-logarithmic graph paper to give the standard curve (see Fig. 1). The concentration of IgE for each of the unknown samples was then read off from this curve and multiplied by the dilution factor to give the total IgE concentration of each sample in Units of IgE per ml.

Results:

The following table shows the results obtained for the standard dilutions shown. These values are used to construct the standard curve, from which the IgE levels of a batch of unknown sera are read off.

TABLE 1.

RESULTS OF A RIST PERFORMED ON 36 SERA SUBMITTED FOR IgE ASSAY BY GLASGOW ALLERGY CLINICS.

Sample.	Diln.	Mean C.P.M.	Units IgE/ml.
Reference sera	neat	349	400.0
"	1:2	447	200.0
"	1:4	626	100.0
"	1:16	1085	25.0
"	1:80	1477	5.0
"	1:160	1619	2.5
"	1:400	1727	1.0
Buffer control.	-	1708	-
Total count.	-	10781	-
E.G.	1:20	1211	320
J.W.	"	1147	400
K.A.	"	1012	600
M.M.	"	541	2600
J.W.	"	1095	480
W.C.	"	281	>8000
M.G.	"	955	740
K.M.	"	813	1120
A.B.	"	833	1080
K.R.	"	1246	286
A.M.	"	1255	280
R.P.	"	1523	84
G.R.	"	279	>8000
A.L.	"	1212	320
K.M.	"	1734	20
R.C.	"	340	>8000
A.H.	"	733	1440
M.Y.	"	1149	400
G.M.	"	303	>8000
A.T.	"	1600	54

Continued overleaf.

TABLE 1 (CONTINUED).

Sample.	Diln.	Mean C.P.M.	Units IgE/ml.
K.A.	1:20	1600	54
A.C.	"	664	1760
T.M.	"	499	3200
S.W.	"	1134	390
J.M.	"	1343	196
S.M.	"	852	1000
M.M.	"	1253	280
G.H.	"	182	> 8000
A.R.	"	447	4000
P.20	"	717	1520
P.11	"	1304	230
P.7	"	1555	70
P.16	"	1384	164
P.23	"	1522	88
P.24	"	1373	176
P.35	"	1487	100

All reference samples + buffer control run in duplicate. Unknown samples were run singly.

If desired by the clinician(or for research purposes), all sera exhibiting IgE levels of >8000 Units/ml. were rerun at a higher dilution to give an actual value.

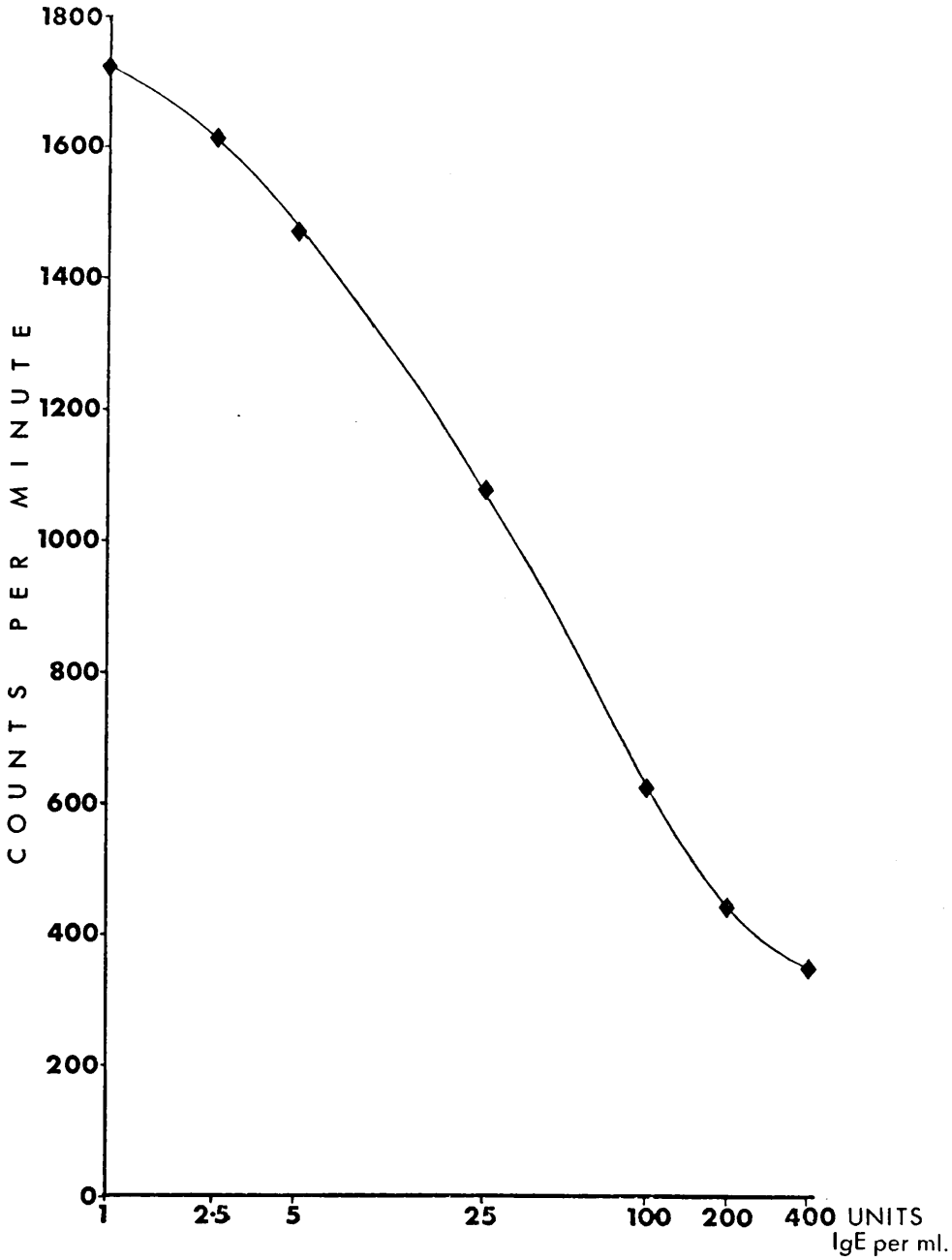


Fig 1. The human RIST. A typical standard curve obtained by plotting the standard IgE dilutions against the concentration of IgE in Units per ml. for each dilution.

Discussion:

The RIST provided a simple, rapid and practicable test for the quantitative measurement of IgE in man, and has contributed greatly to the understanding of the role of this immunoglobulin class in both normal and pathological conditions. As it is known that serum concentration of IgE is significantly raised in many patients with allergic and helminthic diseases, the estimation of IgE may aid the clinical diagnosis of these conditions, and is also essential for the detection of E myeloma cases.

It is, however, important to bear in mind that IgE levels are not elevated in all individuals suffering from IgE mediated allergies. Nor does there appear to be any direct relationship between the level of total IgE and the severity of an allergic condition. In other words some people with high total IgE levels have mild symptoms, whilst others with normal or slightly elevated IgE levels may have severe symptoms. These facts limit the usefulness of the RIST as a paraclinical tool, for the finding of a low IgE level does not exclude the diagnosis of allergy in a dubious case. On the other hand, levels of above 200 Units/ml are unlikely to occur without specific cause, and therefore such levels in patients suspected of being atopic are confirmatory of this diagnosis - provided that parasitic infection has been excluded. Since a diagnosis of allergy is usually made by the clinician on the basis of history, or positive skin or provokation tests, the usefulness of the RIST technique as a laboratory service is largely limited to those cases where high IgE levels are found in individuals who, although having

asthma (for example) are negative to skin or provocation tests. In these cases, so called intrinsic asthma can then be excluded. Unless for research, there is little point in determining the IgE levels of patients whose allergic state has already been diagnosed on clinical grounds.

DETECTION OF IGE IN THE RAT

1. Total Circulating Levels of IgE

(a) The Radioimmunosorbent Test:

Introduction:

The development of a sensitive in vitro assay for circulating levels of rat IgE is a recent one. The discovery by Bazin and colleagues in Belgium of rats producing spontaneous and transplantable IgE immunocytomas (see Introduction to the section on IgE production in the rat), enabled the purification of rat myeloma IgE against which specific antiserum could be raised. The first assays of total IgE levels in the rat were carried out using the Rowe modification of the Mancini single radial Immunodiffusion technique (described below).

Recently, however, it has been possible to set up a rat radioimmunosorbent test using highly purified anti-rat IgE.

The radioimmunoassay used in this laboratory is a 'sandwich' tequnique based on the method of Ceska and Lundkvist (1972).

Principle of the Test:

The test is a solid phase direct 'sandwich' radioimmunoassay.

It utilises solid phase coupled antibodies (immobilised on paper discs) and immunosorbent purified, radiolabelled antibodies. The test is carried out in two stages. The first of these involves incubation of test serum or standard serum dilutions (of known IgE concentrations) with the paper disc-anti-IgE complexes in plastic tubes. After washing, ^{125}I labelled antibodies (immuno-sorbent purified) with specificity for the bound IgE (i.e. ^{125}I-anti-IgE) is added to the tubes. After a further incubation period, the tube contents are washed and their radioactivity content assessed on a gamma counter. The amount of radioactivity bound to the paper discs is directly proportional to the amount of IgE in the test or standard samples.

Materials and Methods:

Paper discs: Filter paper discs with a diameter of 5 mm (~2 mg.) were punched out from Whatman no. 1 filter paper using an office filing punch.

Activation of paper discs: The paper discs were activated using cyanogen bromide (CNBr) according to the method of Ceska and Lundkvist (1972) with some modifications. 5 g of paper discs were allowed to swell for 30 minutes in 100 ml. deionised water. They were then mixed with 100 ml of 3% wt/vol. CNBr. solution. The pH was adjusted to between 10.5 and 11.0 with 1 M NaOH and held there until 20 ml of base was consumed. The solution was aspirated and the discs

washed in 1 litre of ice cold 0.005 M $NaHCO_3$ pH9.0. This washing proceedure was repeated a further 5 times. The discs were then washed twice with 1 litre of ice cold deionised water and then successively with 25%, 50%, 75% and finally 100% acetone in 500 ml portions, two times per given concentration. The discs were dried either by evaporation at room temperature or in a vacuum dissicator at 4°C. The activated discs were stored at 4°C in a sterile air tight container.

Antiserum:

The antiserum used in the test was immunosorbent purified anti-rat epsilon chain specific antibodies prepared against purified rat myeloma IRI62. The antiserum was obtained by immunising rabbits intramuscularly with 0.5 mg of purified myeloma IRI62 in Freunds complete adjuvant. A booster dose in Freunds incomplete adjuvant was given two weeks later, and the rabbits bled two weeks after this. The antiserum was made epsilon chain specific by passage through a column (Sepharose 4B) containing immobilised normal rat serum of very low IgE concentration. Further passage through an immunosorbent column (Sepharose 4B) containing immobilised pure myeloma IR2, with subsequent elution of bound antibodies eliminated idiotype specific antibodies.

Labelling of Rabbit anti-rat IgE Specific Antibodies:

This was carried out as described by Greenwood, Hunter and Glover (1963). The method allows for the radiolabelling of small amounts of protein binding ^{125}I by oxidation with Chloramine T. The reaction is halted by the addition of sodium metabisulphite, and the radiolabelled protein separated from free ^{125}I by filtration through a Sephadex G-200 column.

Labelling Proceedure:

1. 25 µl 0.5 M phosphate buffer pH 7.4 was added to 1 m C ^{125}I in a plastic tube.
2. 25 µg of the rabbit anti-rat IgE anti-body solution was added and the tube contents mixed.
3. Immediately, 25 µl of a 1 mg/ml solution of Chloramine T in 0.5 M phosphate buffer pH 7.4 was added. The tube contents were carefully mixed for between 30 and 90 seconds.
4. Following this 25 µl of a 2 mg/ml solution of sodium metabisulphite in 0.5 M phosphate buffer pH 7.4 was added to the tube and the contents mixed once more.
5. A few crystals of potassium iodide were added in order to give an excess of stable iodide. Finally a drop of bromophenol blue dye in 0.05 M phosphate buffer pH 7.4 plus 0.3% B.S.A. was added, and the solution placed on a G-200 column (20 cm x 1 cm) equilibrated with 0.05 M phosphate buffer pH 7.4 plus 0.3% B.S.A.

6. 0.5 ml eluate samples were collected from the column and measured for radioactivity using a gamma counter. The first radioactive peak to be eluted contained the labelled protein. The subsequent peak contained unbound ^{125}I. The bromophenol blue dye eluted just after the labelled protein and indicated that the latter had been collected. The contents of tubes with gamma counts corresponding to the top of the labelled protein peak were pooled. This contained the labelled anti-rat antibodies. Before use in the RIST and RAST the labelled antibodies were diluted to give ~ 20,000 counts per tube in 0.05 M phosphate buffered saline plus 1% Tween 20 and 0.3% B.S.A.

Coupling Proceedure:

Coupling of anti-IgE antibodies to activated paper discs was based on the method of Ceska and Lundkvist (1972). 100 to 200 μl of specific anti-IgE or 0.5 ml of an IgE fraction of goat anti-rat IgE antiserum was added to 20 ml 0.1 M $NaHCO_3$ buffer pH 9.0. To this solution of anti-IgE in buffer, in the cold, were added 400 activated paper discs. The discs were kept in suspension by vertical rotation at 4°C for 3-4 hours. The discs were then washed three times with 0.5 M $NaHCO_3$ pH 9.0. Any remaining reactive sites on the discs were blocked by incubation with 0.1 M Tris buffer pH 8.0 for 3 hours. The discs were washed

two times in 0.5 M $NaHCO_3$ pH9.0 then three times in 0.1 M Acetate buffer pH 4.0. Finally the discs were washed three times in 0.05 M phosphate buffered saline containing 1% Tween 20 and 0.3% Bovine serum albumin (B.S.A.) and 0.05% Sodium azide. This latter buffer will subsequently be referred to as the incubation buffer.

Rat IgE Standard:

Highly purified rat IgE myeloma IR162 or IR2 at a concentration of 10 µg/ml in incubation buffer was diluted appropriately to give a range of IgE concentrations from 0.05 ng/50 µl to 50 ng/50 µl.

The standard dilutions, once made up, could be used repeatedly in different tests if stored at -20°C.

Dilutions of Unknown Serum Samples:

Unknown sera were diluted 10^{-1}; 10^{-2}; and 10^{-3} in incubation buffer. In the case where sera were expected to contain only a small amount of IgE, dilutions of 10^{-1} and 10^{-2} were used. For sera expected to contain larger amounts of IgE a dilution of 10^{-3} was routinely used but occasionally even higher dilutions were necessary. Dilution of unknown samples is necessary to avoid interference in the test of the high protein concentration of neat serum, and more importantly in this case, to keep IgE

levels within the range of the standard curve.

Test Proceedure:

Lukham plastic disposable tubes (LP3) were used throughout and all IgE or anti-IgE containing samples were dispensed using automatic pipettes with plastic disposable tips.

1. The tubes were labelled and arranged, one tube being set aside for total count assessment.

2. An anti-IgE coated disc was added to all except this last tube using a Pasteur pipette coupled to an aspirator, and 50 µl of incubation buffer was added to each tube.

3. 50 µl of the standard or test serum dilutions were added to duplicate tubes.

4. All the tubes, in their test tube racks, were covered with parafilm and incubated for 4 hours at room temperature with constant motion.

5. Unbound protein was removed by aspiration of the tube contents followed by three washes in incubation buffer using a 2 ml automatic syringe to dispense the buffer and a standing period of between 5 and 10 minutes per wash.

6. 0.1 ml of the labelled anti-rat IgE antibodies in incubation buffer, having a count rate of ~20,000 counts was added to each tube. The total count tube was stoppered and set aside.

7. The rest of the tubes were then covered with parafilm and incubated as in step 4, in a fume cupboard at room temperature for 1⁸ hours.

8. The tube contents were aspirated and the discs washed four times in incubation buffer as outlined in step 5.

9. Finally the radioactivity in all tubes (including the total count tube) was measured in a gamma counter, using a counting time of 1 minute/tube. An empty tube was included to assess background activity.

10. The counts per minute (c.p.m.) for all the tubes were recorded and a mean value for the duplicate tubes taken.

Calculation of Results:

The mean count rates for the standards were plotted against their IgE concentration on lin-log graph paper - IgE concentration (ng/50μl) being on the log scale. The curve obtained should be 'S' shaped. The count rates for the unknown samples were read off from the standard curve and IgE values (in ng/50μl) for the diluted sample obtained. To obtain values in ng, μg or mg/ml. the value obtained was multiplied by 20, then by the dilution factor to give the IgE concentration in the original undiluted serum sample.

Results:

The results of a rat RIST are shown in table 2 and fig. 2 (the standard curve).

TABLE 2. RESULTS OF A RAT RIST PERFORMED ON SERA FROM AN IMMUNOSUPPRESSION EXPERIMENT.

Tube No.	Contents		Dilution	Mean Counts per minute.	IgE concentration.
1	Buffer control		-	327	-
3	Standard IgE.		-	363	0.05 ng/50µl.
5	"		-	401	0.1 "
7	"		-	453	0.2 "
9	"		-	687	0.5 "
11	"		-	947	1.0 "
13	"		-	1523	2.0 "
15	"		-	4475	5.0 "
17	"		-	6940	10.0 "
19	"		-	8966	20.0 "
21	"		-	10000	50.0 "
23	Normal rat serum		1:10	5218	1.30 µg/ml.
25	Before immunisation	1.	"	2664	0.66 "
27	"	2.	"	818	0.15 "
29	"	3.	"	743	0.12 "
31	"	4.	"	686	0.11 "
33	"	5.	"	1140	0.27 "
35	"	6.	"	1276	0.31 "
37	"	7.	"	942	0.20 "
39	D.14 after	1.	"	1377	0.35 "
41	immunisation.	2.	"	1163	0.28 "
43	"	3.	1:50	682	0.23 "
45	"	4.	1:10	883	0.18 "
47	"	5.	"	1250	0.30 "
49	"	6.	"	1277	0.31 "
51	Controls	1.	"	1065	0.24 "
53	"	2.	"	3661	0.84 "

Continued overleaf

TABLE 2 (CONTINUED).

Tube No.	Contents.		Dilution.	Mean counts per minute.	IgE concentration.
55	Controls	3.	1:10	5568	1.40 µg/ml.
57	"	4.	"	3900	0.90 "
59	"	5.	"	6993	2.04 "
61	D.12 after infection.	1.	1:100	9539	59.00 "
63	"	2.	"	8214	30.40 "
65	"	3.	"	9371	52.00 "
67	"	4.	"	8887	39.00 "
69	"	5.	"	7636	25.50 "
71	Controls	1.	1:1000	7696	260.00 "
73	"	2.	"	8103	296.00 "
75	"	3.	"	7103	215.00 "
77	"	4.	"	7113	217.00 "
79	"	5.	"	9064	425.00 "
81	Total count.	-	-	20770	-

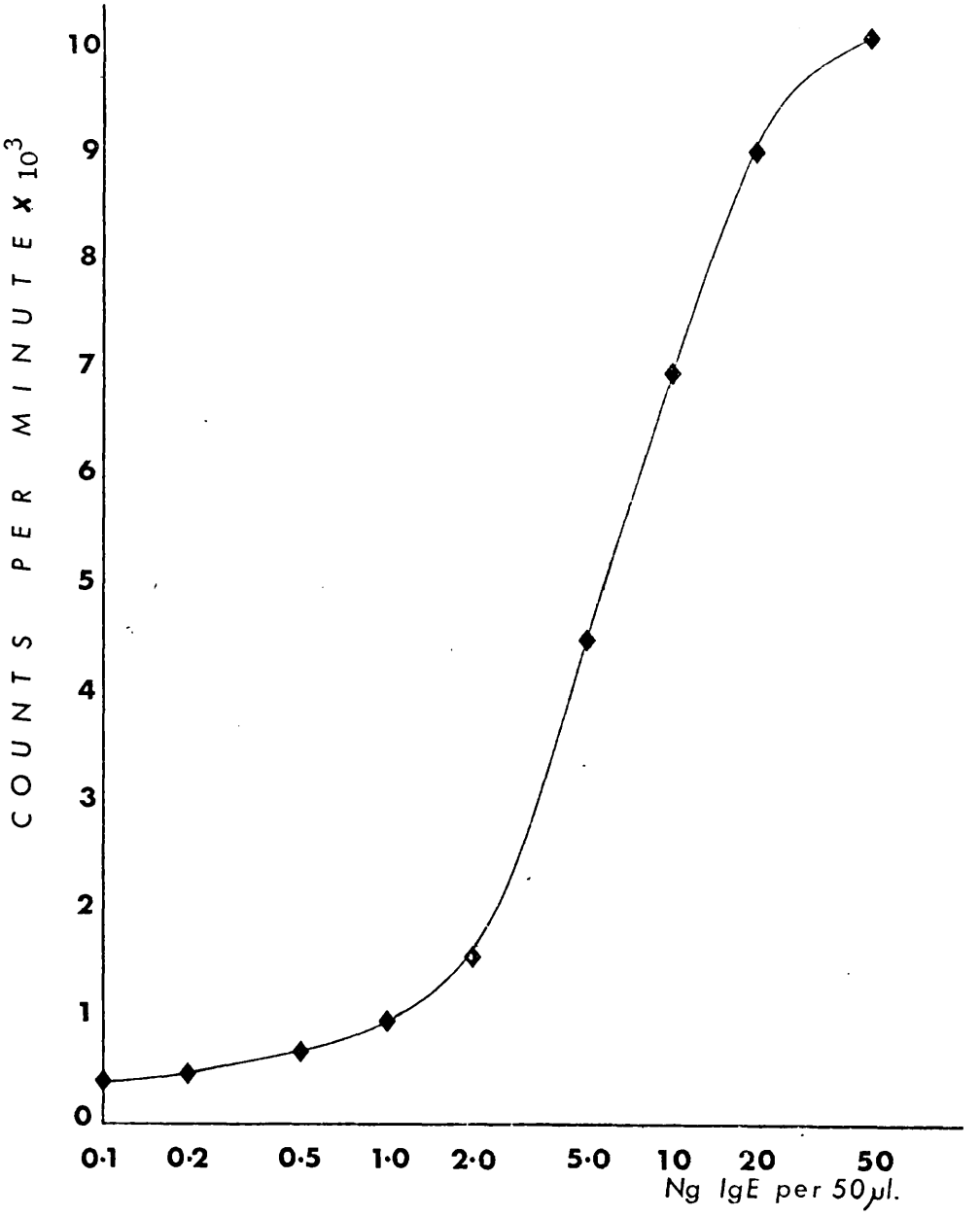


Fig 2. The rat RIST. A typical standard curve obtained by plotting the radioactivity counts for the standard IgE dilutions against the concentration of IgE in ng per 50 µl. for each dilution.

Discussion:

The radioimmunosorbent test described here differs from the human RIST in that it is a direct and not a competitive radioimmunoassay. Thus increased amounts of IgE in test samples were indicated by increased uptake of radiolabelled anti-IgE on to the paper discs. The test is very sensitive and rapid, best results being obtained when unknown sample count rates coincide with the straight portion of the standard curve (see fig. 2). As used in this laboratory, detection of IgE from a lower limit of 40 ng/ml has been achieved, and samples containing high levels of total IgE were diluted until a result coinciding with the straight portion of the standard curve was obtained. A standard normal rat serum sample was run in every test to check reproducibility.

The use of paper discs to insolubilise the anti-IgE has the advantage over particles that centrifugation is not necessary at the washing stages. In addition, the rat RIST can be performed in conjunction with the RAST (see later) the technical steps involved in both tests being identical.

(b) Detection of Total IgE by the Rowe Modification of the Mancini Single Radial Immunodiffusion Technique.

Introduction:

This test was first described for the detection of circulating levels of IgE in man (Rowe, 1969). It has the advantage that highly purified anti-IgE antibodies are not required. With the discovery of rat myeloma IgE the Rowe-Mancini test could then be applied to this experimental animal system, and until the rat RIST was developed was the routine test for the measurement of serum levels of IgE in the rat in this laboratory.

Principle of the Test:

The test as used for the detection of rat IgE is - with a few exceptions - the same as described by Rowe (1969) for the human system. A mixture of agar and diluted goat anti-rat antiserum is poured on to a glass plate. Wells are punched in the agar, and into these are micropipetted standard (myeloma IgE dilutions) or test serum samples. The plate is incubated at 37°C in humid conditions for 48 hours during which time IgE in the test or standard preparations diffuses out and combines with the antiserum in the agar to form invisible precipition rings. After washing, the plate is incubated with radiolabelled diluted rabbit anti goat globulin. After further washing, the plate is

dried out, exposed to X-ray sensitive film, which on being developed, shows ring diameters proportional to the amount of IgE in the original test sample.

Materials and Methods:

Antisera: Goat anti rat IgE serum was obtained from Dr. H. Bazin. This was raised by the injection of purified IR2 myeloma protein into goats. The antiserum obtained was absorbed with a solid immunosorbent prepared from the serum of germ free rats (Bazin, Querinjean, Beckers, Heremans and Dessy, 1974). An IgG preparation of this antiserum was obtained by the Caprylic acid technique (see Materials and Methods).

Rabbit anti-goat IgG was prepared by injecting rabbits intramuscularly with 1 mg goat IgG (prepared by the Caprylic acid technique) along with Freunds complete adjuvant. The animals were bled 14 days subsequent to a booster injection of 0.5 mg goat IgG, and an IgG preparation of the serum obtained was prepared using the Caprylic acid technique (see Materials and Methods).

Standard IgE Preparation:

Purified myeloma IR162 (Temoin) was obtained from Dr. H. Bazin. Purification of the immunoglobulin E myeloma has been described elsewhere (Bazin , Querinjean, Beckers, Heremans and Dessy, 1974). This

reference preparation was estimated to contain 5.7 mg/ml of rat IgE (Jarrett and Bazin, 1974).

Radiolabelling of Rabbit anti-goat Globulin:

Radioiodination of rabbit anti-goat globulin was by the method of McConahey and Dixon which is based on the method of Greenwood and his colleagues (McConahey and Dixon, 1966; Greenwood, Hunter and Glover, 1963). This method was employed as it is easy to carry out, efficient, and reproducible. The principle of the technique (Iodination of proteins using the Chloramine T method) has been outlined above (see Materials and Methods for rat RIST).

Proceedure:

2mC ^{125}I (carrier free preparation) in minimum volume was contained in a stoppered universal.

1. 4 mg of rabbit anti-goat globulin was mixed with 4 ml 0.05 M phosphate buffer pH 7.4.
2. The universal containing the ^{125}I was placed on ice in a fume cupboard and the antiglobulin solution injected with gentle shaking.
3. 200 µg of Chloramine T in 2 ml. buffer was immediately added dropwise with gentle shaking.

4. After a period of 5 minutes, 200 µg of sodium metabisulphite in 2 ml buffer was injected into the universal with gentle shaking.

5. The labelled protein solution was removed from the universal using a 10 ml plastic disposable syringe and aspirating needle, and then injected carefully into dialysis tubing (Visking 18/32).

6. The solution was dialysed with several changes of 0.05 M phosphate buffer pH 7.4 in the cold (4°C) for 24 hours. The resultant labelled protein solution was removed and made up to 20 ml with the above buffer plus 1% B.S.A. and stored at -20°C until required.

Rowe-Mancini test Proceedure:

8 ml aliquots of 3% Noble agar were prepared by dissolving the agar in 0.3 M phosphate buffer pH 8.0 + 0.1 M sodium azide. Goat anti-rat IgE was diluted in the range 1/100 to 1/4,000, the latter dilution giving the greatest sensitivity to the test, enabling IgE estimation in the range 0.69 to 22.24 µg/ml. 8 ml of the relevant anti-IgE dilution was mixed with an 8 ml aliquot of the dissolved agar and carefully poured on to a clean glass plate (8.5 cm. x 10 cm.) and allowed to cool. Using a perspex template, a series of wells, 2.4 mm. in diameter -12 mm apart, were punched giving a total of 42 wells/plate. The IgE standard (IR 162 Temoin) was diluted 1/256 and then doubly diluted

from this up to 1/32,76^{p} using 0.1 phosphate buffered saline (P.B.S.) pH 7.4 plus 1% B.S.A. Unknown sera of low IgE content were tested neat. Potentiated or parasite infected rat sera had to be diluted in the above buffer as otherwise precipitate formation was inhibited by antigen excess. The wells in the agar plate were now filled in duplicate with the standard dilutions and test sera. The plate was then placed in a sealed moist plastic container and incubated at 37^{o}C for 4^{p} hours, to allow precipition rings to form, followed by washing, with stirring, in several changes of 0.1 M P.B.S. pH 7.4 for a further 4^{p} hours. The plate was then transferred to a glass container and flooded with 20 ml. of ^{125}I labelled rabbit anti-goat IgG for 30 minutes. It was then removed from this antiserum and placed in a moist box and incubated at room temperature for approximately 1^{p} hours, followed by a further 4^{p} hours of washing as above. The plate was finally washed in deionised water and allowed to dry in air, then placed in contact with X-ray sensitive photographic film for 4^{p} hours and the film subsequently developed (see fig.3).

Calculation of Results:

By illuminating the film and measuring the diameters of the rings of standard samples, a plot was drawn of ring diameter against IgE concentration (Standard). The ring diameter of each unknown serum was then read off from the standard curve and IgE values calculated.

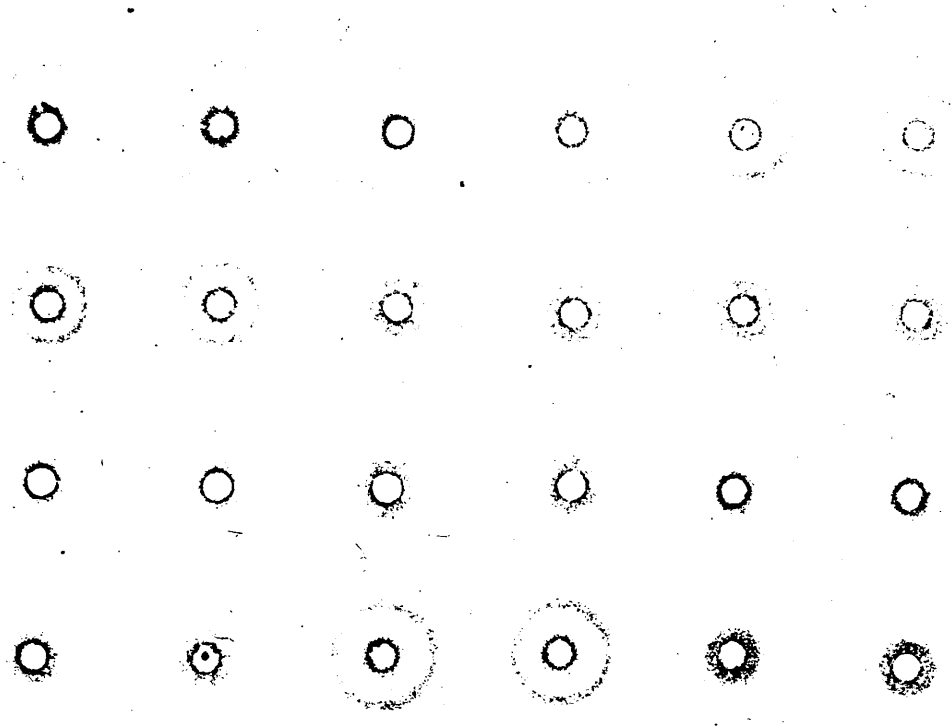


Fig 3. The above photograph shows a plate in which anti-IgE was diluted 1:2000 in the agar. The wells in rows 1 and 2 show duplicate doubling dilutions of the reference preparation starting on the left at a dilution of 1:256. the remaining rows show duplicate assay dilutions of sera from N. brasiliensis infected rats.

2. Detection of Antigen Specific IgE in the Rat

(a) The Radio Allergosorbent Test (RAST)

Introduction:

The radioallergosorbent test was first developed for the in-vitro assay of allergen specific IgE in man (Wide, Bennich and Johansson, 1967). The test is based on the antiglobulin principle of Coombs and his colleagues (Coombs, Howard and Mynors, 1953). In the human system, the test is becoming more frequently used for the clinical assessment of allergen specific IgE levels, being a preferable alternative to direct skin or provocation testing. In the human system, the test is used only in a semi-quantitative manner, distinguishing low, medium or high IgE levels to a given allergen. Owing to the lack of standardisation of many allergen extracts with respect to each other, results in this test for a group of sera can only be compared with respect to any given allergen, and not between different allergens (with any confidence). The RAST has now been adapted here to measure allergen specific IgE levels in the rat. An attempt has been made to make the test more quantitative by comparing the results from unknown sera with a standard curve constructed from values obtained with a range of dilutions of

a standard anti allergen high IgE titre serum. The standard curve consists of counts per minute against dilution of the relevant reference serum (the latter expressed on the log axis of lin-log graph paper).

Principle of the Test:

The RAST is a direct radioimmunoassay (the more IgE in a sample - the more radiolabelled anti-IgE will be bound) differing in principle for the rat RIST in that instead of anti-IgE being coupled to activated paper discs, allergen is coupled. Ideally allergen extracts should be highly purified, and standardised with respect to each other. This would increase the sensitivity and accuracy of the test, and eliminate possible cross reactivity due to impure allergen.

In the first stage of the technique, test sera and a standard (reference) high titre anti-allergen IgE serum diluted appropriately, are incubated with allergen coated paper discs in plastic tubes. After washing ^{125}I anti-IgE is added to all tubes and further incubation period allowed. The tubes are then washed free of unbound material and assessed for radioactivity using a gamma counter. The amount of radioactive antibody bound is directly proportional to the amount of IgE in the serum dilutions. Normal rat serum is included as a negative control in all tests (to assess non specific binding effects).

Materials and Methods:

1. Activation of paper discs:

The paper discs used and the activation proceedure have been described above for the rat RIST.

2. Coupling of allergen to activated discs:

A modification of the method of Axen, Porath and Ernback (1967) was used.

200 activated paper discs were incubated in 20 ml of a 1 mg/ml solution of ovalbumin (Sigma grade V) in 0.1 M bicarbonate buffer pH 9.0. For N.brasiliensis 1 ml of whole worm antigen in 20 ml of the same buffer was routinely used. The discs were kept in suspension by vertical rotation and the coupling process was allowed to proceed overnight at 4°C. Unbound allergen was removed by 3 washes in 0.5 M bicarbonate buffer pH 9.0. Remaining reactive sites on the discs were blocked by incubation for 3 hours in 0.1 M Tris buffer pH 8.0. The discs were washed 2 times in 0.5 M bicarbonate buffer pH 9.0 and then 3 times in 0.1 M Acetate buffer pH 4.0 (to deactivate the discs). Finally the discs were washed 3 times in incubation buffer (0.05 M phosphate buffered saline + 1% Tween 20 + 0.3% B.S.A. + 0.05% sodium azide). The allergen coated discs were stored at 4°C and found to be stable (in the case of egg-albumin coated discs) for 4 months. Before use the discs were washed twice with incubation buffer, to remove any protein dissociated in the interim period.

Antiserum:

The antiserum used in the RAST was the same as used in the rat RIST (see above), and the labelling with ^{125}I was also the same.

Test Proceedure:

Plastic disposable tubes (Luckham LP3) were used throughout. All IgE or anti-IgE containing material was transferred using automatic pipettes with plastic disposable tips.

1. One allergen coated paper disc was added to each tube in the test according to the protocol. A pasteur pipette coupled to an aspitator was used for this purpose. One tube was set aside for total count - receiving no disc.

2. 50 μl of incubation buffer was added to each tube followed by 50 μl of test samples and reference serum dilutions according to the protocol. All samples were run in duplicate. The reference sample was serially diluted through the range, $\frac{1}{2}$ to 1/4096. Unknown sera were also diluted as appropriate in incubation buffer.

3. The tubes in their test tube racks were covered with parafilm and strapped to vertical rotators so that rotation took place around the vertical axes of the tubes. Incubation was allowed to proceed for 4 hours at room temperature.

4. Unbound protein was removed by washing all tubes 3 times in Incubation buffer. Fluid contents were removed using a Pasteur pipette coupled to an aspirator, and buffer was added to the tubes by the use of a 2 ml. automatic syringe.

5. 0.1 ml of the ^{125}I rabbit anti-rat IgE antibody solution was added to all tubes - including the total count tube which received this alone, and was subsequently stoppered and set aside. The anti-IgE solution was previously diluted in incubation buffer to give ~20,000 counts per tube.

6. The tubes were incubated for 1° hours at room temperature in a fume cupboard (as described in step 3).

7. The radioactive anti-IgE solution was aspirated, and the discs washed four times as described in step 4.

8. The fluid contents of the tubes were aspirated, and all tubes (including the one for total count radioactivity count) were assessed for radioactivity by counting for 1 minute in a gamma counter.

Expression of results:

The results of RAST tests have been expressed as follows:

A graph was drawn of counts per minute (cpm), against log2 dilution of the relevant reference preparation (figs. 4,5). The count rates of the test sera were then read off from the standard curve to give a direct RAST score, and this was corrected by subtracting the c.p.m of the log2 dilution of each test serum. The figures which result represent the amount by which the test serum would be required to be diluted (negative figures), or concentrated to bring it to the same activity as the reference preparation. For example, in reference preparations 1 and 2 (PCA titre of

1024 - see below), unknown sera with a RAST score of 0 ought to be of PCA titre 1024.

Reference sera:

Reference serum 1: Pooled high EA PCA titre sera obtained from rats 4 days after a second antigen dose. This preparation has a low total IgE content (15.8 μg/ml). PCA titre is 1024.

Reference serum 2: Pooled high EA PCA titre sera obtained from rats initially immunised with EA and bled at the hight of a potentiated IgE response, i.e. 12-14 days after infection with N.brasiliensis larvae. This preparation has a high total IgE content (58 μg/ml). EA PCA titre is 1024, N.b PCA titre is 0.

Reference serum 3: Pooled anti N.brasiliensis PCA titre sera obtained from rats 6 days after a reinfection N.b PCA titre is 2048. Total IgE is 104 μg/ml.

Reference serum 4: Pooled high anti N.brasiliensis PCA titre sera from rats 20 days after a first and 50 days after a second infection. N.b. PCA titre is 1024 and total IgE is 20 μg/ml.

Results and Discussion:

Fig. 4 (and Appendix 1) show the results of an egg albumin RAST performed on dilutions of reference sera 1, 2 and 3. Serial doubling dilutions of the reference sera were used in the range 1:2 to 1:4096. As a control, dilutions of normal rat serum (low IgE) were also included in the test. References 1 and 2 gave good superimposable curves when count rate was plotted against log2 serum dilution (fig. 4). Reference 3, however, although containing a high total IgE level, does not contain any detectable anti-EA IgE but in the test a decreasing count rate was observed with the initial dilutions of this serum (Appendix 1). This suggests that there is a non-specific binding effect, in which unrelated IgE in the serum sample binds to the EA coupled discs. This assumption is supported by the observation that the normal rat serum (low IgE) recorded a consistent count rate throughout the dilution range employed (Appendix 1). This effect, therefore, appears to be different from the high protein concentration effect which is observed with most sera at low dilution. This latter phenomenon is identifiable as a failure to obtain a maximum count rate until the serum sample has been diluted several times. This RAST inhibitory effect, which has also been observed by Aalberse (1974), could in addition be due to the presence of IgG allergen specific 'blocking' antibodies present in the test sera, which would compete with specific IgE for sites on the allergen coated discs, and give a false low radioactive count in the test. The RAST inhibitory effect is most noticeable in

N.brasiliensis antigen and allergen RASTS (fig. 5).

Fig. 5 (and Appendix 2) show the results of an N.brasiliensis antigen and allergen RAST performed on dilutions of reference serum 3 (high anti-N.b. IgE). To assess non-specific binding effects, dilutions of the reference serum were incubated with EA coated discs. Fig. 5 shows that the N.b. RASTS are not as successful as the EA RAST. They are certainly not as reproducible. This is almost certainly due to the fact that both N.b. antigen and allergen preparations are impure, consisting of a wide array of heterogenous proteins which compete with specific allergen for coupling sites on the discs. In spite of this, certain general patterns are observed in the N.b. RASTS namely; that the serum under test has to be diluted by a greater factor to get a maximum count rate than is observed in the EA RAST. This highlights the protein and/or IgG effect discussed above. Secondly, a high degree of non specific binding occurs, as shown by the high count rates obtained when the N.b. rich IgE serum dilutions are incubated with EA coupled discs (see Appendix 2). This again could be due to the non specific IgE effect (Ref. 3 has high total IgE of 104 μg/ml). In order to compensate for this, corrected N.b. antigen and allergen RAST results were obtained by subtracting the C.p.m. of the EA discs from the values obtained for the N.b. antigen and allergen discs. These ammended results are graphically represented in fig 4 . Finally, it is invariably the case that buffer control count rates for N.b. RASTS are higher than those for EA RASTS (see Appendix 2). The

reason for this is speculative, but could be due to the impurity of the N.b. antigen on the discs facilitating, in some way, non specific binding of the labelled anti-IgE.

The results of an EA RAST performed on experimental sera is shown in Table 3. Also included, as an indication of reproducibility are the results from two tests performed some time apart on the same test sera. For the purposes of this thesis, RAST results have been expressed as a score, obtained by comparing unknown sample cout rates with count rates from a standard curve prepared using a relevant reference serum (see above). This proceedure, although useful is unfortunately quite complicated. An alternative is to present RAST results for unknown sera as the final dilution of the serum still giving a count rate above buffer control levels. This is analogous to the way in which PCA results are expressed (see below), and brings the results of the two tests into direct comparison. A standard curve would not be required for this proceedure, and instead sera of known specific IgE content could be included in each test as a check for reproducibility.

In the RAST shown in table 3, reference serum 1 was used to construct the standard curve. The specific IgE content of unknown samples can only confidently be compared with the standard if the count rate of the former lies on the straight line portion of the standard curve. Sera having count rates corresponding to the upper and lower limits of the standard curve have to be concentrated or diluted accordingly. The reproducibility of the test is only partial (see table 3).

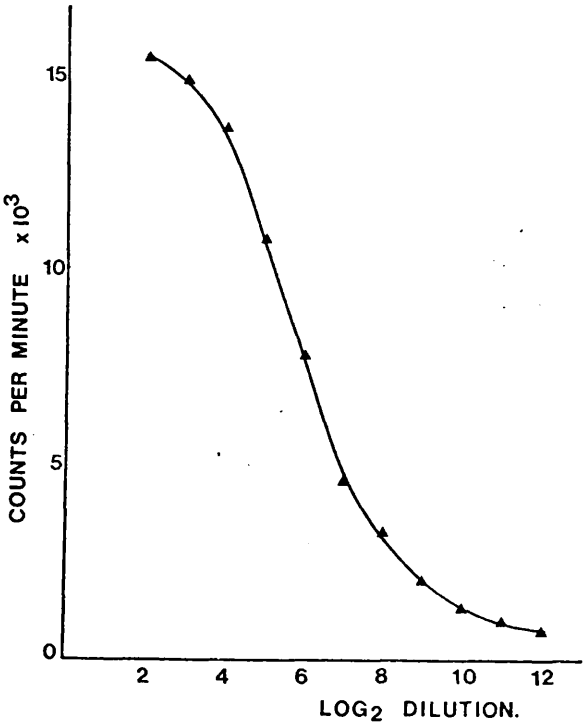
In conclusion, the rat RAST is a sensitive in-vitro tool for the assay of antigen specific IgE. The preliminary experiments published here suggest that the criteria for reproducibility are; highly purified epsilon chain specific anti-IgE, and pure allergen four coupling to activated discs or particles. Reagents (labelled anti-IgE, and to a lesser extent coupled discs), have a limited life span and many tests should be performed consequitively to take advantage of fresh material.

TABLE 3

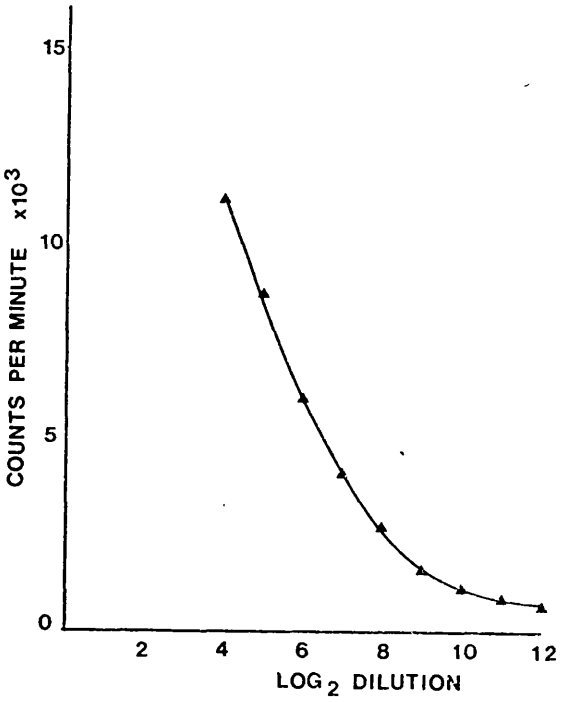
TEST SERA RESULTS FOR AN E.A. RAST (14-10-75) AND RESULTS FOR THE SAME SERA RUN IN A LATTER TEST (20-10-75) FOR A COMPARISON OF REPRODUCIBILITY.

SERUM	log2 dilution.	Mean C.P.M.	RAST Score.	Corrected RAST Score*	Corrected RAST Score (30-10-75)
Potentiation II D.14 after infection sera.	1 8	3095	8.0	0	- 0.30
	4 "	2654	8.5	0.5	- 0.90
	9 "	6671	6.3	-1.7	- 1.40
	12 "	5298	6.8	-1.2	0.35
	2 "	1554	10.0	2.0	0.00
	6 "	571	off scale	-	1.55
High anti-E.A. serum	8	4138	7.3	-0.7	- 0.20
Low anti-E.A. serum	5	6635	6.35	1.35	1.70

* (Score - log2 diln.)



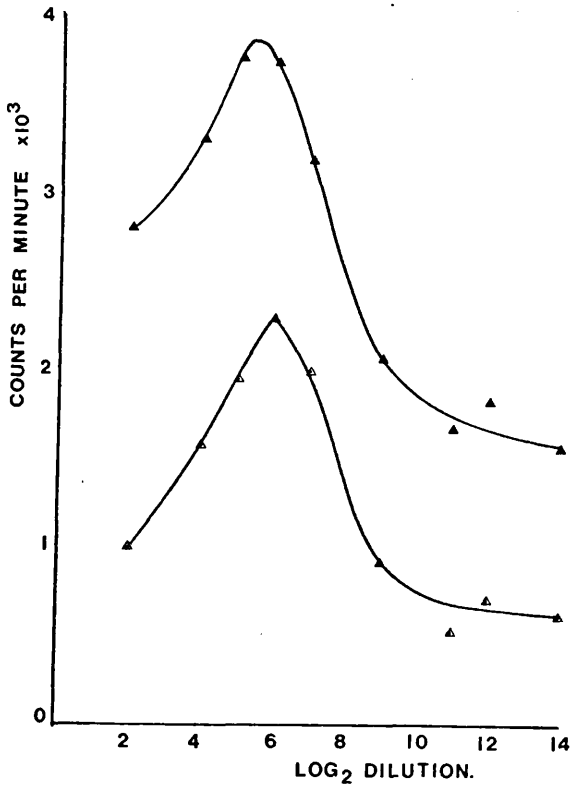
(a)



(b)

Fig.4.

Typical Standard curves obtained from an egg albumin RAST performed on reference sera 1 (fig4 a), and 2 (fig4 b). The results are tabulated in Appendix 1.



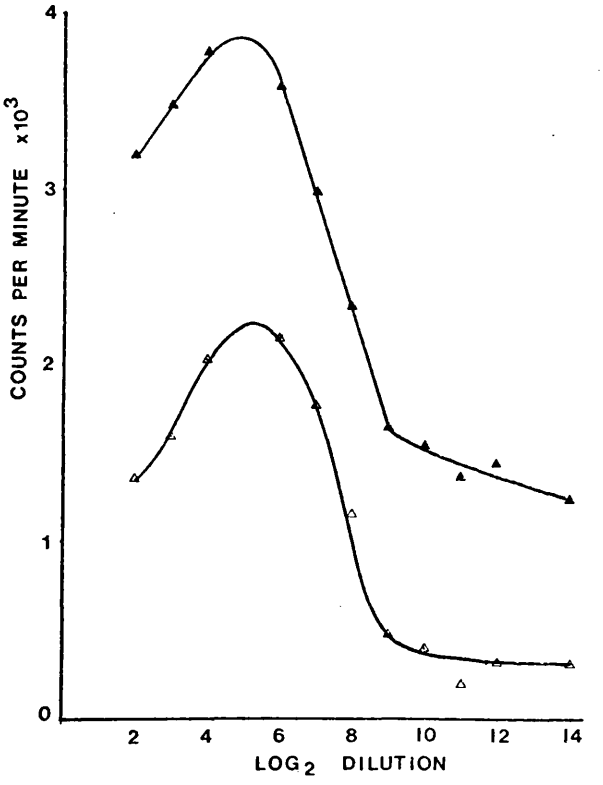


Fig 5 (a) (b)

Standard curves obtained from an N. brasiliensis antigen (a) and allergen (b) RAST. The upper curve in each case (filled triangles) represents unmodified results, whereas the lower curves (hollow triangles) represent results modified by subtracting, from these, C.P.M. values obtained by incubating reference serum dilutions with egg albumin discs (for tabulated results, see Appendix 2).

(b) The Passive Cutaneous Anaphylaxis Test(PCA)

Introduction:

The passive cutaneous anaphylaxis test (PCA) was first described by Ovary (1955; 1964), and is an in vivo quantitative test for the detection of allergen specific IgE. Unlike the RAST, which detects IgE based only on its antigen binding capacity, PCA detects specific IgE by means of both its tissue sensitising and antigen binding capacities. The test is essentially an adaption of the P.K. test (see Introduction to this section). Recipient untreated animals are passively sensitised by the intradermal injection of aliquots of test serum dilutions into skin sites on the shaven back or sides of the animal. A latent period of 48 to 72 hours is then allowed to elapse so that IgE can bind to target cells (mast cells) and all other proteins can diffuse away. The recipient animals are then challenged intravenously with the relevant allergen solution in excess, along with a high molecular weight dye (e.g. Evans blue). The antigen will combine with any IgE bound to mast cells, and evoke a local anaphylactic response resulting in vasodilation and a leakage of the dye into surounding tissue space. This reaction is usually complete within 20 minutes. Thus a blue spot at the skin site of initial sensitisation indicates the presence of IgE antibodies in the original serum samples. The PCA titre is expressed as the reciprocal of the highest serum dilution, giving a positive reaction, conven-

tionally defined as that in which a blue spot of greater than 5 mm occurs. Recipient animals for PCA should generally be of the same species as the serum sample source, because IgE only binds to homologous tissue. Occasionally, closely related species can be used as recipients, e.g. rats for mouse serum or baboons for human serum.

PCA is the most widely used test for the assay of specific IgE in the experimental animal system. This is due to its simplicity and reproducibility, and also because the RAST has only been developed for the human and more recently the rat systems.

Test Proceedure:

Serum samples under test were diluted in 0.85% saline by serial doubling dilutions. 0.1 ml aliquot were then injected intradermally into sites on the shaved back of a recipient Hooded Lister rat. Up to ten such injection sites could be used on any one animal - three down each side and four down the midline of the back. Each sample was run in duplicate on another recipient rat to allow for variations between the two animals. A plan of the injections was kept. In performing the above step, the rats were anaesthetised in trichloroethylene and their backs shaved using electric clippers. Intradermal injections were achieved using 26 guage x $\frac{3}{8}$" needles and 1 ml plastic disposable tuberculin syringes.

The rats were then left for a period of between 24 and 72 hours then challenged with relevant antigen solution and Evans blue dye. Intravenous challenge was achieved by injection into a tail vein which had been dilated by immersion of the tail into warm water for several seconds. The antigens used commonly in the following experiments were egg albumin, and N.brasiliensis antigen. For PCA intravenous challenge, 2.5 mg of EA in 0.25 ml saline was found to be sufficient. The antigen solution was given along with 0.5 ml of a 1% solution of Evans blue dye in the same syringe. 0.5 ml of a solution of N.brasiliensis antigen (1,000 worm equivalents/ml) in saline was routinely used along with 0.5 ml of a solution of 1% Evans blue dye. For this stage, 25 guage x 1" needles and the same syringe type as above were used.

The rats were left for 20-30 minutes after intravenous allergen challenge to allow reactions to develop to a maximum. The rats were then killed and the reactions recorded. Reactions were scored as either positive (+) or negative (-) after studying the inside surface of the skin, if necessary, since small reactions can be more easily seen on the undersurface of this side.

In the PCA test, it is essential to ensure that injection of serum dilutions is intradermal at the passive sensitisation stage, and that the animals are not allowed to come into direct skin contact with trichloroethylene at the intravenous challenge stage as this causes non-specific blueing of the skin, since the anaesthetic has an irritant effect.

SUMMARY

Various methods employed for the quantification of IgE are discussed, and detailed accounts are given of the techniques used in this laboratory.

Total IgE levels in human sera have been estimated using the Phadebas® radioimmunosorbent test (RIST). This is a competitive radioimmunoassay in which IgE in a sample competes with radiolabelled IgE for sites on sephadex-anti-IgE particles. The usefulness and limitations of the test are discussed. A rat radioimmunosorbent test (RIST), and the Rowe modification of the Mancini single radial diffusion test are both described in detail. Both tests measure total IgE levels in rat sera. The rat RIST is a direct 'sandwich' radioimmunoassay in which IgE in a sample binds to anti rat IgE bound to activated paper discs. Finally addition of radiolabelled anti-rat IgE acts as a marker for the amount of sample IgE bound. RIST is more sensitive than the Rowe-Mancini test, detecting IgE levels as low as 10ng/ml. Technical aspects of the tests are discussed in detail.

Antigen specific rat IgE is measured by the radio allergo-sorbent (RAST) and passive cutaneous anaphylaxis (PCA) tests. The RAST is based on the same principle as the rat RIST, but antigen instead of anti-IgE is bound to activated paper discs. The RAST has been used in this laboratory to detect IgE directed against egg albumin and N. brasiliensis antigens, and of the two systems the egg albumin RAST is the more efficient, N. brasiliensis RASTS being hampered by lack of pure antigen preparations. /

The RAST was found to be only partially reproducible, but extremely sensetive. The PCA test is the test of choice in this laboratory for the quantification of antigen specific IgE, and is discussed in detail in this section.

S E C T I O N I I

IgE PRODUCTION IN THE RAT

Introduction:

A rat reaginic antibody, originally called 'mast cell sensitising antibody' was first described by Mota (1963). The quantities of this antibody produced in response to injection of various antigens, given with adjuvant, were small as measured by PCA, the best results being obtained in rats immunised with egg albumin and Bordetella pertussis (Killed organisms) as adjuvant (Mota 1963; 1964 a,b).

Properties:

Rat anaphylactic antibody exhibited properties similar to reaginic antibody previously described in man (see general introduction), namely: heat lability at 56°C; a sedimentation coefficient of between 7S and 19S, and migration as a γ globulin in zone electrophoresis. In vivo, its distinguishing feature was strong skin fixation associated with a high affinity for mast cells (Binaghi, Benacerraf, Bloch and Kourilsky, 1964; Mota 1964 a,b).

Sugsequent studies of rat reaginic antibody by Stechschulte Orange and Austen (1970) showed that specific antiserum to rat homocytotropic antibody failed to recognise rat IgGa-IgGb-IgA, IgM or γ I, but precipitated an electrophoretically fast immunoglobulin with antigen binding capacity. This antiserum, used as an immunoabsorbent, removed the biological activity attributed to rat homocytotropic antibody, as measured by PCA inhibition. The unique immunoglobulin class of rat homocytotropic antibody

was designated rat IgE.

Production:

The rat has latterly been used by several groups as a model for the study of the production of IgE, despite some obvious disadvantages, namely; the apparent requirement for large doses of antigen, the transient and quantitatively poor nature of the IgE response and the inability to evoke significant booster responsees. Those features, originally described by Mota (1964a and Binaghi and Benacerraf (1964), were the general experience of subsequent workers.

Recently, however, it has been discovered that strain variations occur in the rat for the production of this Immunoglobulin, similar to those previously shown by Levine and his colleagues for mice (Levine and Vaz, 1970; Vaz, Vaz, and Levine, 1971). Thus it has been discovered that the Hooded Lister rat is a particularly good producer of reaginic antibody following injection of small doses of antigen, capable also of a marked secondary response following antigen challenge (Jarrett and Stewart, 1974). Murphey and his colleagues (1974) also report variations in IgE production in different rat strains tested.

Recently Bazin and co-workers discovered a strain of rats producing spontaneous and transplantable Immunocytomas (Bazin, Beckers, Deckers and Moriame, 1973). Eight per cent of all these Lou/Wsl rats produce IgE secreting tumours and IgE levels of up

to 50 to 60 mg/ml have been found in these animals (Bazin, Guerinjean, Beckers, Heremans and Dessy, 1974). As a result, it has been possible to purify rat IgE and to raise specific antiserum against the purified antigen. The availability of these reagents makes possible the various in vitro assays for the estimation of total and specific IgE.

These two factors, namely the availability of good IgE producing rats and of anti-rat IgE, have added great potential to the study of IgE production in experimental systems and have made the rat perhaps the most appropriate species for such studies.

Control of IgE Production in the Rat

Tada and Okumura (1971) have studied the control of IgE production in Wistar rats using as standard immunisation technique a footpad injection of Ascaris suum - D.N.P. and intraperitoneal injection of Bordetella pertussis organisms (killed) as adjuvant, followed five days later by a booster dose of antigen alone. In their rats specific IgE levels disappeared rapidly (35 to 40 days) and could not be boosted by a subsequent dose of antigen. In a series of experiments they found that it was possible to inhibit or enhance the production of IgE antibodies by a variety of manoeuvres. Thus passively administered antigen-specific IgG antibodies were found to suppress the IgE response (Tada and Okumura, 1971) as has also been reported for rabbits (Strannegard and Belin, 1970) and mice (Ishizaka and Okudaira, 1972). On

the other hand, enhancement of the IgE response was reported to occur following a non lethal dose of X-irradiation (Tada, Taniguchi and Okumura, 1971), or following the administration of a variety of immumosuppressive drugs such as cyclophosphamide, cortisone or actinomycin D (Taniguchi and Tada, 1971), or small doses of anti-lymphocyte serum (A.L.S.) (Okumura, Tada and Ochiai, 1974).

The broad interpretation of these enhancing effects was that, as a result of these various treatments, there occurred a selective depletion of certain populations of lymphoid cells, probably of thymic origin, which regulated the IgE response. Further work confirmed the regulatory effect of T cells on the IgE response. Both whole cells (Okumura and Tada, 1971 b) or cell free extract (Tada, Okumura and Taniguchi, 1973 a) were able to exert an inhibitory effect on an IgE response, the effect being carrier specific. A T cell factor was subsequently extracted from mechanically disrupted thymocytes of rats hyperimmume to Ascaris-suum - D.N.P. which showed a strong inhibitory effect on anti-hapten IgE responses of X-irradiated rats. This factor was thought to be protein with a smaller molecular weight than other immunoglobulins, and possibly a unique receptor of T cells. An antigen specific T cell factor which augmented anti-hapten IgE responses was also described (Okumura and Tada, 1974). This soluble component when injected into neonatally thymectomised rats, restored their ability to produce both reaginic and haemagglutinating

anti-D.N.P. antibodies. Unlike the inhibitory component, the helper component was described as possessing both Fab and μ chain determinants and was thought to be an IgT-like molecule. On the basis of these findings, Tada and his colleagues postulate that reagin formation in the rat is regulated not only by an IgG feedback effect, but also by two distint antigen specific sub-cellular components of thymus derived lymphocytes.

The events triggering helper or suppressor activity are still unclear but the effect of dose of antigen is clearly of paramount importance. Jarrett and Stewart (1974) have described the effect of antigen dose on primary and secondary reaginic antibody responses in Hooded Lister rats. A primary response could be induced with doses ranging from 1 mg to 1μg of egg albumin injected intraperitoneally with B.Pertussis adjuvant. The level of the primary response was unaffected by the amount of antigen given, as measured by PCA. A sensitising dose of 0.1 μg antigen, although not inducing a detectable primary response, did however prime rats for a secondary response on challenge with antigen alone. A secondary response could be evoked by antigen doses in the range 100 to 0.001 μg egg albumin injected I.P. without adjuvant. The level of the secondary response was found to be determined by the amount of antigen given on the first occasion and was not influenced by the size of the second dose of antigen. Rats receiving small sensitising doses of antigen (e.g. 1 μg) gave significant secondary responses, whereas rats receiving large

sensitising doses of antigen did not. Injection of a challenging antigen dose in excess of 100 μg produced fatal anaphylactic shock in most animals. The primary response was long lived, whereas the secondary response was of short duration. Tertiary booster responses could not be obtained:

The authors explain the above events, namely inhibiting effect of high primary doses of antigen on the secondary reaginic response, the rapid decline of the secondary response and the absence of a tertiary response, in terms of the action of suppressor T cells or γG feedback inhibition or a combination of the two. There is a possibility also that the Hooded Lister rat may have a lesser ability to produce T suppressor cells than Wistar rats, which could explain the greater production of IgE antibodies in rats of this strain.

Other Homocytotropic Antibodies in the Rat

As well as IgE, the rat has also been shown to produce a γG subclass homocytotropic antibody - IgGa or IgG2a (see Bloch and Ohman, 1971). This antibody class can be detected by PCA provided a short (usually four hour) latent period between passive intradermal sensitisation and antigen challenge is allowed. The serum under test can be depleted of IgE antibodies by heating at 56°C for two hours before use.

IgGa appears to bind to mast cells and basophils with a much lower affinity and for a shorter time than IgE. It has been

shown to have a half life in circulation of about five days as compared with 12 hours for IgE, and persists at homologous skin sites for a half life period of 2.4 days, as compared with IgE having a half life of 7.4 days (Tada, Okumura, Platteau, Beckers and Bazin , 1975). The authors postulate that IgGa as well as having a different affinity for target cells (e.g. mast cells), has also a different mode of sensitisation. IgGa fixes complement (Jones, 1969) and complement mediated release of pharmacologically active compounds of immediate hypersensitivity (e.g. SRS-A) from target cells could be the mode of action of this antibody class. (Orange, Valentine and Austen, 196°).

The importance of this immunoglobulin moeity is as yet undefined, and by comparison with IgE it seems to play a minor role as a homocytotropic antibody.

In the work to be reported in this section, the production of reaginic (IgE) antibody in the rat has been studied in the following system:

Production of IgE antibodies following administration of protein antigen.

REAGINIC ANTIBODY RESPONSES TO INTRADERMAL AND ORAL ADMINISTRATION OF EGG ALBUMIN IN THE RAT

Introduction:

In the rat and mouse, intraperitoneal injection of antigen (accompanied by suitable adjuvant) is the most widely used route for the induction of reaginic antibody. Work with various strains of mice, (Levine and Vaz, 1970; Vaz, Vaz and Levine, 1971) and rats (Jarrett and Stewart, 1974) has shown that, when animals are immunised by this route, administration of a small antigen dose is perhaps the most important condition for a sustained and high level IgE antibody response (see introduction to this section). However, the intraperitoneal route is not a natural route of entry for antigen. In the experiments that follow, IgE production has been studied in rats after oral administration and intradermal injection of antigen. These are thought to be routes whereby sensitisation to environmental allergen may occur in man.

Preliminary experiments showed that in rats sensitised with egg albumin and adjuvant, by the intraperitoneal route, intradermal challenge with egg albumin (without adjuvant) resulted in a booster IgE response (Jarrett and Stewart, unpublished results). McDougall (1974) found that Hooded Lister rats could produce both primary and booster IgE responses to egg albumin administered orally in drinking water at a concentration of 100 µg/ml - the animals being allowed to drink ad lib for periods between

24 and 48 hours. In various experiments each animal was calculated to have consumed quantities of water containing between 2 and 5 mg of egg albumin. This was however, thought to be an over-estimate of the amount of antigen actually taken in, since the amount lost by denaturation or spillage of water was not assessed.

In the experiments described here, the minimum dose of antigen necessary to evoke both primary and booster IgE responses, by the intradermal and oral route, was sought. Wistar rats were incorporated into two experiments to compare strain difference.

Materials and Methods:

Antigen administration:

Egg albumin (Sigma gradeV) freshly prepared to a concentration of 10 mg/ml in saline was diluted so that the appropriate dose was contained in 0.5 ml. Intradermal injections were given in 5 sites on the shaven back of the rat, 0.1 ml being injected into each site. A 1 ml plastic disposable syringe and a 26 guage x $\frac{3}{8}$" needle were used for this purpose. In all the following experiments 10^{10} B.Pertrussis organisms in 0.5 ml saline were given intraperitoneally as adjuvant at the same time as the sensitising dose of antigen. For oral immunisation, the desired dose of egg albumin was administered in 0.5 ml saline by stomach tube. This consisted of a 3 cm length of nylon intravenous

cannula (Portex Ltd., Hythe, Kent) 1.65 mm in diameter, attached to a 1 ml. plastic disposable syringe. In all experiments adjuvant was given in the same manner as described above for intradermal immunisation.

In some of these experiments rats were deprived of food, but not water, for 24 hours prior to oral administration of antigen.

The time when the rats in these experiments were bled was determined by previous experience with intraperitoneal immunisation which showed that primary responses appeared around 10 days after immunisation and persisted for 2-3 months. Secondary responses reached peak levels 4 days after challenge and thereafter declined rapidly to former levels (Jarrett and Stewart, 1974).

IgE Produced in the Rat after Intradermal Injection of Antigen.

The following initial experiment was designed to find the lowest dose of egg albumin which could evoke IgE production.

Experiment 1:

3 groups of 6 animals were immunised with 1 mg 100 μg, and 10 μg egg albumin respectively and then challenged after 30 days with 0.1 μg. Both doses were given intradermally and B.pertussis was administered with the first dose but not with the second.

Results:

The results for this experiment are shown in table 4. A primary reaginic (IgE) antibody response was detected in animals from all 3 groups. The magnitude of this response was not dose dependent. Following antigen challenge, however, significantly elevated secondary IgE responses occurred in the animals of groups 2 and 3 (p 0.05 and 0.01 respectively), but not in those of group 1 (to the same extent). This conclusion was supported by χ^2 and paired T-tests.

This experiment demonstrated that 10 µg of E.A. injected intradermally could evoke and IgE response.

Experiment 2:

In order to test the efficacy of even smaller doses of EA in evoking both primary and secondary IgE responses, the experiment set out below was performed:

Group	No. of rats	Amount of EA injected intradermally			
		1st dose (day 0)		2nd dose (day 31)	3rd dose (day 60)
1	16	10 µg	1a	10 ng	1 ng
			1b	1 ng	1 ng
2	15	1 µg	2a	10 ng	1 ng
			2b	1 ng	1 ng

TABLE 4

REAGINIC ANTIBODY RESPONSE AFTER INTRA DERMAL INJECTION OF EGG ALBUMIN(EA) SENSITISING DOSES RANGING FROM 1mg to 10µg, CHALLENGE DOSE 0.1 µg.

EA PCA titres 13 days after 1st dose and 4 days after 2nd dose of EA.

Rat No.	Group 1		Group 2		Group 3	
	1st dose 1 mg.	2nd dose 0.1 µg.	1st dose 100 µg.	2nd dose 0.1 µg.	1st dose 10 µg.	2nd dose 0.1 µg.
1	0	8	256	512	64	64
2	128	512	64	128	64	64
3	256	128	32	128	32	512
4	32	256	32	128	16	128
5	8	64	0	0	256	2048
6	128	512	128	512	4	512

Table 5

REAGINIC ANTIBODY RESPONSE AFTER INTRADERMAL INJECTION OF EA SENSITISING DOSES OF 10 or 1 μg, CHALLENGE DOSES OF 10 or 1 ng.

* Antigen injected on days 0, 31, and 60.

Treatment of rats			EA PCA titres of 8 rats.							
Group	Ag doses injected*	Days bled after each dose.	1	2	3	4	5	6	7	8
1a	1st 10 μg	12	8	128	8	0	4	32	8	0
	2nd 10 ng	4	512	2048	64	4	256	512	512	128
		26	32	64	0	0	32	32	8	2
	3rd 1 ng	4	512	128	0	1	64	16	8	64
1b	1st 10 μg	12	8	1	32	128	0	8	0	16
	2nd 1 ng	4	512	16	512	512	8	512	64	128
		26	32	1	128	128	2	128	2	16
	3rd 1 ng	4	512	8	512	256	8	256	512	256
2a	1st 1 μg	12	0	8	0	0	4	0	4	
	2nd 10 ng	4	0	512	128	2	512	64	1024	
		26	0	16	8	0	16	2	8	
	3rd 1 ng	4	0	Died	256	2	4	128	64	
2b	1st 1 μg	12	8	0	0	1	0	0	0	0
	2nd 1 ng	4	512	32	128	128	0	8	0	4
		26	2	0	0	2	0	0	0	4
	3rd 1 ng	4	1024	2	512	256	8	64	0	64

Results:

The results for experiment 2 are shown in Table 5. The most important feature of the primary IgE response was that it was detectable in 12 of the 16 animals in Group 1 immunised with 10 μg, but in only 5 of 15 animals of Group 2 immunised with 1 μg. Secondary responses not only in the rats which had previously produced reagins, but also in the majority of those which had not. By 26 days after challenge the IgE levels had declined again or had disappeared so that the overall picture in each group resembled that of the primary response. 26 days after the dhallenging dose of EA, PCA titres had declined in all groups to values similar to those of the primary response. This was most marked in animals in groups 1b and 2b, which had been challenged with 1ng EA on both occasions. In these groups, there was a significant difference (both groups p 0.025) between PCA titres 26 days after 1st challenge and 4 days after 2nd challenge. In the other groups, the IgE response was not significantly different.

As part of this experiment, a control group of Wistar rats were included in groups 1 and 2, and all animals challenged with 10 ng EA. It had been expected that Wistar rats would not produce IgE to such low sensitising and challenging doses of egg albumin - as is the case with these rats immunised by the intraperitoneal route (Jarrett and Stewart, 1974). The results for this part of experiment 2 are tabulated in Table 6, and contrary to expectation, both groups of Wistar rats gave an

TABLE 6

REAGINIC ANTIBODY PRODUCTION AFTER INTRADERMAL INJECTION OF EA IN WISTAR RATS.

EA PCA titres 14 days after 1st and 4 days after 2nd dose of antigen.

Rat No.	Group 1.		Group 2.	
	1st dose 10 µg.	2nd dose 10 ng.	1st dose 1 µg.	2nd dose 10 ng.
1	32	512	4	512
2	16	512	1	512
3	32	128	0	64
4	32	128	8	512
5			16	128

IgE response. In fact, there were more responders in the 1μg EA sensitised group of Wistar rats than in the corresponding Similarly treated group of Hooded Listers (compare with Table 5). Following a challenging dose of 10 ng EA, both groups of Wistar rats produced good booster IgE responses.

This experiment prompted a more detailed study on the capacity of Wistar strain rats to produce IgE following intradermal administration of antigen. The next experiment was designed to compare IgE production in Wister strain rats following intradermal and intraperitoneal antigen administration.

Experiment 3:

The individuals of two groups of Wistar rats were immunised with 10μg EA and adjuvant, one group by intradermal and the other by intraperitoneal injection of antigen. All rats were bled as described in Table 7. The challenging dose of antigen was given 29 days after initial immunisation.

The results for this experiment are shown in Table 7. These confirm that the intradermal route of antigen presentation is more favourable to the production of IgE than the intraperitoneal route in Wistar strain rats. Following the first dose of antigen, there was only one responder in the intraperitoneally treated group of rats, whereas all intradermally sensitised rats responded well. Following the second dose of antigen,

TABLE 7.

COMPARISON OF INTRA DERMAL AND INTRA PERITONEAL ROUTES OF ANTIGEN(EA) INJECTION FOR REAGIN PRODUCTION IN WISTAR RATS.

EA PCA TITRES 12 DAYS AFTER 1st AND 4 DAYS AFTER 2nd DOSE OF ANTIGEN.

Rat No.	Group 1 intradermal antigen		Group 2 intraperitoneal antigen	
	1st dose 10 µg.	2nd dose 1 µg.	1st dose 10 µg.	2nd dose 1 µg.
1	8	128	0	32
2	64	2048	0	64
3	4	64	32	64
4	32	128	0	64
5	4	256	0	64
6	32	128		32

booster IgE responses were observed in animals from both groups - group 1 animals, however, having more elevated IgE levels than those of group 2.

IgE Production in the Rat Following Orally Administered Antigen.

In the first experiment in this series a wide range of antigen dosees were administered since it was expected that considerably higher doses might be required for IgE stimulation by the oral route.

Experiment 4:

5 groups of rats were immunised with doses of EA increasing by tenfold increments from 10 μg to 100 mg inclusive, given by stomach tube. The animals were deprived of food (but not water) for 24 hours prior to immunisation. All animals were bled on the days shown in Table 8 following sensitisation and challenge. The rats were given the 2nd dose of EA 31 days after the initial sensitising dose.

The results of this experiment are shown in Table 8. Some animals in each group responded to the initial dose of antigen. The magnitude of this primary IgE response was not dose dependent. Following challenge with 100 μg EA booster IgE responses were observed in animals from groups 3, 4 and 5, the latter group of rats having the most elevated responses. Significant booster

TABLE 8

REAGINIC ANTIBODY TO ORALLY ADMINISTERED EGG ALBUMIN: SENSITISING DOSES RANGING FROM 100 mg to 10 µg, CHALLENGE DOSE 100 µg.

Rat No.	Group 1 100 mg	Group 2 10 mg	Group 3 1 mg	Group 4 100 µg	Group 5 10 µg
	PCA titres 12 days after these doses of EA				
1	8	0	0	8	0
2	0	1	4	1	0
3	32	8	8	0	0
4	0	0	0	1	8
5	0	8	16	0	8
6	0	4	2	0	1
7	2	8	0	0	0
8	16	128	128	0	0
	PCA titre 4 days after challenge of all above rats with 100 µg EA.				
1	0	0	0	128	128
2	0	0	8	128	64
3	128	32	512	64	512
4	0	0	2	64	1024
5	0	16	128	0	128
6	0	4	128	16	512
7	0	32	8	0	512
8	256	32	32	512	0

responses were not observed in animals from groups 1 and 2, which indicated an inhibitary effect of high sensitising antigen dose on secondary responses. This is in agreement with results for intraperitoneal administration of antigen (Jarrett and Stewart, 1974)

In the next experiment, the effect on IgE responses of a 3rd antigen dose was looked for.

Experiment 5.

The experimental protocol is outlined below:

	1st Dose EA	2nd Dose EA	3rd Dose EA
Group 1	1 mg	10 μg	10 μg
Group 2	100 μg	10 μg	10 μg
Group 3	10 μg	10 μg	10 μg

All animals were deprived of food, but not water, for 24 hours prior to immunisation.

The results for this experiment are shown in Table 9. Following initial immunisation, IgE production was evoked in some rats in each group. The magnitude of the response was not dose dependent. After administration of the second antigen dose, significantly boosted IgE levels were found in rats in groups 2

TABLE 9

REAGINIC ANTIBODY RESPONSE TO ORALLY ADMINISTERED EGG ALBUMIN: SENSITISING DOSES RANGING FROM 1 mg to 10 μg CHALLENGE DOSE 10 μg.

PCA titres 12 days after the 1st dose and 4 days after the 2nd and 3rd doses.

Group 1 1st dose 1 mg challenge doses 10 μg*			Group 2 1st dose 100 μg challenge doses 10 μg			Group 3 1st dose 10 μg challenge doses 10 μg		
1st response	2nd response	3rd responce	1st response	2nd response	3rd response	1st response	2nd response	3rd response
0	1	32	0	0	0	16	128	512
0	2	2	8	128	512	16	32	512
1	0	0	1	128	64	4	128	512
64	32	64	0	32	128	0	128	128
8	4	8	4	128	16	2	64	256
0	2	1	16	128	512	8	512	512

* Rats challenged 30 and 51 days after initial immunisation.

TABLE 10

REAGINIC ANTIBODY RESPONSE AFTER ORAL ADMINISTRATION OF EA SENSITISING DOSE 10 μg or 1 μg*, CHALLENGE DOSE 10 μg or 1 μg.

PCA titre 12 days after 1st and 4 days after 2nd dose EA.

Rat No.	1st response to 10 μg.	2nd response to 10 μg	1 μg
1	0	0	
2	0	0	
3	0	128	
4	8	128	
5	2	128	
6	0		16
7	0		32
8	0		0
9	0		2
10	0		16

* Rats immunised with 1 μg EA orally did not produce detectable reagins either as aprimary response or after challenge with the above doses.

and 3. This was not the case for group 1 animals where the high initial antigen dose was presumably inhibitory to a secondary response. Following administration of the 3rd antigen dose, IgE levels were again raised in rats in groups 2 and 3, the latter group of animals having higher IgE levels, than following 2nd dose of antigen.

Experiment 6:

2 groups of 10 rats were given 10μg EA and 1μg EA respectively, split into sub-groups and challenged with 10μg and 1μg EA. The challenging dose was given 27 days after initial immunisation. The results for the experiment are given in Table 10. It is evident from these results that an oral dose of 1μg was insufficient to sensitise rats for IgE production although it could evoke secondary responses in rats previously immunised with 10μg EA.

Discussion:

This series of experiment demonstrates that Hooded Lister rats can be provoked to produce IgE when immunised by oral or intradermal routes.

The characteristics of the primary and secondary reaginic responses were similar to those already described for intra-

peritoneal immunisation (Jarrett and Stewart, 1974). IgE antibodies were present 12 days after the primary intradermal or oral administration of antigen and simultaneous injection of B. Pertussis adjuvant, and the response persisted for at least one month. Secondary responses, the peak levels of which occurred four days after re-stimulation with antigen alone, were characterised by the appearance of IgE antibodies in the serum of animals previously negative, or the elevation of existing levels of the primary response. An incidental finding was that Wistar rats which by comparison with Hooded Lister rats were not thought, by previous criteria, to be a good IgE producing strain (Jarrett and Stewart, 1974), appeared to be more easily sensitised by the intradermal than the intraperitoneal route (Table 7).

In the intradermal immunisation experiments it was found that some rats could produce IgE to a sensitising EA dose as low as 1μg, and that on challenge, good secondary and tertiary responses were produced by these animals. Initial doses of EA of 100μg and more were found to be inhibitary to booster IgE production following antigen challenge. This inhibitary action of high sensitising doses of antigen on secondary IgE responses following antigen challenge is in agreement with earlier findings (Jarrett and Stewart, 1974) for intraperitoneal immunisation. The smallest challenge dose of EA given intradermally was 1 ng - and this was found to evoke a booster IgE response. Smaller doses were not tried.

Following oral administration of egg albumin, rats were found to produce IgE to sensitising doses as low as 10μg EA. Good secondary and tertiary responses were obtained in rats sensitised with the above dose of EA and subsequently challenged. Secondary IgE responses following antigen challenge were not obtained in animals which had been sensitised to doses of EA of 1 mg and more. A challenging dose of 1μg EA was found to evoke secondary IgE responses in animals initially sensitised with low doses of EA. Lower challenging doses were not tried. It is important to point out that the intradermal experiments performed in this laboratory have been reproducible, whereas only 50% of all the oral route of antigen administration experiments have been successful. Failed experiments have been characterised by rats not producing IgE following immunisation with any of the doses of EA in the range used (100 mg to 10μg). The reason for failure in this system is not known, but does not seem to depend on whether or not the animals are fasted prior to immunisation. Other experimental conditions have been to the best of our knowledge, kept constant. The experiments that have been successful, however, do give a clear reproducible pattern.

From the results of these experiments it can be seen that the intradermal route of presentation of antigen requires less antigen to evoke an IgE response at both sensitising and challenging stages than the oral route. This could be explained by the fact

that orally administered antigen is subjected to various denaturing processes in the stomach and intestine before being absorbed into or across the intestinal epithelium and thus evoking an immunological response. It is probable that the administered dose of antigen is much in excess of the actual dose of antigen which is absorbed across the intestinal epithelium. By the intradermal route, a lesser loss of material would be expected.

By contrast with the experiments involving intraperitoneal immunisation (Jarrett and Stewart, 1974) it was found possible to produce tertiary responses, (i.e. following a third dose of antigen) in rats immunised and challenged by the intradermal or oral routes. This difference may not in fact be a function of the site of antigen presentation but of the quantity of antigen which reaches the appropriate immunocompetent cells. It is probable that intraperitoneal immunisation results in a relatively greater proportion of the administered dose being made available for stimulation and inhibition of IgE responses than when it is given by the other routes. Orally administered antigen in particular could be expected to be substantially degraded by the fact that it is subjected to various denaturing processes in the stomach and intestine before being absorbed into or across the intestinal epithelium.

In previous experiments the smallest challenge dose used was 1 ng EA and secondary but not tertiary responses could be

elicited by the intraperitoneal injection of this dose of antigen (Jarrett and Stewart, 1974). The occurrence of tertiary responses in the present experiments promotes the proposition that a challenge dose of even such a small quantity of EA injected intraperitoneally (but not intradermally) is sufficiently large to be inhibitory to the development of the next booster response. Preliminary (unpublished) results support this proposition.

It is possible, therefore, that the occurrence of tertiary responses in the intradermal and oral antigen experiments may be a reflection of the absorption of smaller and therefore less inhibitory amounts of antigen.

Numerous reports in the literature and the success of local prophylactic immunisations, testify to the fact that antigenically intact protein molecules are rapidly absorbed across the intestinal epithelium and may stimulate local or humoral immune responses (Parkins, Dimitriadou and Booth, 1960; Berenstein and Ovary, 196[8]; Korenblatt, Rothberg, Minden and Farr, 196[8]; Crabbe, Nash, Bazin, Eyssen and Heremans, 1969; and see review by Bienenstock, 1974). It has been proposed that one of the functions of secretory IgA is to limit such absorption by combination with antigen (Heremans, 1969) and indeed there is evidence that oral immunisation of rats with human serum albumin leads to a reduced intestinal uptake of the antigen on subsequent challenge (André, Lambert, Bazin and Heremans, 1974). This concept has been extended by the suggestion that the sensitisation of some atopic individuals to common allergens is the result of a transient period of IgA deficiency or the idio-

syncratic possession of low affinity IgA which, by default, allows the absorption via the mucosae of abnormally large quantities of antigen (Taylor, Normal, Orgel, Stokes, Turner and Soothill, 1973).

Paradoxically, the results presented here for the rat show that the larger the dose of antigen absorbed by whatever route, the less likely it is that persistent IgE responses will result. A large dose of antigen results only in a transient IgE response which cannot be boosted. Could it be that the failure of most individuals to develop allergies to dietary or inhaled antigens depends not on the so-called 'mucosal barrier' to the absorption of antigens, but on the very fact that quantities of antigen sufficiently large to be inhibitory to IgE production are normally absorbed? Similarly, while the injection of large quantities of antigen for the purposes of causing hyposensitisation often results in this effect, it may be that the addition of very small quantities of antigen in diagnostic skin tests or in nasal or bronchial provocation tests could for some individuals at some times carry the danger of stimulating or boosting and IgE response. It is, of course, those individuals who, by genetic constitution, are particularly at risk (discussed by Marsh, Bias and Ishizaka, 1974), who most often present themselves in allergy clinics for the latter procedures, but the intradermal route has in fact been used experimentally to induce skin reactivity to Dextran N.279 in both allergic and normal people (Leskowitz and Lowell, 1961).

The role of a possible feedback mechanism on IgE production by IgG class antibodies has not been studied in these experiment, although only low levels have been found following intradermal or oral antigen presentation (Jarrett, unpublished results).

In the rat, as in other experimental animal species, adjuvants appear to be an important if not an essential accompaniement of antigen

for the initiation of a reaginic antibody response (Jarrett, Henderson, Riley and White, 1972). The role of adjuvants in the genesis of naturally occuring IgE responses in man remains to be explored, but by extrapolation from experimental results, particularly those of Ik Chin, Chang and Gottshall (1974) in mice, it is to be expected that sensitisation to environmental or administered allergens might occur shortly after B.pertussis infection or vaccination, and indeed there is some suggestive evidence that this is the case (Stevenet, 1961). Infection with other organisms might have a similar adjuvant effect.

The initiation and maintenance of IgE antibody responses in man may, as in the rat, depend on a critical combination of circumstances, prominently including the absorption by genetically predisposed individuals of minute quantities of antigen, initially in the presence of an adjuvant.

S U M M A R Y

Primary and booster IgE antibody responses were elicited in Hooded Lister rats by the intradermal injection or oral administration of very small quantities of egg albumin. Oral immunization was effected by giving antigen by stomach tube or in the drinking water.

The minimum primary dose of antigen found to be effective was 1 µg intradermally and 10 µg orally, administered together with an intraperitoneal injection of B. pertussis adjuvant. In rats immunized with these doses secondary responses could be evoked by giving even smaller quantities of antigen, thus 1 ng intradermally or 1 µg orally without adjuvant. Smaller challenge doses were not tried.

Large primary doses of antigen (> 100 µg) presented by these routes were, on the other hand, found to be inhibitory to the production of secondary IgE responses, this effect being similar to that observed in previously reported intraperitoneal immunization experiments. By contrast with previous experiments, however, tertiary responses could be obtained following immunization by these routes, and I believe this to be a reflection of the absorption of smaller and therefore less inhibitory quantities of antigen.

The results are discussed in relation to the control of IgE antibody production, current concepts of the control of antigen absorption through mucosal barriers, and possible implications for the genesis of naturally occurring IgE responses in man.

SECTION III

EFFECT OF NAMATODE PARASITE (*N.BRASILIENSIS*) INFECTION ON IgE PRODUCTION IN THE RAT

Introduction:

The gastrointestinal nematode parasite Nippostrongylus brasiliensis provides an excellent laboratory model for the studay of reagin production to helminth parasite infection. It is easy to culture from the egg to the infective larval stage, is the subject of much laboratory research and is not, in relatively small infective doses, lethal to its natural host - the rat.

During the course of infection, certain well defined immunological effects are observed on the parasite. These are namely; a drop in egg production, and an immune expulsion of the adult worms from the gut. These will be described in more detail below.

N.brasiliensis is a member of the superfamily Trichostrongyloidea of the order Rhabditida and is morphologically identifiable as a small coiled reddish coloured worm, the males being three to four millimetres long, and the females four to six millimeters long. It is a naturally occurring parasite in wild rats and its distribution is world wide.

Life Cycle:

The life cycle of N.brasiliensis from egg to adult worm involves five larval stages designated L_1 to L_5. Eggs in the faeces hatch on the ground and undergo two moults to produce the infective third (L_3) larval stage. This penetrates the skin of a new host, and migrates via the bloodstream to the lungs

where a further moult takes place. Finally the parasite reaches the jejunum where it undergoes a final moult to become the young adult worm (Jarrett, 1968; Dunn, 1969; and Murray, 1972).

The adult worms develop to maturity in the small intestine where their numbers remain static. They lay eggs for a number of days, after which they are rapidly expelled from the gut in an exponential fashion, starting around 10-11 days after infection. A drop in worm egg production preceeds the start of expulsion by between 24 and 48 hours (Jarrett, Jarrett and Urquart, 1968a).

IgE in an N.brasiliensis infection

Reaginic antibody was first demonstrated in the serum of rats infected with N.Brasiliensis by Ogilvie (1964). These antibodies were shown to reach high levels from a few days after worm expulsion in a primary infection, remaining detectable in some animals for up to seven months (Ogilvie, 1967).

The reaginic antibody response in these rats could be boosted appearing as high PCA titres on day 6 after reinfection. The presence of live worms, not worm extract, is necessary for the stimulation of high levels of reagin production to N.brasiliensis in the rat (Ogilvie, 1967).

The presence of these antibodies result in the hypersensitivity phenomena which can be demonstrated to occur in an infection, namely;

(a) Induced anaphylaxis in immune rats by administration

of N.brasiliensis antigen (Urquhart, Mulligan, Eadie and Jennings, 1965). This is detectable as a gut associated anaphylaxis, the intestine being the shock organ in the rat (Sanyal and West, 1958).

(b) Dermal weal and flare reactivity by skin testing immune animals with N.brasiliensis antigen injected intradermally. (i.e.)

Although IgE itself has not been shown to have protective properties against N.brasiliensis worms (Ogilvie and Jones, 1969) it has been postulated that the IgE mediated local gut anaphylaxis, in causing increased gut permeability and hence increased macromolecular passage, facilitates the passage of antiworm antibodies (IgG) across the gut mucosa, (Barth, Jarrett and Urquhart, 1966). This results in worm expulsion. On subsequent reinfections, rats have been shown to 'self cure' earlier (Jarrett, 1968).

The Potentiated Reagin Response

Orr and Blair (1969) discovered that an N.brasiliensis infection in rats could potentiate an already existing reaginic antibody response to totally unrelated antigens (egg albumin and conalbumin). Subsequently it has been shown that reaginic antibody to other antigens (eg. Keyhole limpet haemocyanim) can be similarly potentiated, and that N.brasiliensis is not the only parasite capable of having this effect. Potentiation has also been observed in rats infected with the liver fluke Fasciola hepatica (Jarrett, 1972).

The potentiated response is of short duration, reaching a peak fourteen days after an N.brasiliensis infection and dropping to control levels by about day 25 (Orr, Riley and Doe, 1971). Thus the potentiated response precedes the presence, in serum of antiworm IgE antibodies. The effect is immunoglobulin class specific, affecting IgE antibody levels but not IgG or IgGa (Bloch, Ohman, Waltin and Cygan, 1973). Potentiation was best achieved by infecting rats between D10 and D50 after initial immunisation with antigen and adjuvant, no potentiation being observed in rats infected prior to immunisation (Orr, Riley and Doe, 1972). The same authors also demonstrated that reinfection did not result in repotentiation, but merely maintained the potentiated response a short while longer. A potentiated response could be evoked in the rats with live worms only (Orr, Riley and Doe, 1971) and to a lesser extent with the migrating larval stages (Jarrett and Stewart 1973). Total serum IgE levels were raised at the height of a potentiated response (D12 after infection) and unimmunised rats also showed an elevation in total serum IgE levels at the same time after a N.brasiliensis infection (Jarrett and Bazin, 1974). These results indicate that the parasite potentiates IgE levels non specifically.

Thymus deprived rats have been shown to be incapable of mounting a potentiated response after immunisation with egg albumin and B.pertussis followed by N.brasiliensis infection (Jarrett and Ferguson, 1974). This indicates that potentiation is T cell dependent.

In this section, the potentiated response has been studied in more detail, with emphasis on the time course of the event and the relationship between antigen specific IgE levels (as measured by PCA) and total serum IgE levels. The effect of strain of rat on the response has also been studied.

TIME COURSE STUDIES ON RAT IgE PRODUCTION IN N.BRASILIENSIS INFECTION.

Introduction:

This series of experiments was designed to explore the relationship in time between total circulating IgE levels, antigen specific IgE and worm specific IgE following an N. brasiliensis infection in rats.

Jarrett and Bazin (1974) reported that total serum IgE levels were greatly elevated in Hooded Lister rats 12 days after a N.brasiliensis infection, whether or not they had been previously immunised with egg albumin, and before the time when worm specific IgE had appeared in the serum. In the first three experiments described here, levels of total serum and worm specific IgE antibodies were examined at various times after a N.brasiliensis infection in Hooded Lister rats, without prior immunisation with egg albumin. In the final experiments, the above parameters, and also egg albumin specific IgE levels were followed - rats having first been immunised with egg albumin prior to N.brasiliensis infection. The time course of the potentiated, total serum, and anti-worm IgE responses were also studied in Wistar rats to determine whether or not there was a strain difference.

Materials and Methods:

The materials and methods for this section have been described in the General Materials and Methods above. Total IgE estimation was carried out using the Rowe modification of the Mancini single radial immunodiffusion technique described in Section 1.

Experiment 1:

In the first experiment in this series, ten rats were infected with 4,000 N.brasiliensis larvae and bled between days 12 and 24 thereafter. The rats were split into two groups of five and one group was reinfected on day 28 and bled on days 6, 12 and 40 after reinfection. The other group was bled on several occasions between days 34 and 80 of a 1st infection. The results for this experiment are shown in Table 11 (Appendix 3). These show that the total circulating IgE levels rose between 6 and 12 days after infection from 1.28 to 247 μg/ml. Thereafter the level declined at first rapidly, then more slowly to reach a level of 25 μg/ml 80 days after infection. N.brasiliensis specific IgE antibodies were not detectable in sera from animals bled 6 and 12 days after infection. These antibodies, however, appeared in day 18 sera and reached peak levels 2 to 3 weeks after the peak level of total IgE, the latter declining as the parasite specific IgE level rose towards its peak. Total serum IgE rose to a peak 6 days after reinfection (376 μg/ml), thereafter declining. This was closely associated temporally with the rise and fall of parasite specific IgE.

TABLE11. Total IgE and parasite specific IgE in the serum of Hooded Lister rats infected with 4000 N.brasiliensis larvae.

Days after infection.	µg/ml. Total IgE Mean ± S.E.	N.brasiliensis PCA titre - GM (range).
6*	1.28 ± 0.34	-
12*	247 ± 42	-
18*	104 ± 8.0	548 (256-1024)
24*	46 ± 4.7	723 (256-1024)
34	82 ± 18	890 (512-1024)
40	62 ± 8.3	675 (64-2048)
80	25 ± 5.4	147 (64- 512)
Days after reinfection		
6	376 ± 26	2350 (1024-4096)
12	364 ± 29	891 (512-1024)
40	29 ± 3.9	294 (128-1024)

*Group of Ten rats. On D.28 the rats were divided into two groups of 5 and one group reinfected. the same rats were bled repeatedly on the days shown.

In the next experiment, Wistar and Hooded Lister rats were compared for IgE production during an N.brasiliensis infection.

Experiment 2:

10 Wistar and 5 Hooded Lister rats were infected with 4,000 N.brasiliensis larvae and bled between days 13 and 41 after infection. These rats were subsequently reinfected and bled on days 6, 12 and 34 after reinfection.

The results for this experiment are outlined in Table 12 (and Appendices 4 and 5). These show that Wistar strain rats produce lower levels of total circulating IgE than Hooded Listers following N.brasiliensis infection. This level was boosted in both strains of rats following reinfection. Lower levels of parasite specific IgE were also produced by Wistar as compared with Hooded Lister rats.

The next experiment was designed to follow the kinetics of the total IgE response over the period when IgE levels are rising, i.e. between days 8 and 14 after infection with N.brasiliensis.

Experiment 3:

In this experiment, 50 rats were immunised with 10 μg of egg albumin together with 10^{10} B.Pertussis organisms as adjuvant. Ten randomly chosen animals were bled 15 days after immunisation to determine the primary anti-egg albumin IgE response. 29 days after immunisation, all animals were infected with 4,000

TABLE 12. Total IgE and parasite specific IgE in the serum of Wistar and Hooded Lister rats during *N.brasiliensis* (N.b.) infection.

Days after infection.	µg/ml. Total serum IgE Wistar*	Hooded Lister**	N.b. PCA titre G.M.(range) Wistar	Hooded Lister.
13	50 ± 16	226 ± 42	-	-
17	15 ± 2.6	96 ± 16	54 (8-256)	63 (1-256)
24	8.7 ± 2.1	45 ± 7.7	30 (1-512)	76 (32-512)
34	3.9 ± 1.7	11 ± 1.5	26 (1-1024)	511 (32-1024)
Days after reinfection.				
6	163 ± 42	452 ± 72	1096 (128-4096)	2042 (1024-4096)
12	76 ± 22	353 ± 111	548 (128-8192)	776 (128-2048)
34	7.4 ± 1.8	22 ± 2.6	97 (16-512)	512 (512)

* 10 rats)
** 5 rats) bled repeatedly on the days shown.

N.brasiliensis larvae. Groups of 5 animals were exsanguinated daily between [illegible] and 20 days after infection.

The results for this experiment are shown in fig. 6 (and Appendices 6 and 7). Total serum IgE rose to maximum levels between days 12 and 14, the fastest rate of increase occurring between 8 and 10 days after infection. The rate declined between 10 and 13 days after infection and thereafter total serum IgE levels began to decline (see Figure 7). N.brasiliensis specific IgE was not detectable before 14 days after infection, but appeared and rose rapidly in level thereafter. The peak level of egg albumin specific IgE occurred between 11 and 13 days after infection, declining afterwards. However, potentiation unfortunately did not effectively occur in many of the animals for reasons which are not yet understood.

The next experiment was designed to study the relationship in time between total serum IgE, parasite specific IgE and Successfully potentiated egg albumin specific IgE responses following N.brasiliensis infection.

Experiment 4:

5 rats were immunised with 1mg of egg albumin, together with adjuvant. All animals were infected with 4,000 N.brasiliensis larvae each. They were bled between days 6 and 75 after infection and on days 6 and 12 after reinfection.

The results for this experiment are shown in Table 13 (and appendices 8 and 9). Sera obtained from animals 14 days after infection

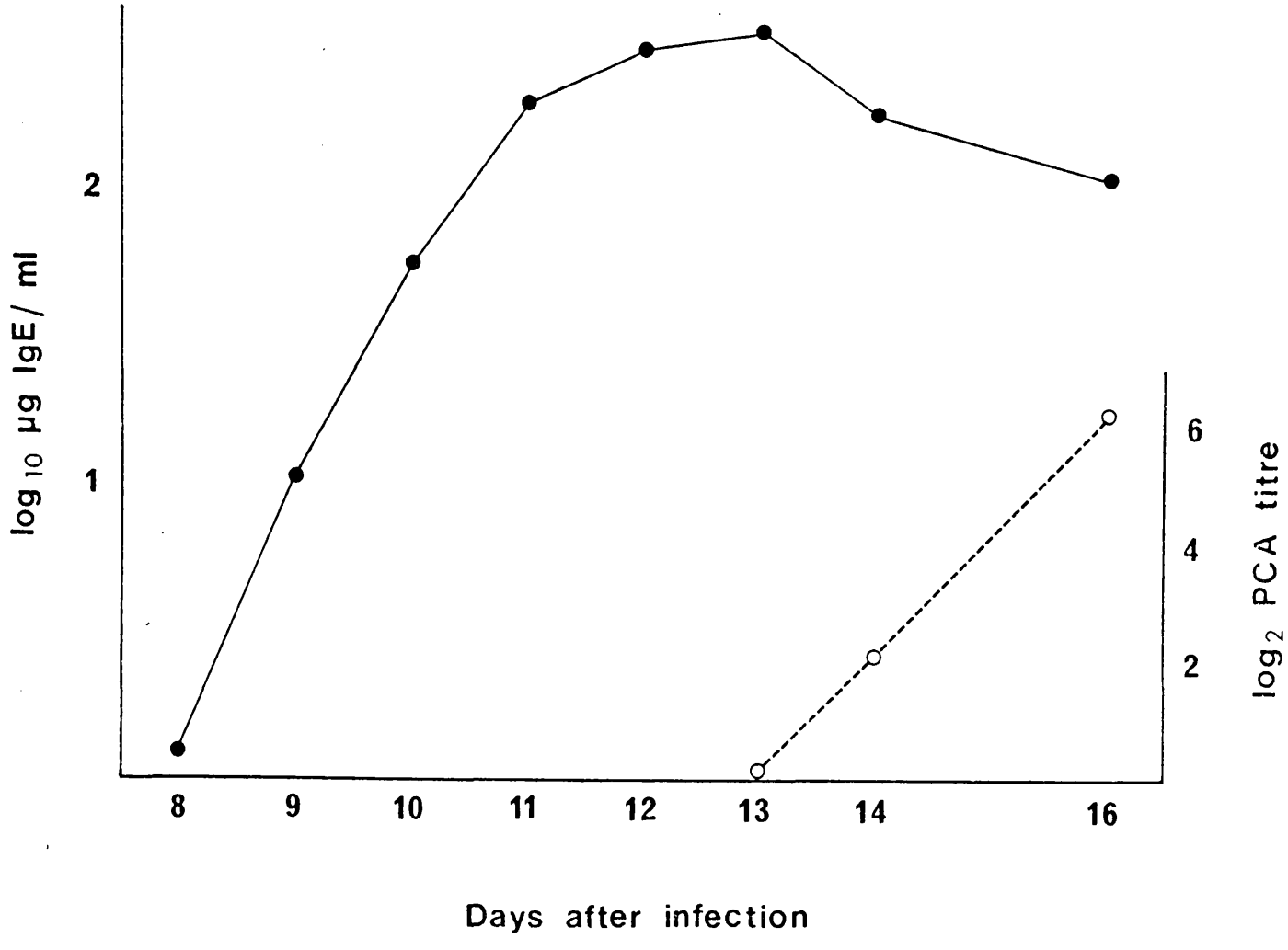


FIG.6. Kinetics of total IgE production (●—●) in N. brasiliensis infected rats, studied by bleeding out groups of 5 rats on the days shown. N. brasiliensis specific IgE (○---○) first detected on day 14 after infection.

TABLE 13 Relationships in time of potentiated, parasite specific and total IgE responses in N.brasiliensis infection(5 rats).

Days after infection.	µg/ml. Total IgE mean ± S.E.	EA PCA titre G.M. (range)	N.b. PCA titre G.M. (range)
6	1.44 ± 0.05	12 (2 - 64)	- (0)
9	12.3 ± 3.18	193 (64-1024)	- (0)
14	334 ± 69	1350 (1024-2048)	- (0)
17	177 ± 34	387 (64 -1024)	147 (8-1024)
21	108 ± 16	97 (32 - 256)	387 (128-1024)
55	48 ± 8	24 (16 - 32)	511 (128-1024)
75	26 ± 6	9 (1 - 16)	293 (16-1024)
Days after reinfection.			
6	227 ± 87	(0)	1551 (1024-2048)
12	332 ± 67	(0)	1023 (512-2048)

contained maximal levels of total IgE (mean 334 µg/ml). This is consistent with the results from previous experiments. This high level declined thereafter, at first rapidly and then more slowly until by 73 days after infection, the mean total IgE value was 26 µg/ml. This level, however, was boosted following re-infection, maximum values appearing in the day 12 sera.

Parasite specific IgE was first detectable 17 days after infection, reaching maximum values on day 55.

Egg albumin specific IgE was maximally potentiated 14 days after infection. Both egg albumin specific and total serum IgE levels started to decline before the appearance, in the circulation, of parasite specific IgE. After reinfection, whereas total IgE levels were boosted, egg albumin specific IgE, as detected by PCA was not.

The final experiment in this series was designed to produce and confirm the findings of the previous experiment.

Experiment 5:

10 rats were immunised with 10 µg of egg albumin together with adjuvant, bled 12 days later, challenged on day 22 of the experiment with 1 µg egg albumin without adjuvant and bled 11 days after challenge. 12 days after the challenging dose of antigen had been administered, all rats were infected with 4,000 N.brasiliensis larvae. They were bled between days 8 and 31 after infection and on days 6, 12 and 24 after reinfection.

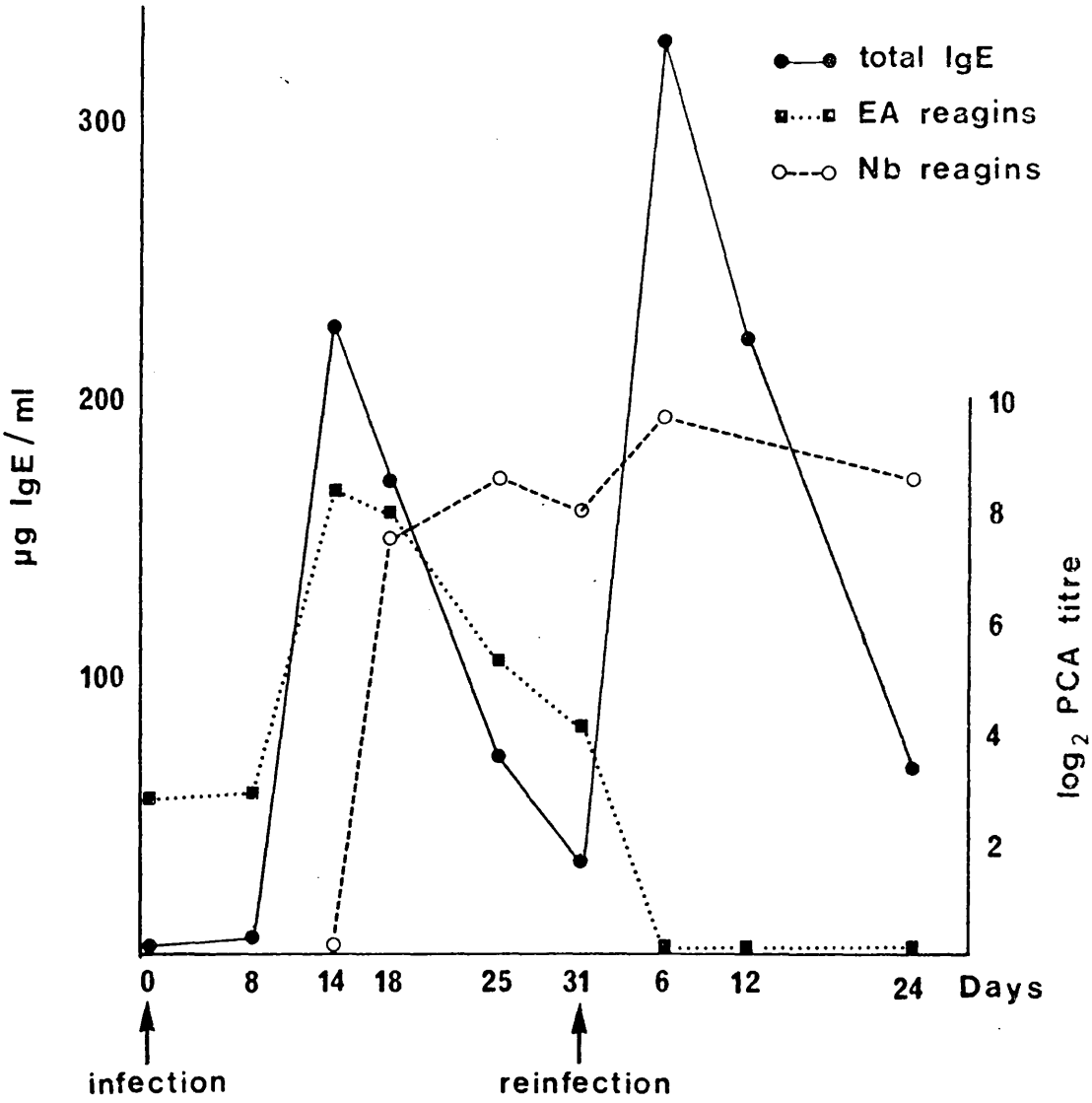


Fig 7. Relationship in time between total IgE and egg albumin and N. brasiliensis specific IgE responses in a first and second N. brasiliensis infection of 10 rats bled repeatedly on the days shown.

The results for this experiment are shown in Figure 7 (Appendices 10 and 11), and confirm the findings of previous experiments, namely; a rapid rise in total IgE from 8 days after infection, which reached a peak level at around 14 days after infection and thereafter declined rapidly. The pattern for the egg albumin specific response was very similar to that for total IgE. Parasite specific IgE appeared in the circulation between 14 and 1[illegible] days and reached peak levels at around 25 days after infection. After reinfection, total IgE rose to higher levels than had occurred in the first infection. This happened by the 6th day after reinfection coincident with the booster parasite specific IgE response. Total IgE however, was raised by a far greater factor than N.brasiliensis specific IgE and declined rapidly, whereas the parasite specific IgE response did not.

Discussion:

The time course studies reported here show that while the peak of the total IgE response occurs on days 12-14 after infection, the fastest rate of increase in IgE levels occurs earlier, between days 8 and 10. It would therefore appear that the maximum stimulating effect of the parasite is exerted just prior to or coinciding with the day 8-10 rapid rise of total IgE. After day 14 of an infection, the total serum IgE level drops at first rapidly and then more slowly. The cause of this

rapid decline could be due, at least in part, to the immune expulsion phenomenon (Jarrett, Jarrett, and Urquhart, 1968a) which removes worms from the intestine of infected animals, starting 10 to 11 days after infection and proceeding exponentially over several days. However, other results suggest that a further factor may operate. Thus, infection of rats before 6 weeks of age results in a form of diminished immunological responsiveness which shows itself in a reduced ability to expel worms (Jarrett, Jarrett and Urquhart, 1966, 1968b). In such animals, total serum IgE levels rise and fall at the same time as in adult rats, despite the continuing presence of a sizeable unexpelled worm burden (Jarrett and Haig - unpublished).

In previous experiments involving modification of the life cycle of the parasite in the host (Jarrett and Stewart, 1973a), it was shown that it was the adult worm which exerted the maximum IgE potentiating effect. Since this stage of the parasite is confined to the gut lumen, it would seem probable that the stimulus for IgE production is a soluble factor secreted by the parasite and absorbed across the intestinal epithelium. Adult worms affected by immunity show structural damage, particularly of the gut cells, which appear before worms are expelled (Ogilvie and Mockley, 1968). The worms present in young rats show similar morphological changes even although they are not subsequently expelled. It could be that the events causing worm 'damage' also result in a cessation of production of the IgE stimulating

factor, and hence in a fall off in the rate of production of IgE.

In a first infection with N.brasiliensis, the egg albumin specific potentiated IgE response occurs simultaneously with the total IgE response. They would therefore appear to be the result of the same stimulus (see Fig.7). Parasite specific IgE does not appear in the sera of rats until about day 16 of an infection - at a time when the potentiated egg albumin specific and total IgE responses are declining. However, previous work has shown that parasite specific IgE can be detected by immediate skin reactions following intradermal injection of N.brasiliensis antigen 10 days after infection. Thus worm specific IgE could be present in the serum before day 16 of an infection, but not detectable by PCA possibly because of the presence of IgE - worm antigen complexes.

Wistar strain rats follow a similar temporal pattern, regarding rise and decline of total and worm specific IgE, following infection as compared with Hooded Lister rats. However, the magnitude of the IgE response is much diminished in Wistar rats when compared with Hooded Listers (see Table 12).

A different sequence of events follows after reinfecting rats with N.brasiliensis. Whereas the total IgE level rises to a maximum around 6 days after reinfection - reaching higher values than those obtained following a first infection - egg albumin specific IgE has never been repotentiated in any of our experiments. The reasons for this are not known. A specific suppressor mechanism could be in operation, or the cells involved

are no longer susceptible to the potentiating stimulus. Parasite specific IgE however, is boosted following reinfection reaching a maximum value after 6 days, thereafter declining at a slow rate. However, total serum IgE rises by a much greater factor than parasite specific IgE, and thereafter the antibody content of a large proportion of this total IgE remains to be accounted for. Jarrett and Bazin (1974) have postulated that, following a first infection with N.brasiliensis, the total IgE response consisted at least in part of a number of potentiated IgE responses to unknown antigens to which the rats had become naturally sensitesed.

It is possible that the lack of repotentiated egg albumin specific IgE following reinfection is an exception, and that re-potentiation of other miscellaneous antigen specific IgE does indeed account for the large rise in total IgE even following reinfection. This point must be explored further.

The peak total IgE response occurs 6 days sooner after reinfection than after a first infection. Thymus derived cells have been shown to be necessary for a successful potentiated IgE response to egg albumin in the rat (Jarrett and Ferguson, 1974) and for the elevation of total serum IgE (Jarrett and Ferguson 1974 - unpublished results). The nature and mode of action of the T cells involved remains unclear. However, as total IgE and parasite specific IgE levels rise simultaneously after reinfection, it is probable that N.brasiliensis specific T cells are involved, being reactivated by the parasite to produce

both the non-specific IgE stimulating factor, and helper activity specific for the anti-parasite IgE response.

SUMMARY

I present here a study of the relationship in time between the elevation of total serum IgE, the parasite-specific IgE response, and the potentiated IgE response to unrelated antigen which occurs in rats following infection with the worm parasite N. brasiliensis.

During a first infection the potentiated IgE response (to egg albumin) and elevation of total IgE occur synchronously rising to a peak on days 12-14 after infection, with the fastest rate of increase occurring between days 8 and 10. N. brasiliensis-specific IgE rises to a peak some 2 - 3 weeks later when both total IgE and the potentiated response have largely declined.

A strain difference is shown in that Wistar rats produce far lower levels of total and parasite-specific IgE than Hooded Listers.

Events following reinfection differ in that total IgE rises more rapidly, very high levels being reached 6 days after reinfection together with a secondary specific IgE response to N. brasiliensis. The total IgE level, however, rises by a far greater factor than parasite-specific IgE and declines rapidly while the parasite-specific response declines slowly over many weeks. The egg albumin response is not repotentiated.

It is proposed that the total IgE response and the potentiated IgE response which forms a small component of it results from the release of a non-specific IgE-stimulating factor produced by N. brasiliensis-specific T cells. In this scheme the same or /

/ similar cells are involved in the production of N. brasiliensis-specific IgE through a separate specific helper function.

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APPENDICES.

APPENDIX 1

RESULTS FOR AN EA RAST PERFORMED ON REFERENCE SERA 1,2,and 3.THE RESULTS FOR REFERENCES 1 and 2 ARE REPRESENTED GRAPHICALLY IN FIG4.

log.2 serum dilution.	MEAN C.P.M.			
	ref.1	ref. 2	ref. 3	Normal rat serum.
2	15466	-	-	574
3	14951	-	-	537
4	13622	11115	2825	561
5	10745	8690	1848	569
6	7914	6064	1261	577
7	4560	4195	1417	513
8	3237	2713	703	482
9	2078	1564	794	-
10	1356	1112	729	-
11	1064	864	698	-
12	808	700	671	-
13	-	560	572	-
Buffer control	602.			

APPENDIX 2

RESULTS OF A N. BRASILIENSIS RAST PERFORMED ON REFERENCE PREPARATION 3(HIGH ANTI N.b.IgE), INCORPORATING EGG ALBUMIN COUPLED DISCS AS CONTROLS TO INDICATE ANY NON SPECIFIC BINDING EFFECTS.

Log 2 diln.	MEAN COUNT RATE (C.P.M.)		
	N.b. antigen discs	N.b. allergen discs	E.A. discs
2	2810	3195	1824
3	2822	3487	1875
4	3318	3791	1741
5	3782	3405	1825
6	3772	3617	1456
7	3215	2998	1206
8	2073	2355	1190
9	2091	1657	1169
10	2517	1577	1167
11	1699	1382	1180
12	1840	1456	1124
14	1588	1266	940
Buffer controls	1919	1175	902

APPENDIX 3
EXPERIMENT I.

TOTAL SERUM IgE LEVELS AND PCA TITRES IN N.BRASILIENSIS INFECTION IN HOODED LISTER RATS.

	DAYS AFTER INFECTION.									
	6	I2	I8		24		34		40	
RAT.	Total IgE µg/ml.	Total IgE. µg/ml.	PCA (log2)	Total IgE. µg/ml.	PCA (log2)	Total IgE. µg/ml.	PCA (log2)	Total IgE. µg/ml.	PCA (log2)	Total IgE. µg/ml.
I	2.94	473	256 (8)	92.6	256 (8)	54.0				
2	<0.69	I70	Io24 (I0)	I53.0	I024 (I0)	25.7				
3	I.39	500	256 (8)	I38.0	5I2 (9)	45.8	5I2 (9)	56.0	I024 (10)	38.0
4	<0.69	I79	I024 (I0)	I27.0	I024 (I0)	69.I	I024 (I0)	I3I.0	64 (6)	66.I
5	0.69	2I2	5I2 (9)	84.5	5I2 (9)	34.2				
6	<0.69	2II	I024 (I0)	79.4	5I2 (9)	63.2	I024 (I0)	II8.0	2048 (II)	70.5
7	<0.69	I7I	256 (8)	85.3	I024 (I0)	54.0				
8	3.58	I47	I024 (I0)	I00.0	I024 (I0)	38.8	I024 (I0)	59.2	I024 (I0)	50.3
9	<0.69	27I	5I2 (9)	I03.0	I024 (I0)	49.8				
I0	<0.69	I4I	5I2 (9)	86.0	I024 (I0)	25.9	I024 (I0)	44.8	I024 (I0)	86.2
MEAN	I.28	247.0	640 (9.I0)	I04.0	793(9.5)	46.05	(9.80)		(9.40)	
S.D.	I.03	I3I.0	346 (0.83)	25.0	305(0.7I)	I4.80	(0.45)		(0.95)	
S.E.	0.34	4I.6	I09 (0.28)	8.0	96(0.22)	4.68	(0.20)		(0.87)	

PCA refers to N.brasiliensis PCA titre.
<0.69 taken as= 0.69.
S.D, = Standard deviation.
S.E. = Standard error.

APPENDIX 3

EXPERIMENT I (CONTINUED).

DAYS AFTER INFECTION.			DAYS AFTER REINFECTION.*								
	80			6			12			40	
RAT	PCA (log2)	Total IgE.**	RAT	PCA (log2)		Total IgE.	PCA (log2)		Total IgE.	PCA (log2)	Total IgE.
3	64 (6)	II.6	I	4096	(12)	288	I024	(10)	416	512 (9)	42.0
4	128 (7)	22.8	2	2048	(11)	448	I024	(10)	376	128 (7)	32.4
6	128 (7)	18.4	5	2048	(11)	432	512 (9)		256	256 (8)	23.0
8	128 (7)	26.4	7	4096	(12)	376	I024	(10)	408	128 (7)	19.8
10	512 (9)	44.0	9	I024	(10)	336	I024	(10)	368	I024 (10)	29.0
MEAN	(7.2)	24.64			(11.2)	376.0		(9.80)	364.80	(8.2)	29.24
S.D.	(1.1)	12.14			(0.84)	66.5		(0.45)	64.15	(1.3)	8.67
S.E.	(0.5)	5.43			(0.37)	25.7		(0.20)	28.69	(0.6)	3.87

*5 rats infected for a second time with 4000 N.brasiliensis larvae 28 days after the first infection.

** In µg/ml.

APPENDIX 4

EXPERIMENT 2.

TOTAL SERUM IgE LEVELS (IN μg/ml.) IN WISTER AND HOODED LISTER (H.L.) STRAIN RATS FOLLOWING N.BRASILIENSIS INFECTION.

	DAYS AFTER INFECTION.										DAYS AFTER REINFECTION.*					
	I3		I7		24		34		4I		6		I2		34	
Rat.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.
I	96.00	240	22.0	88	I4.50	46.8	4.30	I3.8	4.40	25.5	I98.0	307	I72.8	448	II.0	23.0
2	I64.00	I35	25.0	93	7.20	49.0	I.85	6.0	I.90	22.0	I72.8	334	64.0	I93	I0.5	I3.5
3	I.38	243	8.2	43	I9.50	20.0	I8.50	I4.4	I.80	I6.8	320.0	640	96.0	4I6	I2.0	20.0
4	23.20	368	I8.0	I20	I.37	40.0	I.40	9.3	2.60	30.0	72.8	480	49.6	352	I.4	33.0
5	64.00	I47	4.I	I36	5.80	68.0	0.80	9.6	I.38	36.0	137.6	—	52.8	—	18.0	19.0
6	28.80		3.6		20.00		6.60		13.50		67.2		22.4		4.2	
7	87.20		24.0		7.60		1.85		1.38		448.0				1.7	
8	19.20		16.0		2.80		1.38		1.38		38.4				2.0	
9	40.00		7.4		3.60		1.00		1.38		128.0				2.2	
I0	1.38		18.0		4.50		1.38		1.38		48.0				II.0	
MEAN	50.42	226.0	14.63	96.0	8.73	44.76	3.91	10.72	3.11	25.00	163.0	452.0	76.2	353.0	7.4	23.91
S.D.	52.90	93.9	8.16	35.2	6.78	17.30	5.43	3.44	3.77	7.37	131.0	143.0	52.9	III.0	5.8	6.40
S.E.	16.72	42.0	2.58	15.7	2.14	7.74	1.71	1.53	1.19	3.29	41.5	71.7	21.6	55.5	1.8	2.63

*All rats infected again with 4000 N.brasiliensis larvae 45 days after the first infection.

APPENDIX 5

EXPERIMENT 2.

N.BRASILIENSIS SPECIFIC PCA TITRES IN WISTAR AND HOODED LISTER(H.L.)RATS FOLLOWING INFECTION.

	DAYS AFTER INFECTION.						DAYS AFTER REINFECTION.*					
	17		24		34		6		12		34	
Rat.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.	Wistar	H.L.
1	256	128	32	64	32	1024	1024	4096	2048	2048	512	512
2	64	256	64	64	8	1024	4096	1024	512	128	256	512
3	32	1	512	64	1024	32	2048	1024	8192	1024	256	512
4	32	256	32	512	64	1024	2048	2048	512	512	16	512
5	8	64	1	32	8	1024	2048	4096	256	2048	128	512
6	32		64		128		1024		128		32	
7	128		64		8		2048		512		256	
8	32		8		1		128		128		16	
9	256		64		32		1024		2048		512	
G.M.	54	64	30	76	26	512	1096	2046	548	775	97	512
log2 MEAN	5.77	6.00	4.90	6.25	4.70	9.00	10.10	11.00	9.10	9.60	6.60	9.00
S.D.	1.64	2.91	2.33	1.16	2.54	2.23	1.79	1.00	2.00	1.67	2.11	0.00
S.E.	0.54	1.30	0.73	0.41	0.80	1.00	0.57	0.45	0.64	0.74	0.66	0.00

G.M. = Geometric mean.

*All rats reinfected with 4000 N.brasiliensis larvae 45 days after the first infection.

APPENDIX 6

EXPERIMENT 3.

TOTAL SERUM IgE LEVELS(in μg/ml.)IN GROUPS OF 5 RATS BLED ON THE DAYS INDICATED FOLLOWING N.BRASILIENSIS INFECTION.

	DAYS AFTER INFECTION.									
*D.15 after sensitisation.	8	9	10	11	12	13	14	16	18	20
1.38	1.60	9.2	72.0	257	579	297	134	107.5	160	45.3
1.50	2.20	16.4	64.0	188	668	339	164	82.2	128	130.0
1.27	0.69	23.2	80.0	264	368	309	238	109.5	80	78.5
1.45	1.70	8.8	47.2	122	400	389	273	118.0	122	126.0
1.60	0.69	4.8	35.2	185	38	372	149	143.0	170	117.0
1.80										
1.60										
5.20										
2.10										
2.00										
log10 MEAN.	0.091	1.034	1.757	2.290	2.467	2.53	2.260	2.042	2.100	1.96
S.D.	0.240	0.265	0.145	0.135	0.507	0.05	0.134	0.086	0.129	0.19
S.E.	0.110	0.011	0.065	0.061	0.227	0.02	0.059	0.039	0.058	0.09
G.M.	1.23	10.8	57.2	195.0	293.0	339.0	182.0	109.0	125.0	91.0

*10 of 50 sensitised rats randomly chosen and bled to give pre-infection IgE levels.

APPENDIX 7

EXPERIMENT 3(CONTINUED).

EGG ALBUMIN(E.A.)PCA AND N.BRASILIENSIS(NB)PCA TITRES IN GROUPS OF 5RATS BLED ON THE DAYS INDICATED FOLLOWING INFECTION.

D.15 after sens.	DAYS AFTER INFECTION. 8	9	10	11	12	13	14		16		18		20	
E.A.	E.A.	E.A.	E.A.	E.A.	E.A.	E.A.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.
64	16	16	32	0	2048	128	0	128	128	256	0	512	0	0
8	0	64	0	128	0	256	0	4	128	16	0	8192	0	512
32	16	0	64	128	16	128	512	0	128	32	64	16	128	512
2	0	0	0	128	512	2048	128	0	0	32	128	256	0	512
0	0	0	64	128	128	128	16	0	128	512	128	512	64	128
8														
0														
16														
0														
32														
log2 MEAN 3.86	4.0	5.0	5.6	7.0	8.2	8.0	6.6	–	7.0	6.20	6.6	8.6	6.5	8.5
S.D. 1.67										2.16		3.2		1.0
S.E. 0.63										0.96		1.4		0.5

APPENDIX 8

EXPERIMENT 4.

TOTAL SERUM IgE LEVELS(INμg/ml.)IN 5 RATS IMMUNISED WITH EGG ALBUMIN AND SUBSEQUENTLY INFECTED WITH N.BRASILIENSIS LARVAE AND BLED ON THE DAYS INDICATED.

	DAYS AFTER INFECTION.							DAYS AFTER REINFECTION.*	
Rat.	6	9	14	17	21	55	75	6	12
1	1.38	7.8	408	134	144	56	29	269	377
2	1.50	17.0	141	198	147	58	33	186	140
3	1.27	22.5	205	299	77	64	3	544	448
4	1.45	6.0	408	150	68	16	28	74	488
5	1.60	8.2	512	108	104	50	35	62	211
MEAN	1.44	12.30	334.0	177.0	108.0	48	25.6	227.0	332
S.D.	0.12	7.12	155.0	75.0	36.7	19	12.9	106.5	151
S.E.	0.05	3.18	69.4	33.6	16.4	8	5.7	87.0	67

*All rats reinfected with 4000 N.brasiliensis larvae 84 days after the first infection.

APPENDIX 9

EXPERIMENT 4(CONTINUED).

EGG ALBUMIN(E.A.) AND N.BRASILIENSIS(N.B.) PCA TITRES FOR 5 RATS IMMUNISED WITH E.A. AND SUBSEQUENTLY INFECTED WITH N.BRASILIENSIS LARVAE.

	DAYS AFTER INFECTION									* DAYS AFTER REINFECTION.					
	6	9	14	17		21		55		75		6		12	
Rat.	E.A.	E.A.	E.A.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.
1	64	128	1024	512	256	64	1024	16	1024	1	1024	–	2048	–	512
2	8	512	1024	512	1024	128	1024	32	512	16	512	–	2048	–	1024
3	32	1024	2048	1024	512	128	128	32	1024	16	512	–	2048	–	2048
4	2	64	1024	64	64	32	128	16	128	8	16	–	1024	–	1024
5	8	64	2048	512	8	256	512	32	512	16	512	–	1024	–	Dead
G.M.															
Log2 MEAN	3.60	7.60	10.4	8.60	7.2	6.60	8.60	4.60	9.0	3.20	8.20		10.60		10.00
S.D.	1.94	1.80	0.5	1.51	2.7	1.14	1.51	0.55	1.2	1.30	2.39		0.54		0.81
S.E.	0.87	0.81	0.2	0.67	1.2	0.51	0.67	0.24	0.5	0.58	1.06		0.24		0.40

*Reinfected with 4000 N.brasiliensis larvae 84 days after the first infection.

APPENDIX 10

EXPERIMENT 5.

TOTAL SERUM IgE LEVELS (IN µg/ml.) IN 10 RATS IMMUNISED WITH EGG ALBUMIN AND SUBSEQUENTLY BLED ON THE DAYS INDICATED FOLLOWING N. BRASILIENSIS INFECTION.

		DAYS AFTER INFECTION					DAYS AFTER REINFECTION		
Rat.	D.12 after challenge.	8	14	18	25	31	6	12	24
1	1.50	2.60	371	56	48.0	25.6	442	393.0	80.0
2	0.94	1.75	230	256	99.2	41.6	384	118.0	72.0
3	1.62	2.95	250	172	81.6	40.8	320	321.6	57.6
4	1.30	1.72	192	166	38.4	27.2	198	179.0	80.4
5	2.80	3.40	118	142	89.6	36.8	336	84.8	112.0
6	1.30	2.15	204	230	98.0	37.6	448	275.0	55.2
7	1.30	1.80	218	218	112.0	47.2	382	156.8	51.2
8	1.30	1.00	211	204	813.2	36.8	358	313.6	75.2
9	1.30	0.69	256	79	26.2	12.8	230	192.0	72.0
10	1.30	2.20	218	178	30.7	20.0	211	188.0	21.6
MEAN.	1.466	2.026	226.80	170.00	70.69	32.64	330.90	222.00	67.60
S.D.	0.499	0.828	63.39	63.70	31.68	10.80	91.03	98.87	23.60
S.E.	1.579	0.262	20.04	20.16	10.02	3.41	28.78	31.26	7.47

APPENDIX 11

EXPERIMENT 5.

EGG ALBUMIN(E.A.) AND N.BRASILIENSIS(N.B.)SPECIFIC PCA RESULTS FOR 10 RATS IMMUNISED WITH EGG ALBUMIN AND BLED ON THE DAYS INDICATED FOLLOWING SUBSEQUENT INFECTION.

		DAYS AFTER INFECTION.									DAYS AFTER REINFECTION.**			
	D.12 after	8	14		18		25		31		6		13	24
Rat.	challenge.*	E.A.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.	E.A.	N.B.	N.B.	N.B.
1	8	16	128	0	128	16	4	512	4	128	0	1024	128	512
2	16	4	512	0	512	256	128	512	4	–	0	1024	128	1024
3	16	16	1024	0	512	128	128	512	64	1024	0	1024	128	512
4	8	8	128	0	512	256	128	128	64	128	0	512	512	128
5	4	4	512	0	128	128	16	1024	2		0	2048	512	1024
6	4	4	512	0	128	512	0	512	0		0	1024	1024	256
7	4	8	512	0	512	512	16	512	4	512	2	1024	128	128
8	16	8	512	0	512	512	128	1024	64	128	0	1024	128	1024
9	4	4	128	8	128	128	128	128	64		0	32	512	512
10	4	16	512	0	512	128	128	128	128		0	4096	512	128
G.M. log2 MEAN	2.80	2.90	8.50		8.20	7.50	5.77	8.60	4.22			9.70	8.10	8.60
S.D.	0.92	0.87	1.08		1.03	1.51	1.92	1.17	2.38			1.83	1.20	1.26
S.E.	0.29	0.27	0.34		0.33	0.48	0.64	0.37	0.79			0.58	0.38	0.40

*Preinfection PCA titres. ** All rats reinfected with 4000 N.brasiliensis larvae 34 days after the first infection.

No E.A. specific IgE was detected in sera after 6days following reinfection.