

Chapter 9

Purifying and Measuring Immunoglobulin E (IgE) and Anti-IgE

Jamie L. Sturgill and Daniel H. Conrad

Abstract

Immunoglobulins (Igs) are a critical component of the adaptive immune system of both man and mouse. The ability to detect and characterize Igs is an invaluable technique for immunology in either a research or a clinical setting. The advent of enzyme-linked immunosorbent assays (ELISAs) and monoclonal antibody technology has proven instrumental for advancing the science of Ig biology. IgE is of interest as it is the primary Ig responsible for allergic reactions ranging from allergic rhinitis to anaphylaxis. Here, we describe the history behind the IgE discovery and the protocol for purifying IgE and anti-IgE in the mouse. This is followed by our ELISA protocol for mouse IgE detection.

Key words ELISA, Immunoglobulin, IgE, Hybridoma, Antibody purification

1 Introduction

Allergies are defined as the body's response to a normally innocuous substance, such as pollen. Allergic diseases occur in many forms, such as rhinitis, sinusitis, conjunctivitis, eczema, asthma, gastroenteral complications, or in severe cases anaphylaxis or even death. The World Health Organization (WHO) estimates that over 20 % of the world's population suffers from some type of allergic disease with about 150 million people having allergic asthma alone. In the United States, the National Institute of Allergy and Infectious Diseases (NIAID) approximates that between 40 and 50 million Americans suffer from these types of illnesses. With such widespread prevalence in the global population, allergic disease ranks as one of the highest causes of chronic illness and costs billions of dollars annually.

While the general public is all too familiar with the outward signs and symptoms, the underlying biological cause of allergic disease is due to a hypersensitivity reaction of the immune system to an inherently harmless matter. Hypersensitivity reactions were classified

into four distinct groups, or types, by Gell and Coombs in 1963 [1]. Allergic reactions are classified as type 1 because they are mediated by IgE. However at the time, Gell and Coombs referred to Type 1 simply as an “immediate hypersensitivity” because a reaction occurred in minutes. Furthermore, this temporal classification stood because IgE was not officially recognized as the fifth immunoglobulin subtype until 1968.

Although the immediate hypersensitivity phenomenon was officially classified in the 1960s, its existence had been reported since the early 1800s. In 1819, Dr. John Bostock reported the first case of pollen-induced hay fever to the Royal Medical and Chirurgical Society in London and the patient he presented was himself [2]. However, it would take almost another 100 years to link the symptoms of hay fever to a soluble serum factor. In the early 1900s, a French physiologist by the name Richet observed that “while a foreign substance might induce a mild reaction upon first exposure, it could produce severe hypersensitive symptoms and even death when re-introduced late.” [3]. Richet observed that a repeated dose offered no protection, or phylaxis. Thus he coined the term, “anaphylaxis,” meaning without protection. In 1919, Ramirez reported the first case of an asthma attack subsequent to a blood transfusion. In this case, a man by the name of “H.T.,” who had no prior personal or family medical history of allergic disease, received a blood transfusion for anemia. Subsequently, after an encounter with a horse at Central Park, the man suffered a violent asthma attack [4]. While this was an observational report, the first experimental evidence was provided by Prausnitz and Küstner. Küstner, who was a German gynecologist, had previously noted that he developed allergic symptoms after consuming fish. Prausnitz, who was also a fellow German physician, decided to inject some of Küstner’s serum into the skin of his abdomen. After eating some fish himself, Prausnitz’s, who had no prior adverse reactions to fish, suffered from hot, red, swollen skin at the site of the serum injection, confirming their hypothesis that Küstner was indeed allergic to fish. The work of these two men led to the development of the passive transfer of a positive skin test, later coined the PK test [5].

Although these types of hypersensitivities had been described as allergies, a term coined by Clemens Peter Freiherr von Pirquet, two American physicians felt that the “allergy” label was too limiting. Thus, in 1923, Coca and Cooke introduced the word “atopy” into medical vernacular. They felt atopy, which was derived from the Greek word “*ἀτοπία*” meaning placelessness, was a more suitable term to cover all forms of immediate hypersensitivities [6]. They went on to add that atopy was a result of “bodies” they referred to as “reagins.” However, ironically enough they recommended that the term “antibody” should be avoided as they determined that “no evidence of these bodies appear as the result of immunologic stimulation” [7].

It took the next 40 years and the work of two pioneering groups to determine that “reagin” was indeed an antibody. Two Swedish scientists by the name of Bennich and Johansson studied structure and function of immunoglobulins (Ig). Their primary source of human Ig was multiple myeloma serum. In the summer of 1965, they came across a serum of a patient, “N.D.,” whose serum contained an atypical Ig subtype. When compared to IgA, IgM, IgG, or even the newly identified IgD, they saw no similarities. They called this new protein IgX, for the unknown Ig. After further investigation, they discovered that IgX had unique biochemical properties, which were distinct from the other four types. They went on to develop very sensitive assays for the detection of IgX and noted that the normal serum concentration of IgX as compared to IgG was about 200,000-fold less. After collaborating with D.R. Stanworth, it was shown that IgX could block the PK test. Thus, all evidence was pointing to a new class of Ig. Eventually IgX was renamed IgND after the initial patient from which it was isolated. Because of the finding that IgND could inhibit the PK test, Bennich and Johansson began to look at IgND in the context of atopic disease and ultimately went on to develop the radioallergen sorbent test (RAST) [8].

Meanwhile in Denver CO, a husband and wife team were approaching a similar problem, but from a different angle. Kimishige and Teruka Ishizaka were interested in identifying the biological cause of the reagin-mediated histamine release reaction. In 1964, they first reported that the antibody responsible for this was a type of IgA, which they initially called γ A [9]. While this was not widely accepted in the field, they persevered and ultimately identified an antiserum capable of precipitating a serum fraction that could block the PK reaction [10]. This activity did not appear to be similar to any of the other known four Ig types; thus they called it γ E-globulin because it had the ability to cause an erythema reaction. However, despite all their hard work, the Ishizakas were never able to make a purified preparation of γ E-globulin from normal human serum. However, given its extremely low concentration in normal serum, this observation is not too surprising. In early 1967, a fruitful collaboration between the Ishizakas and the Swedish took place. They decided to swap reagents and it was indeed found that IgND and γ E-globulin were one in the same. In February of 1968 at a workshop at the WHO, it was agreed upon that IgE would be the new nomenclature for the newly identified fifth Ig subclass [11].

Like all immunoglobulins, IgE comprises two identical light chains, either κ or λ , and two identical heavy chains, the ϵ chains, which are held together by disulfide bonds. Both light and heavy chains each contain a variable and constant domain. This basic chemical structure of Igs was solved by the work of Edelman and Porter for which they received the Nobel Prize in 1972 [12]. The variable regions of IgE are responsible for antigen binding

specificity, whereas the heavy chain determines effector function. The characteristics that make IgE unique from the other subclasses reside in the ϵ heavy-chain component. IgE has a molecular weight of about 190 kDa, which when resolved under reducing conditions yields two light chains and two heavy chains of about 23 and 72 kDa, respectively [13]. IgE is slightly larger than the monomeric forms of IgG, IgA, and IgD because it has an additional domain in the heavy ϵ chain called C ϵ 4 and it is more heavily glycosylated. Studies with tunicamycin have shown that the glycosylation of IgE is N-linked; however, these additional sugar moieties are not critical for IgE binding to its receptors on mast cells [14].

In addition to its unique structure, IgE has biological activities that are much different than its other Ig counterparts. IgE fails to neutralize, opsonize, participate in antibody-directed cellular cytotoxicity (ADCC), or fix complement. IgE also fails to transport across epithelial surfaces or the placenta and only under instances of widespread inflammation can IgE diffuse into extravascular sites. Serum IgE has a half-life of approximately 3 days, whereas IgG is stable for up to 3 weeks. The reported mean serum levels of IgE are approximately $0.5\text{--}3 \times 10^{-5}$ mg/ml, which is much less than 1.5, 9, or 2.1 mg/ml as seen with IgM, IgG1, or IgA, respectively [15].

Although most equated with unwanted reactions of the immune system, IgE serves a very important evolutionary role in the defense against parasitic disease. Elevated levels of IgE are observed in both man and mouse during parasite infections. Capron et al. have shown a critical role for IgE in the clearance of *Schistosoma mansoni* [16] and it has been reported that IgE-deficient mice have increased worm burden following infection with *S. mansoni* [17], *Brugia malayi* [18], and *Trichinella spiralis* [19]. This protective effect of IgE in the context of microbial pathogens is the basis for the hygiene hypothesis. This theory, originally proposed by the epidemiologist Strachan in the 1980s, states that the declining microbial exposure in industrialized countries is a major causative factor in the increased rise in atopic disease [20].

In this review, we summarize newer methodologies to grow and isolate large amounts of monoclonal mouse IgE and anti-IgE. The use of the purified anti-IgE to measure mouse IgE is also given.

2 Materials

2.1 Antibody Purification

1. The anti-mouse IgE hybridomas B1E3 [21] and R1E4 (kindly provided by M. Kehry) and the mouse IgE anti-DNP hybridoma [22] (kindly provided by F-T. Liu) were all maintained in our laboratory.

2. If needed, antibodies were biotinylated using a 100-fold molar excess of EZ-link Sulfo-NHS-biotin (Pierce, Rockford IL) as per the manufacturer's protocol and dialyzed against 1× PBS.
3. All antibodies were prepared from hybridoma cell culture supernatant using the CL-1000 Adhere CELLline flasks (Integra Biosciences, Switzerland).
4. Cells were grown in complete RPMI-1640 containing 10 % heat-inactivated fetal bovine serum, 2 mM l-glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate, 50 µg/ml amphotericin B, 50 µM 2-mercaptoethanol, 2 µg/ml gentamicin, 100 µM NEAA, and 20 mM HEPES buffer.
5. Cell viability is always measured via Trypan Blue exclusion and if this falls below 50 %, dead cells are removed by density centrifugation over Ficoll-Hypaque.
6. All antibodies were purified by hydrophobic charge induction chromatography using the MEP HypeCel sorbert (Pall Life Sciences, East Hills, NY).
7. Antibody purification is done on a Bio-Rad BioLogic DuoFlow FPLC system.
8. Elution is performed using a series of pH buffers (*see* Table 1).

Table 1
Full eluotropic series

	Buffer/sample	Concentration and amount
1	PBS	25 ml
2	Sample	50–200 ml
3	PBS	Until OD280 < 0.05
4	Water	40 ml
5	2-(<i>N</i> -Morpholino)ethanesulfonic acid (MES)	0.05 M, pH 5.5
6	MES	0.05 M, pH 5.2–50 ml
7	Sodium acetate	0.05 M, pH 4.9–50 ml
8	Sodium acetate	0.05 M, pH 4.6–50 ml
9	Sodium acetate	0.05 M, pH 4.4–50 ml
10	Sodium acetate	0.05 M, pH 4.0–50 ml
11	Sodium acetate	0.05 M, pH 3.0–50 ml
12	PBS	50 ml

PBS is 0.01 M Na phosphate, 0.14 M NaCl, pH 7.4

9. After chromatography was complete, fractions were separated on a 10 % Bis-Tris gel (Invitrogen) by SDS-PAGE under reduced conditions.
10. To visualize proteins, gels were stained with SDS-PAGE stain (2.5 g Coomassie Brilliant Blue R-250, 100 ml glacial acetic acid, 450 ml methanol, and 450 ml dH₂O) for 30 min during continual motion.
11. Gels were then subsequently destained with SDS-PAGE Destain (30 % methanol, 10 % acetic acid, 60 % dH₂O).
12. Protein was concentrated by ultrafiltration with an Amicon filtration unit (Millipore Corporation, Bedford, MA) and dialyzed against 1× PBS.

2.2 ELISA

1. All coating steps are done with borate-buffered saline (0.17 M boric acid, 0.125 M NaCl, pH 8.5, and filter sterilized). Do not attempt to make a 10× stock of BBS as the solution will become saturated.
2. ELISA wash, when indicated, is 1× PBS with 0.02 % Tween-20.
3. Mouse IgE block is 5 ml of 10 mM Hepes, 2 % FBS, up to 500 ml with PBS. Filter sterilize and add 0.02 % Tween-20.
4. Streptavidin AP antibody is purchased from Southern Biotech.
5. Substrate tablets are pNPP substrate tablets (Sigma).
6. Substrate buffer is made by adding to 300 ml water 0.1 g MgCl₂·6H₂O, 0.2 g NaN₃, 50 ml diethanolamine, pH to 9.8, up to 500 ml. It is important to keep this solution away from light.

3 Methods

3.1 Antibody Purification (See Notes 1 and 2)

1. The integra flasks have an outer compartment which holds 1 L of serum-free media and cells are seeded into the inner chamber, which contains 15 ml of the same media plus 10 % heat-inactivated FBS. Flasks were initially seeded with 100 × 10⁶ cells.
2. Two times per week, the outside media is replaced and the cells removed from the inner chamber. The inner chamber supernatant is collected and frozen and 1/3 of the cells are returned to the inner chamber with fresh media.
3. Cell viability is always measured via Trypan Blue exclusion and if this falls below 50 %, dead cells are removed by density centrifugation over Ficoll-Hypaque.
4. When supernatants were harvested, they were centrifuged at 400 × *g* for 5 min and stored at −20 °C until purification.

5. When at least 200 ml has been collected, just prior to purification, supernatants were pooled and further clarified by centrifugation at $2,400 \times g$ for 30 min.
6. All antibodies from hybridomas were purified by hydrophobic charge induction chromatography using the MEP HypeCel sorbert, which is immunoglobulin selective and binds a broad range of Ig subtypes as previously described both due to hydrophobic and affinity reasons, virtually all immunoglobulin classes bind to this absorbent [23] (*see Note 3*).
7. This purification is done on a Bio-Rad BioLogic DuoFlow FPLC system and the program is adjusted so as to wash with PBS until OD from applied supernatant (up to 200 ml can be applied at a time—the flow rate is maintained at 2/ml/min) drops below 0.05 OD280 (*see Note 4*).
8. The column is then washed for 10 min with water, which helps elute bound albumin—then the protein is eluted with a low pH buffer. If using a new monoclonal and the eluting pH is not known, then a series of pH buffers are applied (*see Table 1*) and the pH at which the monoclonal Ig elutes is recorded.
9. Subsequent purifications can then use the eluting pH plus the pH 3.0 buffer. The latter elutes bound light chain, which is a common contaminant in monoclonal supernatants. An example of the FPLC purification of mouse IgE anti-DNP is shown in Fig. 1 and IgG anti-mouse IgE (R1E4) [24] in Fig. 2.
10. After chromatography was complete, fractions were separated on a 10 % Bis-Tris gel by SDS-PAGE under reduced conditions (*see Note 5*).
11. To visualize proteins, gels were stained with SDS-PAGE stain for 30 min during continual motion.
12. Gels were then subsequently destained with SDS-PAGE Destain.
13. After the fractions which were determined to contain purified antibody were pooled together, protein was concentrated by ultrafiltration with an Amicon filtration unit and dialyzed against $1 \times$ PBS.
14. In each case the insert shows the SDS-PAGE analysis of the purified protein and as can be seen, a purity of about 95 % is accomplished in just this single step. The mouse IgE anti-DNP yield, as well as most monoclonal IgG yields, is about 50–75 mg per 200 ml of Integra supernatant.

3.2 ELISA for IgE Detection

1. Coat a Maxisorp ELISA plate with 100 μ L/well of the 10 μ g/ml of the rat anti-mouse IgE mab clone B1E3 in borate-buffered saline (*see Note 6*).
2. Incubate either for 1 h at 37 °C or O/N at 4 °C (*see Note 7*).

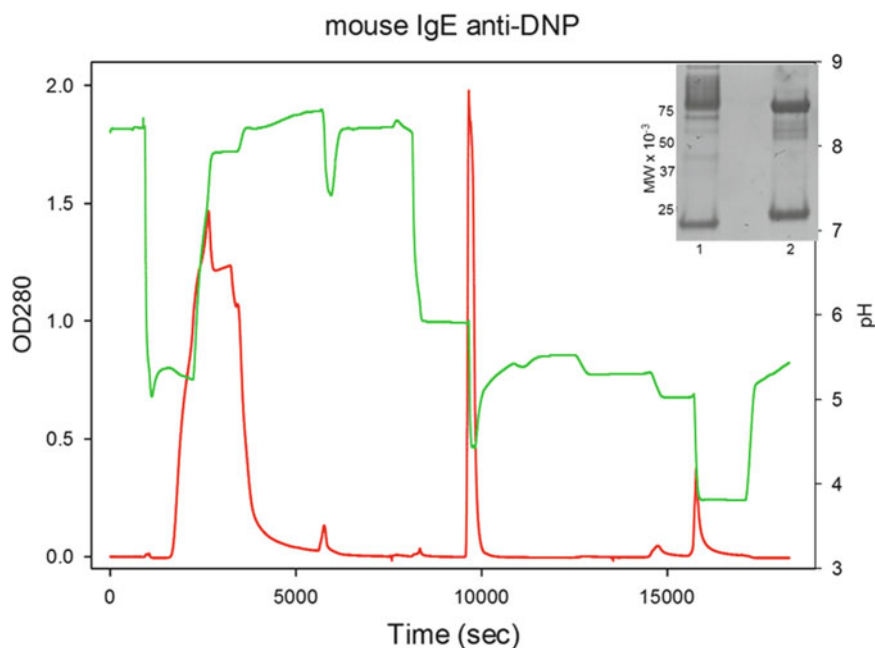


Fig. 1 Mouse IgE anti-DNP purification on MEP-Hypercel. 200 ml of Integra collected sample is applied to a 20 ml MEP-Hypercel column ((red solid line) OD280, (green solid line) pH). The column is then washed with PBS until the OD280 is less than 0.05. 50 ml of distilled water is followed by 50 ml of PBS and then the IgE is eluted using 0.05 M Na acetate, pH 4.9. After the IgE peak is completely off the column, residual light chain is eluted using the Na acetate buffer at pH 3.0. The MEP-Hypercel is then washed with 50 ml of PBS and reused for additional IgE purifications as needed. The *insert* shows (*Lane 1*) rat IgE, IR162, purified by conventional chromatography (*ref*) and (*Lane 2*) the mouse IgE anti-DNP after pooling the peak and concentrating. 10 μ g of protein was applied per lane

3. Wash the plate twice with diH_2O .
4. Block the plate with mouse IgE block 200 μL /well.
5. Incubate either for 2 h at 37 $^\circ\text{C}$ or O/N at 4 $^\circ\text{C}$ (*see Note 7*).
6. Wash the plate twice with diH_2O .
7. Add 100 μL /well of the samples, blanks, and standards. Standard curves were generated with mouse IgE anti-DNP beginning at a concentration of 1,000 ng/ml and diluted 1:2 across the plate.
8. Incubate either for 1 h at 37 $^\circ\text{C}$ or O/N at 4 $^\circ\text{C}$ (*see Note 7*).
9. Wash plates 2 $\times$ with diH_2O , 2 $\times$ with ELISA wash, and 2 $\times$ with diH_2O .
10. Detection is done by incubation of the plates with 100 μL /well of the biotinylated rat anti-mouse IgE mab R1E4. We purify from hybridoma in house and use at a concentration of 1:4,000.
11. Incubate for 1–2 h at 37 $^\circ\text{C}$.

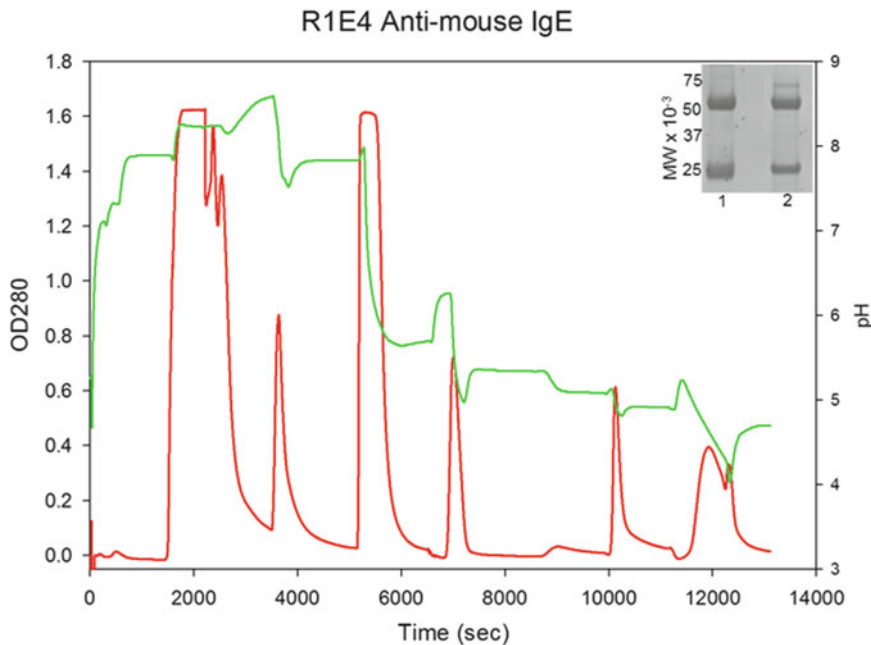


Fig. 2 Monoclonal anti-mouse IgE (R1E4) purification on MEP-Hypercel. 200 ml of Integra collected sample is applied to a 20 ml MEP-Hypercel column as in Fig. 1; *lines* are also presented as in Fig. 1. The column is then washed with PBS, water, and PBS again. Following the second PBS wash, the R1E4 is eluted by using the 0.05 M MES, pH 5.2 buffer. Some additional R1E4 is eluted with the pH 4.9 buffer, the second peak is less pure (not shown). Residual light chain and other contaminants are eluted with the pH 3.0 buffer and washed with PBS. The *insert* shows commercial rat IgG (*Lane 1*) and the pH 5.2 R1E4 peak after pooling and concentrating (*Lane 2*). 10 μ g of protein was applied per lane

12. Wash plates 2 $\times$ with diH₂O, 2 $\times$ with ELISA wash, and 2 $\times$ with diH₂O.
13. Add 100 μ L/well of streptavidin-AP at 1:400 for 1 h at 37 $^{\circ}$ C.
14. Wash plates 2 $\times$ with diH₂O, 2 $\times$ with ELISA wash, and 2 $\times$ with diH₂O.
15. To develop plates add 100 μ L/well of substrate. Substrate is pNPP substrate tablets diluted in substrate buffer. Add one tablet for every 5 ml of substrate buffer.
16. Read at OD 405 nm.

4 Notes

1. Given the relatively low concentration of IgE in serum and the fact that IgE is only made after multiple rounds of cellular division in vitro, detection of IgE needs to be specific and sensitive. This protocol has been used in our previous publications

and the source of the various monoclonal proteins is noted there as well.

2. This strategy for monoclonal antibody growth and purification has considerable advantages over other purification strategies. Use of the Integra flasks avoids the need for using animals for ascite production. Ascite production is now banned in many areas due to animal discomfort considerations and even when allowed, strict regulations are required for animal monitoring.
3. We reuse the MEP-hypercel multiple times for purifying the same monoclonal. While the same adsorbent could also be used for different monoclonal Igs, this is not recommended due to low levels of contamination from the original protein.
4. The final Ig product is free from any contamination by normal mouse or rat Ig. In addition, this single-step FPLC purification procedure has clear advantages over previous IgE purification protocols, which required salt precipitation, ion exchange chromatography, and gel filtration.
5. Indeed, as is shown in the SDS-PAGE insert in Fig. 1, the IgE purity is superior to the multistep protocol [24] that was used for the IR162 rat myeloma IgE.
6. The ELISA plates we utilize are the Nunc Maxisorp Plates which ensure high protein binding.
7. This step in the ELISA protocol can be done overnight.

References

1. Gell P, Coombs R (1963) Clinical aspects of immunology. In: Blackwell K (ed) 1st edn. Oxford, England
2. Hurwitz SH (1929) The lure of medical history: John Bostock (1773–1846): author of the first clinical description of hay fever. *Cal West Med* 31(2):137–138
3. Tan SY, Yamanuha J (2010) Charles Robert Richet (1850–1935): discoverer of anaphylaxis. *Singapore Med J* 51(3):184–185
4. Ramirez M (1919) Horse asthma following blood transfusion: report on a case. *J Am Med Assoc* 73(13):984–985
5. Prausnitz D, Kustner H (1921) Studien uber die Ueberempfindlichkeit. *Zentrabl Bakteriol [A]* 86:160–175
6. Coca A, Cooke R (1923) On the classification of the phenomena of hypersensitiveness. *J Immunol* 8:163–182
7. Johansson SG (2006) The discovery of immunoglobulin E. *Allergy Asthma Proc* 27(2 Suppl 1):S3–S6
8. Stanworth DR, Humphrey JH, Bennich H, Johansson SGO (1968) Inhibition of Prausnitz-Kustner reaction by proteolytic-cleavage fragments of a human myeloma protein of immunoglobulin class E. *Lancet* 2:17–18
9. Ishizaka K, Ishizaka T, Hathorn EM (1964) Blocking of Prausnitz-Kustner sensitization with reagin by ‘A Chain’ of human gamma1A-globulin. *Immunochemistry* 1:197–207
10. Ishizaka K, Ishizaka T (1967) Identification of gamma-E-antibodies as a carrier of reaginic activity. *J Immunol* 99(6):1187–1198
11. Bennich HH, Ishizaka K, Johansson SG, Rowe DS, Stanworth DR, Terry WD (1968) Immunoglobulin E: a new class of human immunoglobulin. *Immunology* 15(3):323–324
12. (2010) Nobel Prize. <http://www.nobelprize.org>
13. Bennich H, Johansson SGO (1971) Structure and function of human immunoglobulin E. *Adv Immunol* 13:1–55
14. Conrad DH (1985) Structure and synthesis of IgE. In: Kaplan AP (ed) *Allergy*. Churchill Livingstone, New York, pp 3–21
15. Janeway CA, Travers P (1996) *Immunobiology: the immune system in health and disease*, 2nd edn. Current Biology Ltd., New York

16. Capron M, Capron A (1994) Immunoglobulin E and effector cells in schistosomiasis. *Science* 264:1876–1877
17. Conrad DH, Tinnell SB, Kelly AE (1998) Immunoglobulin E. In: Kaliner MA (ed) *Current review of allergic disease*. Blackwell Science, Philadelphia, pp 39–50
18. Spencer LA, Porte P, Zetoff C, Rajan TV (2003) Mice genetically deficient in immunoglobulin E are more permissive hosts than wild-type mice to a primary, but not secondary, infection with the filarial nematode *Brugia malayi*. *Infect Immun* 71(5):2462–2467
19. Gurish MF, Bryce PJ, Tao H, Kisselgof AB, Thornton EM, Miller HR, Friend DS, Oettgen HC (2004) IgE enhances parasite clearance and regulates mast cell responses in mice infected with *Trichinella spiralis*. *J Immunol* 172(2):1139–1145
20. Strachan DP (1989) Hay fever, hygiene and household size. *Br Med J* 229:1259–1260
21. Keegan AD, Fratazzi C, Shopes B, Baird B, Conrad DH (1991) Characterization of new rat anti-mouse IgE monoclonals and their use along with chimeric IgE to further define the site that interacts with Fc ϵ RII and Fc ϵ RI. *Mol Immunol* 28:1149–1154
22. Liu F-T, Bohn JW, Ferry EL, Yamamoto H, Molinaro CA, Sherman LA, Klinman NR, Katz DH (1980) Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J Immunol* 124(6):2728–2737
23. Schwartz W, Jiao J, Ford J, Conrad D, Hamel JF, Santanbien P, Bradbury L, Robin T (2004) Application of chemically-stable immunoglobulin-selective sorbents: harvest and purification of antibodies with resolution of aggregate. *BioProcess J* 3(5):53–62
24. Isersky C, Kulczycki A Jr, Metzger H (1974) Isolation of IgE from reaginic rat serum. *J Immunol* 112:1909–1919