

An automated particle-counting immunoassay (PACIA) for serum IgE

C. G. M. MAGNUSSON, D. COLLET-CASSART, T. G. MERRETT* and P. L. MASSON

*Unit of Experimental Medicine, International Institute of Cellular and Molecular Pathology, Université Catholique de Louvain, Brussels, Belgium, and *RAST Allergy Unit, Benenden Chest Hospital, Cranbrook, Kent.*

(Received 11 February 1981; accepted for publication 24 March 1981)

Summary

Total IgE was determined in 107 sera using a novel, automated, non-isotopic immunoassay called PACIA (particle-counting immunoassay) based on agglutination of anti-IgE coated latex particles by IgE. The IgE values ranged from 10 to 50 000 iu/ml and were compared with results obtained by a conventional radioimmunoassay (RIA) which used a fast double antibody separation technique—the coefficient of correlation was 0.985 and the regression line $y = 0.82x + 130.00$.

PACIA had several advantages over the RIA technique: using a sampling rate of 50/hr, results were obtained in 35 min compared to 16–20 hr, no labelled IgE was required and the separation step, which relied on measuring the number of non-agglutinated particles by an optical cell counter, was fully automated. The threshold of sensitivity was 10 iu/ml and the maximal coefficient of variation for between assay precision was 12.9%.

Introduction

The assay for serum immunoglobulin E (IgE) and particularly the antibody IgE is an important laboratory measurement for the diagnosis of atopic allergy. However, the radioallergosorbent test (RAST) is so work-intensive, slow and uses such costly reagents, that some authorities question its value in the clinic as compared to the skin-prick test (Adkinson, 1980).

The first step towards the automation of IgE antibody testing is the development of an automated, fast, sensitive assay for IgE. Our purpose was to adapt the particle-counting immunoassay (PACIA) (Cambiaso *et al.*, 1977) technique for this measurement and to validate the accuracy of the results by reference to a routinely used conventional double antibody radioimmunoassay (RIA) technique.

Correspondence: Dr Carl G. M. Magnusson, Unit of Experimental Medicine, International Institute of Cellular and Molecular Pathology, ICP 7430, Avenue Hippocrate, 75B-1200 Brussels, Belgium.

PACIA is based on agglutination of latex particles coated with the antibodies directed against the antigen of interest. The extent of agglutination due to the antigen, is estimated by a cell-counter electronically modified only to count residual non-agglutinated particles. Their number will vary inversely to the antigen concentration.

Materials and methods

Immunoglobulin E

Serum from patient Des with IgE-K myeloma (Vaerman, 1979) was the source for purified IgE. The final product contained less than 0.5% (w/w) of the other immunoglobulins as estimated by immunonephelometry (AIP-system, Technicon, Tarrytown, N.Y., 10717). The isolation and characterization of Fc'' (pepsin)- and Fc (papain) fragments used to raise specific IgE antisera will be described in detail elsewhere.

Anti-IgE sera

Antisera against the Fc'' fragment of IgE Des were raised in three New Zealand white rabbits by intradermal injections at multiple sites every 2 weeks of 100 μ g antigen in 500 μ l of physiological saline emulsified with 500 μ l of complete Freund's adjuvant. The animals were bled 10 days after the third injection and then 10 days following each booster injection. Anti-light chain activity was removed by passage through an IgG-sepharose immunosorbent (Cambiaso *et al.*, 1975). Such antisera reacted in double immunodiffusion with both F(ab')₂ and Fc fragments of IgE Des. The absence of spurs of F(ab')₂ and Fc fragments over Fc'' fragment indicated that the antiserum was exclusively directed against the Fc'' fragment. To check for possible residual anti-light chain activity the agglutinating ability of serial tenfold dilutions of polyclonal IgG (10 mg/ml), monoclonal IgM K-type (5 mg/ml) and cord sera were tested on the anti-Fc'' coated latex. No agglutination was observed further indicating the specificity of the antisera. Antisera against Fc fragment were prepared as above.

F(ab')₂ fragments from anti-IgE serum

The IgG fraction, which was isolated by ammonium sulphate precipitation and chromatography on DEAE-cellulose, was digested by pepsin in 0.1 M acetate buffer, pH 4.5, for 24 h at 37°C in an enzyme/protein ratio of 1/50 (w/w). The reaction was stopped by adding solid tris (hydroxymethyl) methylamine and the F(ab')₂ fragments were recovered by filtration on a column of Ultrogel AcA 44 (LKB, Bromma, Sweden) in 1/15M phosphate buffer, pH 7.3 containing 1 M NaCl. After concentration and dialysis in physiological saline, aliquots of 3–4 mg/ml were stored at 4°C with 1 mg/ml sodium azide prior to the adsorption with latex particles.

Latex coating

A 100 μ l sample of a 10% latex suspension (0.8 μ mol) (Rhône-Poulenc, Courbevoie, France) was added to a mixture of 300 μ l F(ab')₂ fragments (1.2 mg) and 800 μ l of fivefold diluted glycine buffered saline (GBS—0.17 M glycine in a 0.1 M NaCl adjusted to pH 9.2 with NaOH, and containing 0.4 g/l sodium azide as bacteriostat). After 30 min incubation at room temperature, the anti-IgE coated latex was washed once with 2 ml of fivefold diluted GBS, and twice with 2 ml of GBS-BSA (1% bovine serum albumin in GBS), using centrifugation at 10 000 rev/min for 5 min. After resuspension

in 2 ml of GBS-BSA and sonication for 10 sec (Branson Sonifier B12, Danbury, CT, 06810) the latex was lyophilized in 200 μ l aliquots. Stored at 4°C in stoppered tubes, this was stable for at least 6 months. Before use the latex was resuspended in 200 μ l of distilled water, diluted with 1.8 ml of GBS-BSA and sonicated for 10 sec. Such an aliquot was sufficient for 60 tests. The quantity of latex needed was prepared daily from the same lyophilized batch.

IgE measurements

PACIA. The Technicon PACIA system (Holy, 1979) (Technicon International Division, Geneva, Switzerland), as marketed for the measurement of immune complexes, has been used for this assay and is shown schematically in Fig. 1. After centrifugation for 5 min at 5000 rev/min, the serum was diluted tenfold with NRS-BSA buffer (1 volume of normal rabbit serum and 9 volumes of GBS-BSA) in a sample cup which was then placed in the inner row of the sampler tray. The procedure outlined in Fig. 1 was then followed. Additive is GBS containing 50 mM EDTA and half-saturated with NaCl to reduce serum interferences due to protein-protein interactions and 60 g/l polyethylene glycol 6000 (Merck, Darmstadt, FRG) to enhance the antigen-antibody reaction (Hellsing, 1975).

RIA. The conventional RIA used a fast double antibody separation technique, and has been described in detail elsewhere (Merrett & Merrett, 1978). Briefly, an assay tube containing 100 μ l of test (or standard) serum diluted in a 1:1 mixture of horse serum, 50 μ l of ^{125} I-labelled IgE (Phadebas IgE Test Kit: Pharmacia GB Limited, Hounslow, Middlesex, England) and 100 μ l of rabbit anti-IgE (Fc) (Hoechst U.K. Limited, Hounslow, Middlesex, England) was incubated at room temperature overnight. Antibody-bound tracer was separated from the free fraction by adding to the assay

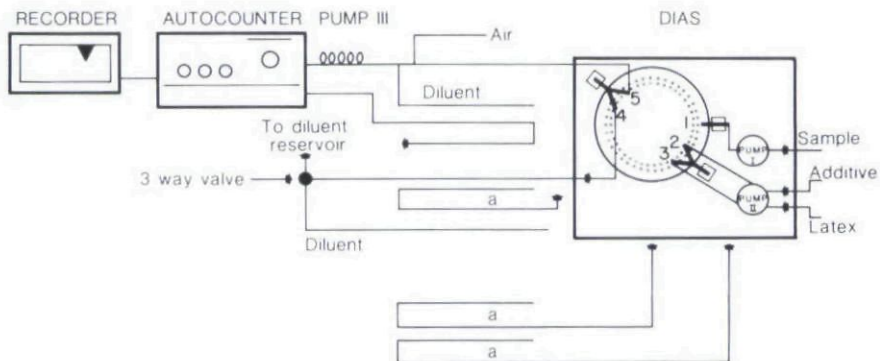


Fig. 1. Flow diagram of PACIA when applied to the determination of IgE. (a) = wash solution to DIAS probe reservoirs. Probe 1 rises out of the wash reservoir and drops into the sample cup. Pump I aspirates 30 μ l of the diluted sample. Probe 1 rises out of the sample, dips into the wash solution to remove external droplets of serum and dips into an empty reaction tube in the outer row of the sampler tray. Pump I discharges the sample and returns to its wash position. The sampler tray indexes forward and comes under probe 2, where, by the action of pump II, 30 μ l of additive is dispensed. Three indexed positions further, a probe 3 delivers 30 μ l of latex also through pump II. After 26 min incubation at 37°C under continuous vortex mixing, 0.88 ml of GBS is released into each tube under probe 4 by pump III and a three-way valve. Three indexed positions further the aspirating probe 5 dips into each reaction tube in turn and the pump III aspirates 0.5 ml of prepared sample, air segments the stream, further dilutes with twenty times the volume of GBS containing 1 ml/l Tween 20 (Technicon) and, after debubbling, pumps the resulting stream through the optical cell counter. Fifty analyses/hr are performed on this apparatus.

mixture 50 μ l of a suspension containing donkey anti-rabbit IgG covalently linked to polymaleic anhydride particles, shaking for 10 min and, finally, centrifuging for 2 min at 1500 *g* before decanting the supernatant solution and counting radioactivity associated with the residual particles. All sera were assayed in duplicate at dilutions of 1/5 and 1/50, and the inter-assay coefficients of variation over the 6-month period for quality control sera of 3.5, 14 and 75 iu/ml were 7.1, 6.3 and 8.3 respectively.

IgE reference standards

All standards were stored at -20°C , and thawed only once before use.

PACIA. The standard (Behring Institute, Marburg/Lahn, FRG—Batch number 046505A) contained 8800 iu/ml, where one unit is approximately 2.4 ng (Bazalar & Hamburger, 1972), and was diluted in NRS-BSA buffer to obtain standards of 1 to 200 iu/ml.

RIA. The standards were prepared from a pool of sera obtained from 20 Phadebas IgE test kits and diluted with 50% horse serum to final concentration of 0.5, 2, 5, 10, 20, 50, 100, and 200 iu/ml.

Serum samples

One hundred and seven sera which had been assayed by RIA were selected so as to provide a range of values from 10 to 50 000 iu/ml for quantification by PACIA. Sera had been stored at -20°C for a period not exceeding 6 months and were obtained from patients whose symptoms were possibly related to atopic allergy. They included eight haemolysed, six icteric and thirty-four turbid samples. All were assayed blind.

Results

Calibration curves

A plot of peak heights (number of non-agglutinated particles) *vs* log IgE concentration formed a sigmoidal curve extending from 1 to 200 iu/ml (Fig. 2).

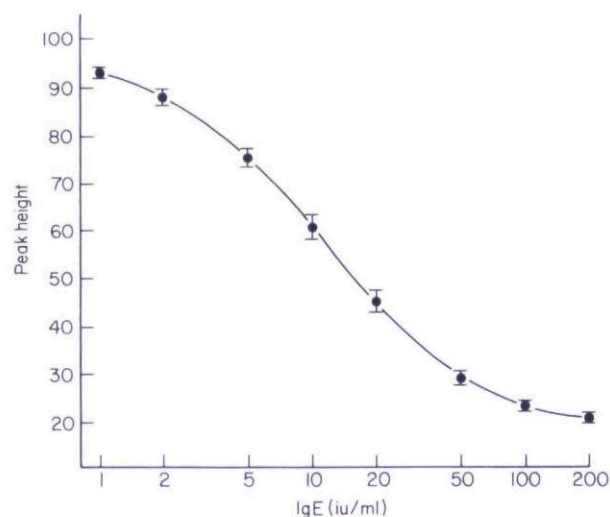


Fig. 2. Standard curve for the determination of IgE by PACIA. This is a composite of thirty-five curves run over 20 days. Vertical bars = 1 s.d. Peak height is proportional to the concentration of non-agglutinated particles flowing through the autocounter.

Non-specific agglutination

The agglutinating activity of the 107 test sera was estimated with latex coated with $F(ab')_2$ fragments from a non-immunised rabbit using the same concentration of $F(ab')_2$ fragments as for the preparation of anti-IgE latex. The sera were diluted ten times as for the IgE determination. The coefficient of variation for the mean concentration of non-agglutinated particles reached 2%, expressed as peak heights, which corresponds to less than 5 iu/ml in the sera. This indicated that non-specific agglutination and/or errors inherent in the whole system were negligible.

Analytical recovery

Nineteen patients' sera containing 10 to 267 iu were mixed with an aliquot of the IgE reference serum to get a supplement of 200 iu IgE in each sample. The mean IgE recovered was 101.9% with an s.d. of ± 13.4 . As a check for parallelism five sera were serially diluted (1/10, 1/20, 1/40 and 1/80). The coefficients of variation on the calculated values ranged from 2.3 to 8.2%.

Accuracy

Each serum was assayed in duplicate and when necessary further dilutions were made so as to be sure of interpolating the IgE levels from a reliable part of the standard calibration curve. The coefficient of correlation for values between 10 and 50 000 iu/ml was $r=0.985$, with a linear regression line of $y=0.82 \times + 130.00$ (Fig. 3), and included some discrepant results (Table 1). The corresponding sera were analysed again with PACIA using the anti-Fc' and the anti-Fc antisera. Interestingly enough two samples gave relatively low values in RIA and PACIA with the anti-Fc antiserum compared to the results obtained with the anti-Fc' antiserum. The presence of proteolytic fragments of IgE reacting with the anti-Fc' antibodies and not or less with the other antisera could be responsible for the discrepancy. The low slope seen in the correlation is attributed to the high influence of some IgE-rich sera. If eight values, those exceeding 10 000 iu/ml, are omitted from the correlation the regression line becomes $y=0.95 \times - 7.50$ with a correlation coefficient of $r=0.989$. The discrepancies observed for the high values can be explained by the increased possibility of dilution errors and are not due to major differences in the standards used.

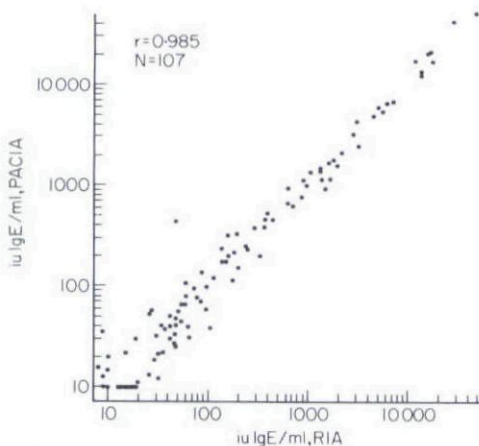


Fig. 3. Correlation between the RIA and PACIA results for IgE.

Table 1. Discrepant results (iu/ml between RIA and PACIA)

		PACIA (anti-FC')				PACIA (anti-Fc)			
		Serum dilutions				Serum dilutions			
RIA		1/10*	1/10	1/20	1/40	1/10	1/20	1/40	
106	ND	38	40	ND	ND	17	<20	<40	
37	34	425	370	350	ND	70	70	68	
157	161	320	315	285	304	200	182	178	
9	ND	36	<10	<20	ND	13	<20	<40	
189	ND	330	170	155	182	175	168	187	
134	ND	240	167	190	ND	139	153	178	

* Values obtained on a first series of aliquots and used for the correlation study (Fig. 3). The PACIA assay was repeated on a second series of aliquots taken from the same samples.

ND=not done.

Table 2. Precision of PACIA in its application to the determination of IgE

Within run*		Between run†	
Concentrations iu/ml $\pm$ s.d.	Coefficient of variation (%)	Concentrations iu/ml $\pm$ s.d.	Coefficient of variation (%)
12.5 $\pm$ 1.5	12.0	30.5 $\pm$ 3.9	12.8
62.0 $\pm$ 8.0	12.9	132.2 $\pm$ 13.0	9.8
115.0 $\pm$ 5.0	4.3	192.0 $\pm$ 16.3	8.5
372.0 $\pm$ 20	5.4	311.2 $\pm$ 27.6	8.9
1275.0 $\pm$ 75	5.9	956.5 $\pm$ 106.1	11.1

* Twenty assays repeated on the five samples within a day with one standard curve.

† Assay repeated during 20 days with a new latex suspension taken every day from the same lyophilized batch.

Precision

The PACIA precision was studied using five sera with concentrations covering the range of the calibration curve (Table 2). Maximal intra-assay and inter-assay coefficients of variation were 12.9% and 12.8% respectively.

Discussion

Accuracy, precision and sensitivity

We have measured IgE levels ranging from 10 to 50 000 iu/ml in 107 serum samples using PACIA and RIA. The correlation coefficient ($r=0.985$) was comparable to those

reported by Merrett & Merrett (1978) in a study of four different RIAs. When comparing two RIAs based on different principles, i.e. the conventional double antibody system with the sandwich technique, they obtained correlation coefficients of $r=0.995$ between two personal methods and $r=0.973$ between a commercial technique (Phadebas IgE Test, often called RIST) and the personal sandwich technique (analogous to Phadebas IgE PRIST, but using particles instead of paper discs). The precision of PACIA also is satisfactory as the maximal inter-assay CV for a concentration of 30.5 iu/ml was 12.8% which has to be compared to the 11.6% and 14.2% values which the authors reported for a similar IgE level using Phadebas RIST and PRIST respectively.

However, the precision for an automated system was expected to be better. In another study performed on the assay of α_1 -foetoprotein by PACIA, we also observed about the same coefficient of variation (Collet-Cassart *et al.*, 1981). In that work we used a special protocol allowing for the evaluation of carry-over and drift. The carry-over was weak, as it did not exceed 1.3%, but the drift was found to be relatively important and probably accounts for most of the variations observed in PACIA.

PACIA, like RIA, was sensitive to the low picomolar range and 10 iu/ml could be measured with confidence. Therefore, the technique was adequate for the routine estimation of IgE levels since the geometric mean for an adult U.K. population is 21 iu/ml (Nye *et al.*, 1975).

Main advantages of PACIA

These are particularly evident when the operation list for RIA is considered. Automation, which was facilitated by the absence of separation steps, reduces the work for PACIA to the predilution step, placement of the sample in the tray, and calculation of results. A short centrifugation (5000 rev/min for 5 min) of the samples, although not always necessary, is, however, recommended to avoid the presence of particles which may interfere with latex counting, e.g. chylomicrons. The simplicity of these operations contrasts with the many steps required by RIA and enzymeimmunoassay (Weltman *et al.*, 1976) of IgE. Moreover, in most of these techniques, the incubation time is 20 hr, whereas the complete throughput in PACIA is 35 min. The absence of radioisotopes and purified IgE, which can be difficult to obtain, are other obvious advantages.

Precautions to be observed with PACIA

Competing antigen is absent, and so the specificity of the antiserum used to coat the particles must be carefully checked. In this work, the specificity of the anti-IgE antiserum was checked for both its reaction with other serum proteins and also for the various antigenic determinants of the IgE molecule. Our antiserum was clearly directed against determinants which are common to $F(ab')_2$ and Fc fragments, and characteristic of the Fc'' fragment (not shown). Such an antiserum should be superior to antisera directed against either whole IgE, Fc, or ϵ -chain because the specificity is restricted to determinants which are resistant to heat (Dorrington & Bennich, 1978) and to some proteases. Hence, this antiserum can be used for IgE assay even in samples which have been stored under bad conditions, e.g. transportation without cooling, or, perhaps, contamination by bacteria.

Another possible inconvenience of PACIA is the risk of a prozone which corresponds to a decrease of agglutination in the presence of an excess of agglutinator.

However, with the preparation of latex used in this work, no prozone was observed even when the IgE concentration reached 2000 iu/ml in the standard curve.

Techniques which use insolubilized antibodies such as PACIA, RIST- and PRIST-like assays, various factors e.g. antibodies in patients' sera can react with the insolubilized anti-IgE antibodies and, by masking them, decrease their binding capacity. In RIST-like assays, which are based on a competition for the insolubilized anti-IgE between labelled IgE and the sample IgE, the results will be abnormally elevated since less radioactive IgE will be bound (Merrett & Merrett, 1978; Gleich & Dunnette, 1977). In sandwich assays like PRIST, the insolubilized anti-IgE antibodies are used to extract the IgE to be determined and to present it, in a second step, to labelled anti-IgE antibodies. In this case, the results tend to be too low (Merrett & Merrett, 1978). In PACIA, interaction of antibodies against the animal immunoglobulin used to coat latex can result in non-specific agglutination or inhibition of the specific agglutination. Two main types of antibodies could interfere by reacting with the insolubilized IgG: (i) rheumatoid factor, which is an anti-IgG auto-antibody mainly reacting with aggregated IgG, particularly those of human and rabbit origin; (ii) anti-bovine IgG antibodies which occur in many human sera and often cross-react with sheep IgG. Interferences due to such cross-reactions have been described for the RIST and RAST (Foucard *et al.*, 1975); In PACIA, the risk of interference by rheumatoid factor is avoided by the use of $F(ab')_2$ fragments to coat latex. To prevent the effect of putative anti-rabbit IgG antibodies which could occur in patients' sera, samples were diluted in buffer containing normal rabbit serum. It should be noted that antibodies against $F(ab')_2$ fragments have been described in various patients, but these antibodies do not react with $F(ab')_2$ of heterologous IgG (Waller & Duma, 1972).

In addition to antibodies, substances such as complement or any other serum protein having some affinity for $F(ab')_2$ could interfere in the test. To decrease the risk of these non-specific interactions, we used conditions preventing activation of complement and protein-protein interactions. These conditions were pH 9.2, high ionic strength (1 M NaCl) and chelation of bivalent cations by EDTA. The addition of polyethylene glycol to the reaction medium was to enhance the antigen-antibody reaction and the specific agglutination process.

In conclusion, PACIA is a technique that appeals instantly, because of its simplicity and ease of automation. For the quantification of serum IgE it requires only anti-IgE latex particles, which are stable for at least 6 months; no additional reagents such as complement, labelled tracers, competitive antigen or separating agent are necessary. Furthermore, it is fully automated, requires only a short incubation time, and yet can achieve a sensitivity in the low picomolar range making it a suitable screening test for total IgE.

Acknowledgments

We are grateful to Dr J. P. Vaerman in this laboratory for providing serum from myeloma E patient Des, Dr J. C. Daniel of Rhône-Poulenc, Courbevoie, France for supplies of latex, Dr H. Holy for reviewing the manuscript, Mrs T. Vandeberghe-Briet, and Mr J. Knops for their skilful technical assistance. We are particularly indebted to C.L. Cambiaso for his continuous advice and unvaluable help.

This work was supported by Technicon Instruments Corp., Tarrytown, N.Y., and the Post Office and Civil Service Sanatorium Society.

References

- ADKINSON, N.F., JR (1980) The radioallergosorbent test. Uses and abuses. *Journal of Allergy and Clinical Immunology*, **65**, 1.
- BAZARAL, M. & HAMBURGER, R.N. (1972) Standardization and stability of immunoglobulin E (IgE). *Journal of Allergy and Clinical Immunology*, **49**, 189.
- CAMBIASO, C.L., GOFFINET, A., VAERMAN, J.P. & HEREMANS, J.F. (1975) Glutaraldehyde-activated aminoethyl-derivative of Sepharose 4B as a new versatile immunosorbent. *Immunochemistry*, **12**, 273.
- CAMBIASO, C.L., LEEK, A.E., DE STEENWINKEL, F., BILLEN, J. & MASSON, P.L. (1977) Particle counting immunoassay (PACIA). I. A general method for the determination of antibodies, antigens and haptens. *Journal of Immunological Methods*, **18**, 33.
- COLLET-CASSART, D., MAGNUSSON, C.G.M., RATCLIFFE, J.G., CAMBIASO, C.L. & MASSON, P.L. (1981) Automated particle counting immunoassay for alpha-fetoprotein. *Clinical Chemistry*, **27**, 64.
- DORRINGTON, K.J. & BENNICH, H.H. (1978) Structure-function relationships in human immunoglobulin E. *Immunological Reviews*, **41**, 3.
- FOUCARD, T., BENNICH, H., JOHANSSON, S.G.O. & LUNDQVIST, U. (1975) Human antibodies to bovine γ -globulin. *International Archives of Allergy and Applied Immunology*, **48**, 812.
- GLEICH, G.J. & DUNNETTE, S.L. (1977) Comparison of procedures for measurement of IgE protein in serum and secretions. *Journal of Allergy and Clinical Immunology*, **59**, 377.
- HELLSING, K. (1975) Letter to the editor. *Immunochemistry*, **9**, 753.
- HOLY, H.W. (1979) Technicon PACIA system monograph 1. Instrumentation. Technicon International Division, S.A., Geneva, Switzerland, 1979.
- MERRETT, T.G. & MERRETT, J. (1978) Methods of quantifying circulating IgE. *Clinical Allergy*, **8**, 543.
- NYE, L., MERRETT, T.G., LANDON, J. & WHITE, R.J. (1975) A detailed investigation of circulating IgE levels in a normal population. *Clinical Allergy*, **5**, 13.
- VAERMAN, J.P. (1979) A new case of IgE myeloma (Des) ending with renal failure. *Journal of Clinical and Laboratory Immunology*, **2**, 343.
- WALLER, M. & DUMA, R.J. (1972) Increased antibodies to IgG fragments: correlations with infection due to gram-positive bacteria. *Journal of Infectious Diseases*, **125**, 45.
- WELTMAN, J.K., FRACKELTON, A.R., SZARO, R.P. & ROTMAN, B. (1976) A galactosidase immunosorbent test for human immunoglobulin E. *Journal of Allergy and Clinical Immunology*, **58**, 426.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.