

DETECTION OF IgE(IgND) SPECIFIC ANTIBODY (PROBABLY REAGIN) TO CASTOR-BEAN ALLERGEN BY THE RED-CELL-LINKED ANTIGEN-ANTIGLOBULIN REACTION

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Summary Tests have been performed on the sera of patients with allergic asthma to castor-bean allergen. The patients lived in an environment where the atmosphere was polluted with dust from a castor-bean mill. IgE-specific antibody to castor-bean allergen could be detected by the red-cell-linked antigen-antiglobulin reaction in the unheated serum of patients with allergic asthma to castor-bean allergen. The probability is that the antibody measured was reagin. This follows from what is known about IgE, its heat-lability, and the correlation with the clinical state of the patients and with passive cutaneous anaphylaxis reactions in the baboon.

Introduction

THE red-cell-linked antigen-antiglobulin reaction (R.C.L.A.A.R.) was introduced in prototype by Coombs et al. (1953) and Coombs and Fiset (1954). Further studies were reported by Steele and Coombs (1964) and Coombs et al. (1965). The reaction was originally developed to answer the need for a sensitive passive hæmagglutination procedure capable of detecting "incomplete" or non-precipitating antibodies to soluble antigens or allergens. A special stimulus in its development was the possibility that it would detect reagin antibody in the serum of atopic patients.

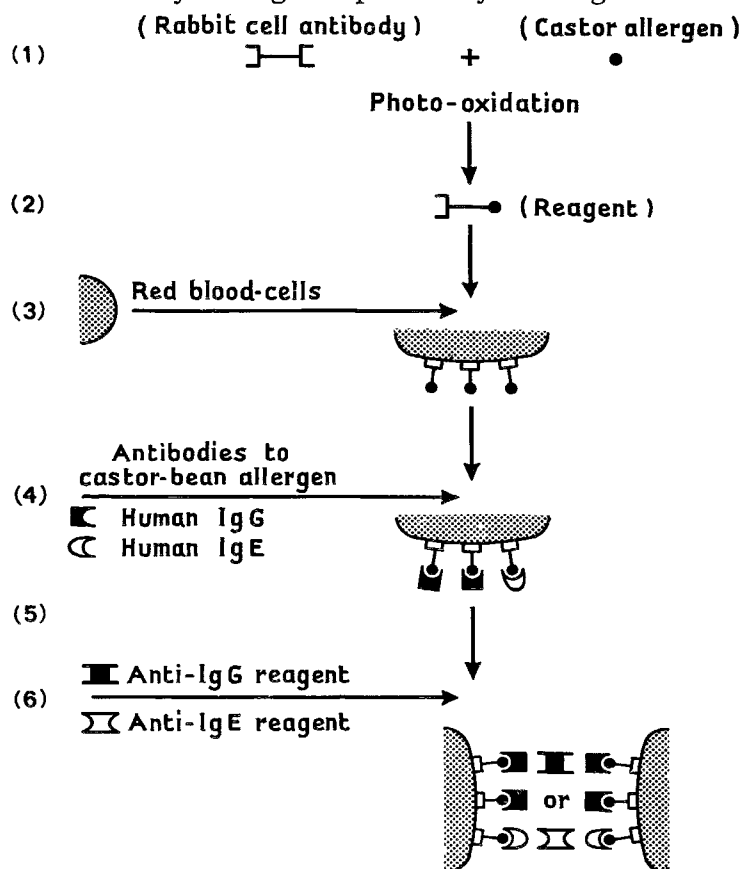
In one modification of the reaction the allergen or antigen is coupled by photo-oxidation to rabbit antibodies to human red-cells. Rabbit red-cell antibodies so coupled no longer agglutinate human red-cells but combine with the red-cells, and in so doing link the coupled antigen to the cells (see figure). These allergen-linked red-cells behave well in that they do not absorb human globulin non-specifically—as do red-cells tanned and coated with protein allergens or red-cells directly covalently linked with protein allergens by tetrazotised benzidine (Friedmann, Hunter, and Coombs 1968)—but may be agglutinated by specific antibody to the attached allergen. Also, specifically reacting but non-agglutinating antibody to the linked allergen may be detected by a subsequent antiglobulin reaction—i.e., the antigen-linked cells after exposure to the human test serum are washed, exposed to rabbit anti-human globulin serum, and then read for subsequent agglutination. With use of antiglobulin sera specific for each of the known immuno-

globulin heavy chains it is possible to recognise the immunoglobulin class of the reacting antibodies in the human serum under examination.

The experiments reported here were begun in 1965 when the prevailing opinion was that human reagin was IgA. Sera were obtained from patients in the South of France living near a castor-bean factory. Many of these patients had allergic asthma due to inhalation of dust containing castor-bean protein allergen.

The protein of the castor-bean proved to be easy to couple by photo-oxidation (Steele and Coombs 1964) to rabbit antibody to human red-cells, allowing the sera to be examined for IgG and IgA antibodies to castor-bean protein. It was not surprising that IgG antibodies could be detected, often to high titre. IgA antibodies were also detected at a lower titre but there seemed to be no obvious correlation with the clinical status of the patients. A year later the same sera were re-examined with μ -specific and δ -specific antiglobulin reagents, but again with no significant results in relation to likely reagin antibody content.

Recently a myeloma protein has been detected (Johansson and Bennich 1967) which has been found to represent a new class of human immunoglobulins, provisionally called IgND (Johansson, Bennich, and Wide 1968). IgND and the E-globulin of Ishizaka et al. (1966) share antigenic determinants, and at a meeting at the World Health Organisation international reference centre for immunoglobulins in Lausanne, in February, 1968, it was stated that they belong to a previously unrecognised class



Diagrammatic representation of the R.C.L.A.A.R.

- (1) Photo-oxidation to make dimer of rabbit cell antibody and castor-protein allergen.
- (2) The reagent, which can be stored at -20°C .
- (3) Immersing red-cells in diluted reagent links allergen to the red-cell surface.
- (4) Exposure of allergen-linked red-cells to human serum under test.
- (5) Antibodies adsorbed and cells washed.
- (6) Agglutination of sensitised cell suspension by selective specific antiglobulin reagents.

of human immunoglobulins which should be designated IgE (γ E) (World Health Organisation 1968).

There is increasing evidence that reaginic antibody is IgE: isolated E-myeloma ND inhibits specifically the Prausnitz-Küstner reaction (Stanworth, Humphrey, Bennich, and Johansson 1967) and ragweed antibodies of γ E-type are in many respects similar to reagins (Ishizaka and Ishizaka 1967).

We report here further examination of these same sera from castor-bean-sensitive patients by the R.C.L.A.A.R. using an antiglobulin serum specific for IgE; the results suggest that reaginic antibody can be detected by this test if a specific anti-IgE antiglobulin serum is used.

Material and Methods

Human Sera

Blood was withdrawn by venepuncture from allergic patients (whose clinical history is summarised in table 1) living in the South of France. The separated sera were freeze-dried for transportation to the U.K. and were then reconstituted and stored at -25°C till tested. The sera were not heated or absorbed in any way, unless stated in the text.

Castor-bean Allergen

Dericated castor-bean allergen was kindly supplied by Dr. F. H. Milner of the Bencard Allergy Unit, Brentford, Middlesex. This had been prepared as follows: 50 g. of

TABLE 1—TITRE OF CASTOR BEAN PROTEIN-REACTING ANTIBODIES OF DIFFERENT IMMUNOGLOBULIN CLASSES IN THE SERA OF PATIENTS LIVING IN THE NEIGHBOURHOOD OF A CASTOR-BEAN OIL MILL

Patient no.	Clinical history *	D.A.	Antibody titre measured by the R.C.L.A.A.R.					Reagin titre measured by P.C.A. reaction in baboon skin
			IgG	IgA	IgM	IgD	IgE	
A	1 2 3 5	0	32	8	4	0	8	..
B	1 2 3 5	8	>521	32	32	≤8	..	..
C	1 2 3 5	0	32	8	0	0	4	..
D	1 2 3 5	0	128	8	0	0	16	..
E	1 2 3 5	4	256	16	8	≤4	64	..
F	1 2 3 5	2	32	8	2	≤2	64	125
G	1 2 3 5	2	32	8	2	≤2	8	..
H	1 2 3 5	4	>512	16	8	≤4	16	>25
I	1 2 3 5	4	64	16	8	≤4	32	..
J	1 2 3 4	2	64	8	2	≤2	8	..
K	1 2 3 4	2	256	8	8	≤2	16	..
L	1 2 3 5	0	256	8	4	0	16	..
M	1 2 3 4	0	32	0	0	0	4	..
N	1 2 3 5	8	2000	128	64	≤8	256	>125
O	1 2 3 5	0	8	0	0	0	0	0 (? neat)
P	(1 2 3) 6	0	256	8	0	0	0	0 (? neat)
Q	1 7 8	0	4	0	0	..	0	..
R	1 4 7 8	0	4	0	0	..	0	..
S	1 8	0	64	4	0	..	0	0
T	1 7 8 9	0	2000	128	2	..	0	0
U	11	0	0	0	..	..	0	0
V	7 10	0	(2)	0	..	..	0	0
W	11	0	0	..	..	..	0	0
X	7 10	0	4	..	..	..	0	..
Y	7 10	0	4	..	..	..	0	..
Z	7	0	4	..	..	..	0	..

0=2 except for patients O and P which were tested undiluted for IgE antibody.
(..)=Very weak reaction.
D.A.=Direct agglutination.
P.C.A.=Passive cutaneous anaphylactic reaction.
* Footnote for clinical history: (1) patient living in the vicinity of castor-bean mill or warehouse or works in oil mill, (2) clinical asthma to castor bean, (3) positive skin test to castor-bean extract, (4) has received injections of castor pollen extract (not bean extract) for hyposensitisation, (5) patient has not received hyposensitising injections, (6) patient thought to have developed "blocking antibody" by continuous contact with mill, as clinical cure but never received hyposensitising injections, (7) patient has allergic asthma to other inhalants but not to castor-bean protein, (8) negative skin test to castor-bean extract, (9) patient has worked in castor-oil mill since 1936, (10) hayfever patient, (11) non-allergic healthy controls.

castor-bean seed is crushed to a fine meal, and placed in a glass flask with 200 ml. of distilled water, the flask and its contents are heated for 4 hours on a boiling water bath, and the resulting extract after cooling, is separated from the insoluble solids by filtration through a fluted Whatman no. 1 filter-paper. To the extract is added one quarter of its volume of 95% ethanol (or industrial methylated spirit prepared from 19 volumes of 95% ethanol and 1 volume of methanol). The resulting precipitate is removed by centrifugation and discarded. To the supernatant liquid is added at least four times its volume of 95% ethanol or industrial spirit. The precipitate so produced is separated by centrifugation, washed with a further small quantity of 95% ethanol, and dried in a vacuum desiccator over calcium chloride or silica gel.

For our own experiments 1 g. of the powder was extracted in 10 ml. saline solution. The extract was centrifuged free of insoluble material, dialysed, and then absorbed repeatedly with washed red-cells to remove any residual ricin. By Folin estimation the extract gave a protein concentration of 30 mg. per ml.

Reagents for R.C.L.A.A.R.

The principles, methodology, and standardisation of such reagents have been described in detail by Steele and Coombs (1964). Briefly, a 1% solution of a globulin fraction (40% ammonium-sulphate precipitate) of rabbit anti-human red-cell serum was mixed with an equal volume of a saline extract (1% protein) of dericated castor-bean meal and photo-oxidised using rose-bengal as the sensitiser.

After tests for coupling, the suitable dilution for coupling castor seed allergen to human red-cells could be gauged.

Antiglobulin Reagents Specific for IgG, IgA, IgM, IgD, and IgE

Anti- γ -chain (specific for IgG), anti- α -chain (specific for IgA) and anti- μ -chain (specific for IgM) were prepared as described by Coombs et al. (1965). Anti- δ (specific for IgD) was kindly supplied by Dr. David Rowe, department of experimental pathology, University of Birmingham. It was raised against an IgD myeloma protein and absorbed with a human serum deficient in IgD but containing normal quantities of IgG, IgA, and IgM. Anti-IgE was raised in rabbits using the 5 S Fc-fragment (Bennich and Johansson 1967) of E-myeloma ND, and it was absorbed with excess of a serum from a healthy, non-allergic, individual with low IgE concentration. The antiserum was found to be specific for the Fc-fragment of E-myeloma ND as revealed by immunoelectrophoresis and gel diffusion.

All the antiglobulin reagents were heat-inactivated and absorbed free of human red-cell agglutinins with human red-cells which had been washed six times.

R.C.L.A.A.R.

The test has been fully described by Steele and Coombs (1964) and Coombs et al. (1965). In brief, castor-bean allergen was coupled to human red-cells by incubating equal volumes of a 4% suspension of group-O human red-cells and castor-bean-allergen-coupled red-cell antibody reagent diluted 1/10. The cells were then washed and, as a 2% suspension, added to serial dilutions of the unheated human sera under test. After 1 hour's incubation at room temperature the cells in each tube were washed twice to remove uncombined serum-proteins, and adsorption of specific antibody was tested for by adding test drops from each tube to tubes containing reagents specific for human IgG, IgA, IgM, IgD, and IgE, all at their optimal dilutions. With the antiglobulin sera used the optimal dilutions were 1/1000, 1/200, 1/40, 1/200, and 1/500, respectively. Drops of serum-treated antigen-linked red-cells were also placed in a 1/100 dilution of normal rabbit serum as controls. The contents of the tubes were mixed and subsequent agglutination read by the pattern of sedimentation of the cells.

Passive Cutaneous Anaphylaxis in Baboon Skin

This was performed according to the procedure outlined by Layton et al. (1963). After tranquillisation, 0.1 ml. amounts of

dilutions of sera were injected intradermally. 48 hours later, again after tranquillisation, a mixture of 2½ ml. 1% Evans-blue in saline solution and 2 ml. castor-bean extract containing 40 mg. protein was injected intravenously. Strong reactions—seen as blueing of the skin came up within minutes. The final reaction was read after 20 minutes. The castor-bean extract had also been absorbed with human red-cells to remove any residual ricin.

Results

Tests for Specific Antibodies Using the R.C.L.A.A.R.

The unheated sera from patients sensitive to castor-bean protein were examined by the R.C.L.A.A.R. using castor-bean-protein-linked red-cells and the immunoglobulin class of the reacting antibodies measured with antiglobulin reagents specific for heavy-chain determinants. The results, together with the clinical status of the patients, are recorded in table I.

Examination of Reaginic Antibody by Passive Cutaneous Anaphylaxis

Three sera showing IgE antibody to castor-bean allergen and six sera without such antibody were titrated in a passive transfer test as described by Layton et al. (1963). The results are recorded in table I. The strong reaction obtained with serum F was abolished when the serum was heated at 56°C for 1 hour.

Observations on the Results of the Above Tests

None of the sera had strong agglutinating activity for the allergen-linked red-cells but many sera possessed combining IgG, IgA, and IgE antibodies which were revealed by the antiglobulin test.

In all cases IgG antibody was present to the highest titre and was present in some patients (especially patients S and T) who had no clinical asthma to the castor allergen. The tests for IgA antibody were of interest two years ago when they were first performed because of the then supposition that reagin may have been IgA, but again patients S and T possessed this antibody but had no clinical allergic manifestations. Also the IgA antibodies as well as the IgG antibodies were stable to 4 hours heating at 56°C. The sera were also tested for IgM antibodies and the results are recorded in table I; no special comment is needed. The sera were screened for possible IgD-specific antibodies but no unequivocal positive results were obtained.

The results obtained with the specific antiglobulin serum-anti-IgE were of great interest. Nearly all the patients with allergic asthma to the castor allergen had measurable IgE reacting antibody, to quite high titre in certain cases (i.e., patients F and N). No IgE antibody was found in six sera from persons living in Cambridge (U, V, W, X, Y, and Z), although three of these suffered with hayfever. Of special interest was the absence of specific IgE antibody in patients Q, R, and T who lived near the castor-bean mill, had allergic asthma to house dust, yet had no clinical or skin sensitivity to castor-bean allergen. Patient T had worked at the mill since 1936, had an IgG antibody to a titre of 2000, an IgA titre of 128, yet he had no detectable IgE antibody.

The results of the passive cutaneous anaphylaxis tests correlated extremely well with the findings of the R.C.L.A.A.R., using the anti-IgE serum. Three sera, F, H, and N with high IgE antibody all gave powerful blueing reactions in baboon skin. Titrations were performed in the baboon skin but the reactions were so strong with the more concentrated serum samples that they spread and interfered with the test sites of the more diluted samples. The skin test was probably somewhat more

TABLE II—TITRATION OF SERA FROM TWO CASTOR-BEAN-SENSITIVE PATIENTS USING DIFFERENT DILUTIONS OF THE ANTI-IgE SERUM

Antiglobulin reagent	Dilution	Titre of IgE antibody to castor allergen *	
		Patient F	Patient I
Normal rabbit serum ..	1/40	2	2
Anti-IgE	1/40	8	8
	1/200	16	16
	1/500	64	32
	1/1000	32	32
	1/2000	8	32

* In neither serum did heating at 56°C for 4 hours lower the IgG antibody, but it did completely inhibit the IgE-reacting antibody. In serum F the IgE antibody lost reactivity by heating for half an hour at 56°C.

sensitive than the passive agglutination test. All the sera which failed to show IgE antibody in the R.C.L.A.A.R. also failed to give a blueing reaction in passive cutaneous anaphylaxis.

Two sera call for special mention: patient O, a farmer with asthma and allergic rhinitis on contact with castor-bean pomace, also having a positive skin test; and patient P who had asthma to castor allergen but who underwent natural hyposensitisation while working in the castor mill. Both sera were negative for IgE antibody by the R.C.L.A.A.R., but were also negative when tested by passive cutaneous anaphylaxis although at the test site with undiluted serum the weakest suspicion of blueing could be seen.

Optimal Dilution of Anti-IgE Serum and Heat-stability of the IgE Antibody

Two sera, F and I, were serially diluted and tested against red-cells linked with castor allergen. After incubation and washing, the cells were tested for adsorbed IgE antibody using different dilutions of the anti-IgE antiserum. Optimal results were obtained with the anti-IgE serum diluted in the order of 1/500 (table II). This dilution was used in the tests reported.

Heating the two human sera for 4 hours at 56°C destroyed the detectable combining activity of the IgE antibody although it did not affect the IgG antibody. 56°C for half an hour was sufficient to destroy the detectable combining activity of the IgE antibody in patient I.

From these findings we cannot conclude whether the antibody structure or the E(ND)-determinants or both are heat-labile. Other results (Ishizaka et al. 1967, Johansson 1968) seem to indicate that the antigenic determinants of IgE are more heat-labile than its antibody activity.

Discussion

There is increasing evidence that reaginic antibodies are identified as IgE, which has recently been established as a new class of human immunoglobulins. Allergen-specific antibodies of the IgE (IgND) type have been detected in serum of patients with atopic allergy (Wide, Bennich, and Johansson 1967). A very good correlation (96% agreement) was found between the presence of such antibodies and the results of provocation tests. It is very important to be able to verify this finding in another laboratory using another method. The R.C.L.A.A.R. is most suitable in this respect since it is strictly immunoglobulin specific and the main objective in its development was to get a test which might afford a simple procedure for detection of reaginic antibody (Coombs et al. 1953, 1965).

By coupling the allergen to a red-cell antibody and by

this means fixing the allergen to the red-cell surface no damage is done to the red-cell membrane which may allow non-specific absorption of γ -globulin. Thus any uptake of immunoglobulin on to the antigen-linked red-cells as detected by an antiglobulin reaction becomes meaningful. The full potential of the method has only become realised with the development of antiglobulin reagents specific for the different immunoglobulin classes and now the development and characterisation of specific anti-IgE (Bennich and Johansson 1967) is of the greatest significance.

The correlation of IgE antibody titres to castor allergen in the patients studied, with their clinical status, with the passive cutaneous anaphylaxis reactivity in baboon skin and the heat-lability of the reacting antibody strongly support the reaginic nature of the measured antibody.

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IMMUNOGLOBULIN LEVELS IN ETHIOPIAN PRESCHOOL CHILDREN WITH SPECIAL REFERENCE TO HIGH CONCENTRATIONS OF IMMUNOGLOBULIN E (IgND)

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Summary The immunoglobulins G, A, M, and D were determined in three series of Ethiopian preschool children. The levels of IgG and IgD were significantly higher than those found in a group of Swedish children of the same age; the mean levels of IgD being five to six times higher than in the Swedish series. The concentrations of the new immunoglobulin class, IgE, were also studied. The levels of IgE were between sixteen and twenty times higher than in Swedish children, and in a group with verified infection with *Ascaris lumbricoides* the level was twenty-eight times higher. These findings favour the hypothesis that parasitic infestations are important factors in stimulating IgE production.

Introduction

THE frequent reports of high incidences of raised serum-immunoglobulin levels in Africans compared with Europeans have been reviewed by Turner and Voller (1966) and Rowe et al. (1968). It is usually suggested that the

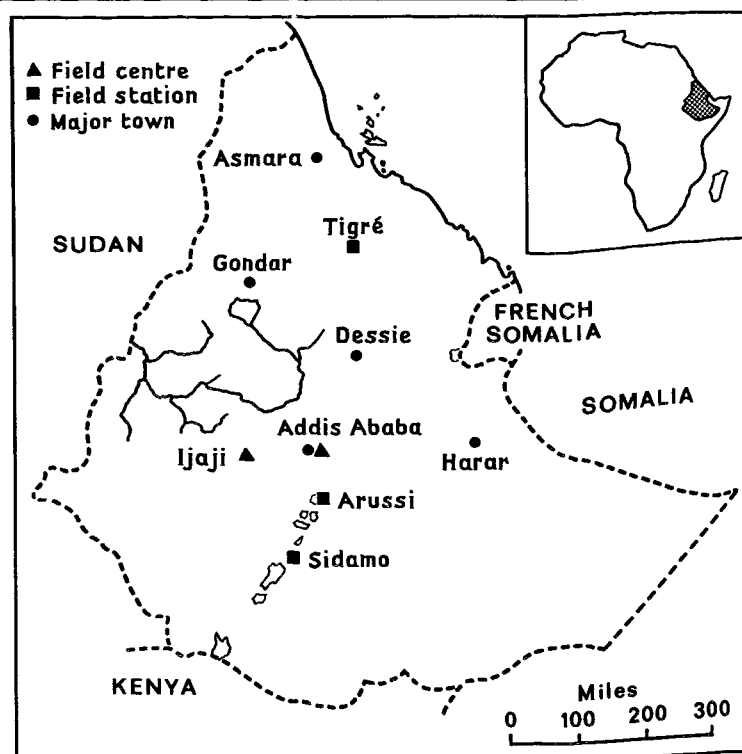


Fig. 1—Field centres and stations belonging to the Children's Nutrition Unit.

raised levels are due to an intense challenge with bacterial, viral, protozoal, and helminthic infections and only to a minor extent, if at all, to racial differences. Very few studies using immunological techniques have been carried out on children (Rowe et al. 1968). Since conditions may vary from one African region to another, a further comparative study, of the levels of IgG, IgA, IgM, and IgD in Ethiopian and Swedish children, seemed warranted.

Strikingly high levels of a new immunoglobulin, IgE* (Johansson, Bennich, and Wide 1968) which seems to be of importance in atopic allergy (Johansson 1967, Stanworth, Humphrey, Bennich, and Johansson 1967, Wide, Bennich, and Johansson 1967) have been found in cases of asthma and hay fever (Johansson 1967, Johansson and Bennich 1967), while bacterial and viral infections do not seem to influence the levels (Johansson and Bennich 1968). It seemed of especial interest to analyse the situation in African children with respect to this new immunoglobulin fraction.

The serum samples were collected as part of the activities at the Children's Nutrition Unit (C.N.U.) in Addis Ababa. This unit, which represents a technical assistance programme between Sweden and Ethiopia, came into operation in 1962 (Ågren et al. 1966) and has carried out investigations directed especially towards the incidence and type of undernutrition in children from birth up to and including the age of ten years. The investigations have also included an analysis of the general state of health of the children.

Material

The C.N.U. has started field activities in various parts of Ethiopia, representing different cultures and living conditions (fig. 1). For this investigation, series of serum samples were chosen from Ijaji a typical medium-sized market village in the central Ethiopian highlands, and from Sidamo village, a more isolated rural community in the southern part of the country. Except for the age selection (table 1) and the time period (autumn, 1964) the specimens from Ijaji and Sidamo were

* At a nomenclature meeting at the World Health Organisation immunoglobulin reference centre at Lausanne in February, 1968, the designations γ E-globulin and IgND were replaced with the designation IgE (γ E) (World Health Organisation 1968).