

Research Paper

Assessment of determinants of optimum performance of the CAST[®]-2000 ELISA procedure

Cathrine Van Rooyen^{a,*}, Ronald Anderson^b

^a*Department of Medical Virology, Faculty of Health Sciences,*

University of Pretoria and Tshwane Academic Division of the National Health Laboratory Service, P.O. Box 2034, Pretoria 0001, South Africa

^b*Department of Immunology/Medical Research Council Unit for Inflammation and Immunity, Faculty of Health Sciences,*

University of Pretoria and Tshwane Academic Division of the National Health Laboratory Service, South Africa

Received 7 August 2003; received in revised form 22 October 2003; accepted 4 December 2003

Available online 4 February 2004

Abstract

The Cellular Antigen Stimulation Test (CAST[®]), a recent innovation in the laboratory detection of allergen sensitivity, is a functional assay based on the release of sulphidoleukotrienes (LTs) from allergen-activated circulating basophils. The current study was undertaken to establish optimum determinants of this procedure. The results demonstrate that treatment of blood with preservative-free heparin in polypropylene containers, and removal of erythrocytes by selective lysis with 0.83% ammonium chloride are excellent alternatives to the use of CAST[®] venepuncture tubes and dextran, respectively. Similarly, decreasing and increasing the leucocyte concentration by 0.5- and 4-fold, respectively, as well as omission of interleukin-3 (IL-3) were also without significant effect. However, treatment of leucocyte suspensions with bacterial endotoxin (>1 µg/ml) activated the release of sulphidoleukotrienes from basophils and/or other types of leucocytes. The findings of this study are clearly relevant, both with respect to cost and performance, when implementing the CAST[®] in clinical immunology laboratories.

© 2004 Published by Elsevier B.V.

Keywords: Basophils; CAST[®]; Dextran; Heparin; Endotoxin; Interleukin-3; Sulphidoleukotrienes

1. Introduction

Avoidance strategies are the cornerstone of optimum management of the allergic patient, and are dependent on acquisition of accurate clinical histories verified, where possible, by objective assessment of

allergen sensitivity. In clinical practice, laboratory measurement of circulating allergen-specific IgE antibodies is reasonably sensitive and specific for some categories of allergens, such as inhalant allergens and insect venoms, but is less reliable with respect to confirmation of food and drug allergies (Wedi et al., 1996; Humbert et al., 1999; Beckett, 2000). This limitation has been attributed to the existence of IgE-independent mechanisms of mast cell and basophil activation (Humbert et al., 1999; Beckett, 2000; Douwes et al., 2002; Kraneveld et al., 2002; Boyce, 2003). Clearly, reliable and safe laboratory procedures,

Abbreviations: CAST[®], Cellular Antigen Stimulation Test; EDTA, ethylenediaminetetracetic acid; IL-3, interleukin-3; LT, sulphidoleukotriene.

* Corresponding author. Tel.: +27-12-319-2670; fax: +27-12-325-5550.

E-mail address: cvrooyen@medic.up.ac.za (C. Van Rooyen).

which detect both IgE-mediated and IgE-independent sensitivity, have an important role to play in both the diagnosis and management of allergic individuals.

Theoretically, assays based on the measurement of mediator release from circulating basophils following *in vitro* exposure to test allergens should detect both IgE-dependent and -independent basophil activation, and could be used in combination with, or as an alternative to, measurement of allergen-specific IgE (Czech et al., 1996; Ferrer et al., 1996; Maly et al., 1996). Early assays based on measurement of mediator release were not, however, adopted in routine clinical practice, largely as a consequence of their technical complexity, lack of standardisation, and poor cost effectivity (Crockard and Ennis, 2001).

The Cellular Antigen Stimulation Test (CAST®)-2000 ELISA is a novel, commercially available, diagnostic laboratory procedure, which is based on the release of the sulphidoleukotrienes (LTs) C₄, D₄ and E₄ from allergen-activated circulating basophils *in vitro*. This procedure appears to have eliminated many of the limitations which restricted the clinical application of earlier functional assays (De Weck, 1997). Initial clinical evaluation has demonstrated good sensitivity and specificity, underscoring the potential usefulness of this procedure in detecting sensitivity to various categories of allergens, including inhalants, foods and drugs (Ferrer et al., 1998; Busse et al., 2000; Vila et al., 2001).

In the current study, we have attempted to identify patient and laboratory variables which may affect performance of the CAST®-2000 ELISA test and/or reliability of data, as well as modifications which may improve cost effectivity and speed of acquisition of results.

2. Materials and methods

2.1. Blood specimens

Venous blood (35 ml) was taken from six different healthy, non-atopic adult volunteers (four females, two males; average age 41 years) and dispensed in 5-ml volumes into tubes containing ethylenediaminetetraacetic acid (EDTA; for measurement of total and differential circulating leucocyte counts; SA Scientific Products, Johannesburg, South Africa), or preserva-

tive-free heparin (5 units/ml blood, Sigma, St. Louis, MO, USA), as anti-coagulants, and the remaining 25 ml into CAST® venepuncture tubes (Bühlman Laboratories, Allschwil, Switzerland) containing an undisclosed anti-coagulant. The CAST® venepuncture tubes are purchased separately from the CAST®-ELISA kits and are recommended by the manufacturer for use in the CAST®-ELISA. Blood was also taken from seven individuals (five females, two males; average age 38 years), with known sensitivity to house dust mite, confirmed by skin prick testing.

2.2. CAST®-2000 ELISA

Test kits containing all the necessary components for performance of this test according to the manufacturer's recommendations were purchased from Bühlman Laboratories. The CAST®-2000 ELISA, which quantifies the release of LTs C₄, D₄ and E₄ after treatment of circulating leucocytes with test allergen(s), is performed in three stages. These are: (i) leucocyte isolation following removal of most of the erythrocytes by dextran sedimentation, (ii) successive addition of interleukin-3 (IL-3) and test allergen to the cells to achieve sensitisation ("priming") and activation respectively; and (iii) measurement of LTs in the cell-free supernatants using an ELISA procedure. LT release by sensitised cells in the absence of added allergen represents the background value, while maximal release is achieved by treating sensitised cells with an anti-IgE receptor antibody (provided by the manufacturer; concentration and origin undisclosed). The test was performed according to the manufacturer's specifications and the results expressed as picograms sulphidoleukotrienes/ml, a composite measure of LTs C₄, D₄ and E₄.

2.3. Experimental design

Experiments were designed to investigate the influence of each of the following on basal and maximal release of LTs from circulating leucocytes of each non-atopic blood donor: (i) the apparent requirement for CAST® venepuncture tubes, (ii) the necessity for the lengthy (90 min) dextran sedimentation step, (iii) the requirement for IL-3 priming, (iv) endotoxin contamination of buffers and (v) variations in the leucocyte count concentration.

2.4. Requirement for CAST® venepuncture tubes

This was determined by comparing the basal and maximally stimulated LT release responses of basophils isolated from blood collected in CAST® venepuncture tubes, with those of cells isolated from EDTA- or preservative-free heparin-treated blood collected in polypropylene tubes.

2.5. Requirement for dextran sedimentation

In these experiments, the basal and maximally activated LT responses of leucocytes prepared from blood collected into CAST® venepuncture tubes, following dextran (provided by the manufacturer) sedimentation by mixing dextran and blood at a ratio of 2:0.5 for 90 min at room temperature, were compared with those of matched leucocytes prepared by NH₄Cl (0.83%; Merck, Darmstadt, Germany)-treatment of whole blood at 4 °C for 10 min (2 ml blood + 48 ml NH₄Cl).

2.6. Requirement for IL-3

Following dextran sedimentation of blood collected into CAST® venepuncture tubes, the resultant leucocyte-enriched populations were suspended in stimulation buffer with and without IL-3 (provided by the manufacturer) and basal and maximal LT release responses compared.

2.7. Effects of added endotoxin

For these experiments, leucocytes were prepared by dextran sedimentation of blood collected into CAST® venepuncture tubes and resuspended in endotoxin-free stimulation buffer, or in stimulation buffer spiked with bacterial endotoxin (lipopolysaccharide from *Escherichia coli* 0127:B8, Sigma) at final concentrations of 0.1, 1 and 10 µg/ml. The basal and maximally activated LT responses of the endotoxin-treated and -untreated cells were then compared.

2.8. Variations in leucocyte counts

For these experiments, leucocytes isolated from dextran-treated blood collected in CAST® venepuncture tubes were resuspended in stimulation buffer to

the recommended concentration (i.e. equivalent to the circulating leucocyte count), as well as to concentrations which were lower (0.5-fold) and higher (2- and 4-fold) than this value. The basal and maximally activated LT responses of these cell suspensions were then compared.

2.9. Comparison of the modified CAST®-ELISA test in the detection of allergen sensitivity

In these experiments, we evaluated the potential influence of combinations of variables, as opposed to the single variables described in Sections 2.4, 2.5 and 2.6, on basal and maximally activated LT responses of leucocytes, as well as on the detection of sensitivity to house dust mite, using matched leucocyte suspensions from seven different subjects with established reactivity to this allergen. Two modified procedures were used and compared with the conventional CAST®-ELISA procedure. Modified procedure 1 used preservative-free heparin collection tubes, NH₄Cl lysis and standard CAST®-ELISA buffer. Modified procedure 2 used preservative-free heparin collection tubes, NH₄Cl lysis and IL-3 free buffer. Allergen (*Derma-atophagoides pteronyssinus*) was obtained from the manufacturer of the CAST®2000-ELISA (Bühlman Laboratories).

2.10. Expression and analysis of results

The results of each series of experiments are presented as the mean values ± standard deviations (S.D.). Statistical analyses were performed using ANOVA, with the Tukey–Kramer Multiple Comparisons Test where appropriate. A *P* value of <0.05 was considered significant.

3. Results

3.1. Requirement for CAST® venepuncture tubes, dextran sedimentation and interleukin-3

The results of experiments designed to investigate the effects of collection of blood into preservative-free heparin, or removal of erythrocytes by NH₄Cl lysis, or omission of IL-3 on the performance of the CAST® 2000-ELISA procedure, using leucocytes from non-

Table 1

Effects of altering single parameters on basal and maximal leukotriene production by leucocytes from non-atopic donors in the CAST® 2000-ELISA

System	Leukotriene production (pg/ml)	
	Basal	Maximal
Conventional CAST® procedure	325 ± 90*	2753 ± 1095**
Tubes containing preservative-free heparin	252 ± 131	2642 ± 1101**
Ammonium chloride lysis	137 ± 72	2750 ± 1104**
Omission of IL-3	308 ± 148	2740 ± 1127**

*The results are presented as the mean values ± S.D. for paired leucocyte suspensions isolated from six different subjects.

** $P < 0.05$ for comparison between the basal and maximal values for each system.

atopic donors are shown in Table 1. In comparison with the results obtained with the conventional CAST® procedure, none of these modifications affected the maximal LT responses, although collection of blood into preservative-free heparin, and especially lysis of erythrocytes with NH_4Cl , decreased the basal responses; this would have the effect of increasing the stimulation indices.

3.2. Endotoxin contamination

Bacterial endotoxin, at concentrations of 0.1 and 1 µg/ml did not affect either basal or maximal production of LTs by leucocytes from non-atopic donors (results not shown). However, treatment of the cells with 10 µg/ml endotoxin resulted in an increase in basal responses which did not achieve statistical significance. The mean (± S.D.) values for basal and maximal LT production for the control system

were 325 ± 90 and 2753 ± 1095 pg/ml, respectively, while the corresponding values for cells treated with 10 µg/ml endotoxin were 1015 ± 1094 and 2766 ± 1064 pg/ml, respectively.

3.3. Variations in leucocyte concentrations

The mean total and differential circulating leucocyte counts of the non-atopic donors used were all within the normal ranges for our laboratory, with a mean (± S.D.) basophil count of $0.03 \pm 0.02 \times 10^9/\text{l}$ blood.

Using the conventional CAST® procedure, adjusting the leucocyte suspensions from these non-atopic donors to concentrations which were 0.5-fold less, or 2- and 4-fold greater than those of the unadjusted cell suspension did not significantly alter the clear distinctions between basal and maximally stimulated LT responses (results not shown).

3.4. Comparison of the standard and modified CAST®-ELISA procedures

The validity of the modified CAST® procedure in the clinical setting was evaluated by comparing its performance relative to that of the standard procedure in the determination of basal, allergen-activated and maximally activated LT production by leucocytes isolated from seven individuals with a known sensitivity to *D. pteronyssinus*. These results are shown in Tables 2 and 3. As opposed to the decrease in basal LT values observed when a single parameter (preservative-free heparin or NH_4Cl lysis, Table 1) was varied, combining two (preservative-free heparin + NH_4Cl

Table 2

Comparison of the standard and modified CAST®-ELISA procedures in the detection of sensitivity to house dust mite

	Leukotriene production (pg/ml)			Stimulation indices	
	Basal	Allergen-activated	Maximal	Allergen activated	Maximal
Standard procedure	242 ± 134*	2937 ± 669	3148 ± 137	16 ± 9	17 ± 9
Modified procedure 1	85 ± 36	2130 ± 1089	2382 ± 963	46 ± 63	49 ± 63
Modified procedure 2	102 ± 46	1265 ± 839**	1703 ± 524**	13 ± 7	20 ± 8

⁺ Mean stimulation indices (allergen-activated/maximal values divided by the corresponding basal values) shown in parentheses.

*Results from seven different individuals with known sensitivity to house dust mite are expressed as the mean values ± S.D. Modified procedure 1 uses preservative-free heparin collection tubes, NH_4Cl lysis and standard CAST®-ELISA buffer, containing IL-3. Modified procedure 2 uses preservative-free heparin collection tubes, NH_4Cl lysis and IL-3 free buffer. The differences between the basal and corresponding allergen-activated and maximally activated responses for each procedure all achieved statistical significance ($P < 0.05$).

** $P < 0.05$ for comparison with the standard procedure.

Table 3

Basal and allergen-activated LT values for each atopic individual using the conventional CAST®-ELISA and the two modified procedures

Individual	Procedure used to determine production of LTs (pg/ml)					
	Conventional		Modified 1		Modified 2	
	Basal	Allergen	Basal	Allergen	Basal	Allergen
1	498	3139	105	652	38	483
2	168	3200	78	2279	106	1735
3	273	1421	118	553	126	541
4	229	3200	110	2988	151	753
5	113	3200	60	2400	64	805
6	297	3200	103	2883	159	2701
7	116	3200	17	3154	71	1841

lysis) or especially three variables (preservative-free heparin + NH₄Cl lysis + omission of IL-3) lowered not only the basal, but also the allergen-activated and maximally activated LT responses (relative to those of the conventional CAST®-ELISA procedure, Table 2). In spite of these differences in absolute values, the stimulation indices (allergen-activated and maximally activated values divided by the corresponding basal values) for each of the modified procedures did not differ significantly from one another, or from those of the conventional procedure (Table 2). Moreover, the distinction between basal and allergen-activated responses was clearly evident for each of the seven subjects irrespective of the procedure used (Table 3).

4. Discussion

In the current study, we observed that CAST® venepuncture tubes could be replaced by tubes containing preservative-free heparin as anti-coagulant with no adverse effects on results. Indeed, basal values for LT production by leucocytes isolated from blood collected in tubes containing preservative-free heparin were lower, suggesting that this modification is not only a suitable alternative to CAST® venepuncture tubes, but may even be preferable.

We were concerned about the use of dextran in the CAST®-ELISA procedure for two reasons. Firstly, dextran-treatment of circulating leucocytes has been reported to sensitise and/or activate the pro-inflammatory activities of these cells (Sher et al., 1974; Berends et al., 1994; Iba et al., 2002). Secondly, the

recommended time of 90 min for erythrocyte sedimentation adds significantly to the time required to complete the procedure. In the current study, we observed that a brief (10 min) exposure of blood to NH₄Cl resulted in leucocyte preparations which produced similar overall results to those prepared from matched dextran-treated blood. Results showed comparable stimulation indices and positivity rates, although both basal and maximal LT responses were marginally lower, indicating a lesser degree of non-specific cellular stimulation during leucocyte isolation. Clearly, NH₄Cl lysis of erythrocytes significantly reduces the time taken to perform the test, without compromising the quality of results. Moreover, decreasing or increasing the leucocyte concentration in the CAST®-ELISA procedure by 0.5-fold and up to 4-fold, respectively, did not significantly alter the discrimination between basal and maximally activated LT responses.

Inclusion of IL-3 in the stimulation buffer to sensitise circulating basophils for LT production on exposure to allergen is also recommended by the manufacturer. Although the mechanism of sensitisation of the cells remains to be established, stimulatory effects of the cytokine on cytosolic phospholipase A₂ activity is a possibility, as opposed to interference with basophil apoptosis or other mechanisms involving de novo protein synthesis which are unlikely to be operative during the 40-min exposure of the cells to IL-3 (Miura and MacGlashan, 2000; Sin et al., 2001; Zheng et al., 2002). We were unable to detect significant effects of inclusion of the cytokine on either basal or maximal LT production by the cells using blood from non-atopic donors.

We then compared two modified procedures, in which two or three parameters (preservative-free heparin + NH₄Cl lysis ± IL-3) were varied, with the conventional CAST®-ELISA procedure in the detection of sensitivity to house dust mite, as well as on basal and maximally activated LT responses. The values for basal, allergen-activated and maximally activated LT responses were lower with both modified procedures, particularly procedure 2 (preservative-free heparin, NH₄Cl lysis, omission of IL-3), suggesting that the combination of collection of blood into CAST® venepuncture tubes, dextran sedimentation of erythrocytes and inclusion of IL-3 all interact to sensitise the cells for increased spontaneous and stimulated

production of LTs. None of these parameters when evaluated individually (Table 1), impacted significantly on either basal or maximal responses. This decrease in LT production observed with the modified procedures did not, however, significantly influence the discrimination of the CAST®-ELISA, with clear distinctions observed between basal, allergen-activated and maximal responses, as well as comparable stimulation indices for all three systems.

The manufacturers of the CAST®-ELISA recommend that all buffers and containers used in the CAST®-ELISA are endotoxin-free, presumably to avoid false-positive results due to endotoxin-activation of LT production by basophils, eosinophils and monocytes in the leucocyte population (Krausfilarska et al., 1998; Boyce, 2003). In agreement with this recommendation, we observed that addition of endotoxin to the stimulation buffer (40-min exposure), resulted in a dose-related increase in basal production of LTs by the cells, emphasising the importance of avoidance of endotoxin contamination. In this context, it is noteworthy that certain commercial allergen preparations may contain endotoxin (Trivedi et al., 2003), which is clearly to be avoided in functional assays of allergen sensitivity such as the CAST®-ELISA procedure.

In conclusion, in addition to establishing factors which optimise test performance, we have also identified modifications of the CAST®-ELISA procedure which have the potential to decrease costs and expedite the acquisition of results. All of these are relevant to the discerning and efficient implementation of the CAST®-2000 ELISA as a diagnostic procedure in clinical immunology laboratories.

References

- Beckett, W.S., 2000. Occupational respiratory diseases. *N. Engl. J. Med.* 342, 406.
- Berends, C., Dijkhuizen, B., Demonchy, J.G.R., Gerritsen, J., Kauffman, H.F., 1994. Induction of low density and up-regulation of CD11B expression of neutrophils and eosinophils by dextran sedimentation and centrifugation. *J. Immunol. Methods* 167, 183.
- Boyce, J.A., 2003. Mast cells: beyond IgE. *J. Allergy Clin. Immunol.* 111, 24.
- Busse, A., Czech, W., Schopf, E., Simon, J.C., 2000. Cellular antigen stimulation test (CAST)—its relevance in the diagnosis of pseudoallergic reactions to food additives and acetylsalicylic acid. *Allergologie* 23, 110.
- Crockard, A.D., Ennis, M., 2001. Basophil histamine release tests in the diagnosis of allergy and asthma. *Clin. Exp. Immunol.* 31, 345.
- Czech, W., Busse, A., Wedi, B., Kapp, A., 1996. Pseudoallergic reactions to food additives and non-steroidal antiinflammatory drugs. *Allergologie* 19, 442.
- De Weck, A.L., 1997. Sellulärer Allergen-Stimulierings-Test (CAST). *Allergologie* 20, 487.
- Douwes, J., Gibson, P., Pekkanen, J., Pearce, N., 2002. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 57, 643.
- Ferrer, M., Sanz, M.L., Prieto, I., Oehling, A., 1996. Antigen-specific sulphidoleukotriene production and histamine release in pollinic patients. *J. Investig. Allergol. Clin. Immunol.* 8, 271.
- Ferrer, M., Sanz, M.L., Prieto, I., Oehling, A., 1998. In vitro antigen-specific sulphidoleukotriene production in patients allergic to *Dermatophagoides pteronyssinus*. *Clin. Exp. Allergy* 28, 709.
- Humbert, M., Menz, G., Ying, S., Corrigan, C.J., Robinson, D.S., Durham, S.R., Kay, A.B., 1999. The immunopathology of extrinsic (atopic) and intrinsic (nonatopic) asthma. *Immunol. Today* 20, 528.
- Iba, Y., Sugimoto, Y., Kamei, C., 2002. Participation of mast cells in colitis inflammation induced by dextran sulfate sodium. *Meth. Find. Exp. Clin. Pharmacol.* 24, 15.
- Kraneveld, A.D., van Der Kleij, H.P.M., Kool, M., van Houwelingen, A.H., Weitenberg, A.C.D., Redegeld, F.A.M., Nijkamp, F.P., 2002. Key role for mast cells in non-atopic asthma. *J. Immunol.* 169, 2004.
- Krausfilarska, M., Matolepszy, J., Medrala, W., Dobek, R., 1998. Bacterial lipopolysaccharide-induced sulfidoleukotriene release from peripheral blood leukocytes in patients with asthma and chronic obstructive pulmonary disease. *J. Investig. Allergol. Clin. Immunol.* 8, 94.
- Maly, F.F., Martiwyss, S., Blumer, S., Cuhastark, I., Wuthrich, B., 1996. Interleukin-3 facilitated blood mononuclear cell sulfidoleukotriene generation and whole blood histamine release in honey bee and yellow jacket venom allergy. *Allergy Clin. Immunol. Int.* 8, 11.
- Miura, K., MacGlashan Jr., D.W., 2000. Dual phase priming by IL-3 for leukotriene C₄ generation in human basophils: difference in characteristics between acute and late priming effects. *J. Immunol.* 164, 3026.
- Sher, R., Anderson, R., Rabson, A.R., Koornhof, H.J., 1974. Standardization of the nitroblue tetrazolium test. *S.A. Med. J.* 48, 209.
- Sin, A.Z., Roche, E.M., Togias, A., Lichtenstein, L.M., Schroeder, T.J., 2001. Nerve growth factor or IL-3 induces more IL-13 production from basophils of allergic subjects than from basophils of nonallergic subjects. *J. Allergy Clin. Immunol.* 108, 387.
- Trivedi, L., Valerio, C., Slater, J.E., 2003. Endotoxin content of standardized allergen vaccines. *J. Allergy Clin. Immunol.* 111, 777.
- Vila, L., Sanz, M.L., Sanchez, G., Uasuf, C.G., Ferrer, M., Barrio, M., Dieguez, I., 2001. Study of the in vitro sulphidoleukotriene production in food-allergic patients. *J. Investig. Allergol. Clin. Immunol.* 11, 247.

- Wedi, B., Elsner, J., Kapp, A., 1996. In vitro diagnosis of Pseudo-Allergic reactions—new aspects. *ACI International* 8 (4), 113.
- Zheng, X.Y., Karsan, A., Duronio, V., Chu, F., Walker, D.C., Bai, T.R., Schellenberg, R.R., 2002. Interleukin-3, but not granulocyte colony-stimulating factor and interleukin-5, inhibit apoptosis of human basophils through phosphatidylinositol 3-kinase: requirement for NF κ B-dependent and -independent pathways. *Immunology* 107, 306.