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MONOCLONAL DINITROPHENYL-SPECIFIC MURINE IgE ANTIBODY: PREPARATION, ISOLATION, AND CHARACTERIZATION[1]

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A murine hybridoma secreting monoclonal IgE antibodies of anti-2,4-dinitrophenyl (DNP) specificity was generated by fusion of SP2/0 tumor cells and spleen cells from DNP-*Ascaris*-hyperimmunized mice. Hybridomas secreting anti-DNP antibodies of other heavy chain classes, i.e., μ, γ_1 and γ_{2b}, were also obtained from the same fusion experiment. Large quantities of IgE antibodies were obtained from ascites of mice in which the IgE-secreting hybridoma was propagated *in vivo*. The IgE antibodies were isolated by precipitation with ammonium sulfate followed by afinity chromatography on DNP-bovine serum albumin (BSA)-Sepharose-4B and further purified by ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-200. The isolated IgE has an approximate m.w. of 184,000, a total carbohydrate content of 13.3%, and its amino acid composition has been determined. The antibody has an association constant with DNP-lysine of 1.4×10^8 M^{-1} at 25°C and 7.1×10^7 M^{-1} at 37°C. Rabbit and goat antibodies against the hybridoma IgE were prepared and the antisera were made specific for IgE by adsorption on normal mouse serum protein-Sepharose-4B. Solid phase radioimmunoassays for measuring murine antigen-specific and total IgE were developed and were shown to have high specificity and sensitivity. Finally, the isolated hybridoma IgE can mediate antigen (DNP-BSA)-induced release of mediator (serotonin) from rat basophilic leukemia cells.

The discovery of immunoglobulin E (IgE) has contributed significantly to the understanding of human hypersensitivity reactions mediated by reaginic antibodies (1). Unlike most other immunoglobulin (Ig) classes, IgE is normally present in very low concentrations in serum. For some years, this made the isolation and characterization of antibodies of this class very difficult. Our current knowledge of the physical and chemical properties of IgE has substantially benefited from the discovery of rare IgE-producing myelomas of the human and rat, and characterization of the isolated proteins from such myelomas (2–6). However, none of the available human or rat IgE myeloma proteins has a known antigenic specificity.

Studies of murine IgE have heretofore been limited due to the lack of any known spontaneous IgE-producing murine myelomas. The seminal work of Köhler and Milstein and their colleagues (7, 8) in developing the first successful technology for fusing normal antibody-secreting plasma cells to murine plasma cell tumors has opened new avenues for creating a variety of stable cell lines secreting monoclonal antibodies of known classes and specificities. Böttcher *et al.* (9) reported the successful production of a hybridoma secreting ovalbumin-specific murine IgE antibodies. Because of the potential usefulness of homogeneous IgE antibody specific for a defined determinant, we have generated a murine hybridoma that secretes large amounts of IgE antibodies with anti-2,4-dinitrophenyl (DNP) specificity. This hybridoma was created by fusion of murine myeloma cells with spleen cells from DNP-*Ascaris*-hyperimmunized mice. We have obtained large quantities of DNP-specific IgE antibodies from ascites of mice in which this IgE-secreting hybridoma was propagated *in vivo*.

Herein, we describe the preparation of this hybridoma, the purification and characterization of the DNP-specific murine IgE secreted by it, and the development of highly sensitive radioimmunoassays for quantitating murine IgE utilizing heterologous antibodies prepared against the hybridoma product.

MATERIALS AND METHODS

Chemicals, proteins and hapten-protein conjugates. *N*-DNP-glycine, *N*-ε-DNP-L-lysine and *N*-carbobenzoxy (CBZ)[4]-glycine were obtained from Sigma Chemical Company, St. Louis, Mo. [^{3}H]-DNP-L-lysine (4.9 Ci/mole) was obtained from New England Nuclear, Boston, Mass. Polyethylene glycol (PEG) 1000 was purchased from J. T. Baker Chemical Company, Phillipsburg, N. J. Sodium dodecyl sulfate (SDS) and

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[4] Abbreviations used in this paper: ASC, *Ascaris suum* proteins; Alum, aluminum hydroxide gel; CBZ, carbobenzoxy; CNBr, cyanogen bromide; DME, Dulbecco's modified Eagle's medium; GAME, goat anti-mouse IgE; GARG, goat anti-rabbit globulin; ICFA, incomplete Freund's adjuvant; NMS, normal mouse serum; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, 0.01 M phosphate buffer, pH 7.2, 0.15 M NaCl; PCA, passive cutaneous anaphylaxis; RAME, rabbit anti-mouse IgE; RBL, rat basophilic leukemia; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; NRS, normal rabbit serum.

polyacrylamide were obtained from Bio-Rad, Richmond, Calif. Lactoperoxidase was purchased from Worthington Chemicals, Freehold, N. J.

Bovine serum albumin (BSA) was purchased from Sigma Chemical Company, St. Louis, Mo. and *Ascaris* proteins (ASC) were extracted from *Ascaris suum* (10) and kindly provided by Dr. Kurt Bloch, Massachusetts General Hospital, Boston, Massachusetts. $DNP_{2.1}$-ASC, DNP_{16}-BSA and DNP_{28}-BSA were prepared as previously described (11, 12). Subscripts refer to the number of moles of DNP per mole of BSA and moles of DNP $\times 10^{-7}$/mg of ASC.

Human myeloma IgE (Sha) and rat myeloma IgE (IR162) were kindly provided by Dr. Hans Speigelberg, Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California.

Animals. (BALB/c × A/J)F_1 (CAF_1) mice (8 to 12 weeks old) were obtained from the Scripps Clinic and Research Foundation mouse breeding colony and from Jackson Laboratories, Bar Harbor, Maine. Retired breeder male albino rats (500 g or more) were purchased from Simonsen Laboratories, Gilroy, Calif. New Zealand red rabbits (2 to 5 kg) were obtained from the Scripps Clinic and Research Foundation husbandry breeding facilities. Saanen goat (140 lb.) was obtained from Burley Ranch, Bonita, Calif.

Immunoadsorbents. Cyanogen-bromide (CNBr)-activated Sepharose-4B was purchased from Pharmacia, Piscataway, N. J. and was utilized for the preparation of immunoadsorbents according to the manufacturer's instructions. DNP-BSA-Sepharose-4B was prepared by coupling 20 mg of DNP_{16}-BSA to 2 g dry weight of CNBr-activated Sepharose. Normal mouse serum (NMS) protein-Sepharose-4B was prepared by conjugating 2 ml of NMS to 4 g dry weight of CNBr-activated Sepharose-4B. Murine IgE-Sepharose-4B was prepared by coupling 1 mg of purified hybridoma IgE (see below) to 0.3 g dry weight of CNBr-activated Sepharose-4B.

Preparation of Hybridomas: 1. Sensitization of DNP-specific IgE-secreting murine spleen cells. CAF_1 mice were hyperimmunized with DNP-ASC in the following manner: Primary immunization consisted of 10 μg of DNP-ASC adsorbed on 4 mg of aluminum hydroxide gel (alum). All mice were boosted twice with 10 μg of DNP-ASC in saline 3 weeks and 11 weeks after the primary immunization. One week after the second booster immunization, spleen cell suspensions were prepared from these sensitized donor mice and transferred (35×10^6 cells/recipient) to sublethally irradiated (650 rads) syngeneic recipients. All recipients were boosted with 10 μg of DNP-ASC in 2 mg of alum shortly after cell transfer. Seven days later, all recipient mice were bled for determination of serum IgE anti-DNP antibody titers, and their spleens were removed for fusion (see below).

2. Construction of hybridomas. The parental cell line used in this study was SP2/0, a non-Ig-secreting tumor cell line of BALB/c origin, described by Shulman *et al.* (13). It is resistant to 1×10^{-4} M 8-azaguanine and is maintained in Dulbecco's modified Eagle's medium (DME, Gibco H-21,Gibco Biologicals, Santa Clara, Calif.), supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 5 μg/ml gentamycin, nonessential amino acids, glutamine, and 8-azaguanine.

The procedure used to obtain hybridomas is a modification of that described by Gefter *et al.* (14). Briefly, parental tumor cells were washed twice in DME and 7.2×10^6 tumor cells were mixed with 1×10^8 spleen cells from which red blood cells had been previously removed by lysing with Tris-buffered (10 mM, pH 7.2) ammonium chloride (0.17 M). The cells were spun down and all liquid was carefully aspirated away. The pellet was then resuspended in a solution of 30% PEG 1,000 in DME. The cells remained in this solution for a total of 8 min, during which time they were centrifuged for 3 min at 800 rpm. After the 8 min had elapsed, 5 ml of DME were added to the tube and the cells were washed twice in this medium. They were resuspended in 15 ml of DME supplemented with 10% FCS, gentamycin, nonessential amino acids, and glutamine and incubated overnight at 37°C in a 10% CO_2-in-air atmosphere. The following day, the cells were pelleted by centrifugation and resuspended in 30 ml of this same medium containing hypoxanthine (1×10^{-4} M), aminopterin (4×10^{-6} M), and thymidine (1.6×10^{-5} M), and were distributed into 192 wells of 96-well, flat-bottomed microplates (Linbro, Hamden, Conn.). Cultures were fed after 1 week by the addition of 1 drop of this same medium without aminopterin.

3. Screening of DNP-specific IgG and IgE hybridoma clones. Hybridomas secreting anti-DNP antibody activity in their supernatants were detected by radioimmunoassay (RIA), as described below. Positive clones secreting IgE antibodies were identified by 1) failure to react in solid-phase RIA with antibodies specific for the other heavy chain Ig isotypes, and 2) positive reaction detected by the passive cutaneous anaphylaxis (PCA) technique in rats; PCA reactions were conducted as described previously (15). Other heavy chain Ig isotypes of other anti-DNP antibody-secreting clones were determined by solid-phase RIA as described below.

4. Passage of hybridoma cells in vivo. CAF_1 mice were injected i.p. with 0.5 ml of pristane (Aldrich Chemical Company, Milwaukee, Wis.). One week or more later, these mice were injected with hybridoma cells (1×10^6 per mouse) i.p. The tumor cells were allowed to grow as ascites in the peritoneal cavity for approximately 2 weeks, after which the ascites fluids were collected in the presence of heparin and centrifuged. The cell pellets were resuspended and either passaged again *in vivo* into pristane-primed CAF_1 mice or frozen in 10% dimethylsulfoxide and preserved in liquid nitrogen. The supernatants recovered from such ascites fluids were kept frozen at −70°C until used.

Purification of monoclonal DNP-specific IgE antibodies. Cold ammonium sulfate (pH 7.0) was added to the hybridoma ascites fluid to 30% saturation. The precipitates were removed and the IgE antibodies were precipitated from the supernatant at 60% saturation of ammonium sulfate at 4°C. The precipitates were redissolved in 0.01 M phosphate-buffered, 0.15 M NaCl (PBS, pH 7.2) and dialyzed against PBS overnight. The solution was then applied to a column of DNP-BSA-Sepharose-4B; unbound material was eluted with PBS, followed by 0.05 M CBZ-glycine (pH 8.0). The bound, DNP-specific IgE was eluted with 0.02 M DNP-glycine (pH 8.0). Free DNP-glycine was removed by dialysis against several changes of PBS.

IgE was further purified by ion-exchange chromatography on DEAE-cellulose (DE-52, Whatman, Clifton, N. J.); the column was eluted with a linear gradient of 0.01 M Tris-HCl, pH 8.0, to 0.01 M Tris-HCl, pH 8.0, 0.2 M NaCl. The IgE peak was pooled, concentrated, and further purified by gel filtration on Sephadex G-200 (Pharmacia) equilibrated in PBS and eluted with the same buffer.

All procedures were performed at 4°C; the concentration steps were achieved by using Millipore immersible CX ultrafiltration units (Millipore, Corp., Bedford, Mass.).

Physicochemical characterization: 1. Analytical ultracentrifugation. High-speed equilibrium ultracentrifugation (16) was carried out in a Spinco Model E ultracentrifuge (Beckman Instruments, Fullerton, Calif.) equipped with absorption optics. Samples of purified IgE (250 μg/ml, 100 or 200 μl) in PBS were

centrifuged at 10,000 rpm for 68 hr. For estimation of m.w., partial specific volume was calculated (17) from the amino acid and carbohydrate composition determined for this protein as described below. The calculated partial specific volumes of sugar residues were taken from the literature (18). Sedimentation velocity measurements were made in the same instrument. Samples (250 or 500 μg/ml, 600 μl) in PBS were centrifuged at 56,000 rpm at 25°C. The sedimentation coefficient was corrected to the viscosity and density of water at 20°C.

2. Amino acid analysis. Amino acid analysis was carried out on a Beckman Spinco Model 121-M amino acid analyzer; protein samples were hydrolyzed in 6 N HCl in sealed and evacuated tubes at 110°C for 24 hr, 48 hr, and 72 hr. Triplicate samples were run for each hydrolysis time. Serine and threonine values were corrected to 0 hydrolysis time. Cysteine and methionine were analyzed as the oxidized products (performic acid oxidation of protein at 0°C for 2 hr). For the analysis of tryptophan, triplicate protein samples were hydrolyzed in 5 N NaOH for 22 hr at 110°C in the presence of 15 mg of hydrolyzed potato starch (19).

3. Extinction coefficient, $E^{1\%}_{280nm}$. This was estimated from $E = A/1c$, where A is the absorbance at 280 nm, 1 is the length of optical path (1 cm), and c is the concentration of IgE (mg/ml) determined by the sum of amino acid composition and carbohydrate content.

4. SDS-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE analysis was conducted with the Laemmli system (20), running purified IgE under reducing conditions on a 7 to 17% gradient slab gel or a 10% tube gel.

5. Carbohydrate analysis. Total hexose was determined by the phenol-sulfuric acid reaction (21) by using an equimolar mixture of galactose and mannose as standard. Fucose was determined by Dische-Shettles cysteine-sulfuric acid reaction (22). *N*-acetyl neuraminic acid (sialic acid) was measured by the thiobarbituric acid assay (23). Hexosamine was determined with a Beckman Spinco amino acid analyzer after hydrolysis in 4 N HCl in sealed and evacuated tubes at 110°C for 4 hr.

6. Equilibrium dialysis. Equilibrium dialysis measurements were performed by a previously described modification (24). Dialysis bags containing 0.5-ml samples of a) anti-DNP IgE, b) normal mouse IgG, or c) PBS were placed in 125-ml bottles containing [^{3}H]-DNP-lysine in the range of 0.625 to 10×10^{-8} M. IgE and IgG were used at a concentration of 25 μg/ml; each sample was run in duplicate. The bottles were rotated at 25°C for 48 hr or at 37°C for 12 hr. After equilibration, triplicates of 50-μl samples from each dialysis bag were counted; counting errors for all samples were less than 2%.

Antisera: 1. Rabbit anti-mouse IgE (RAME) antiserum. RAME was prepared by immunizing New Zealand red rabbits in the footpads with 500 μg of purified IgE in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, Mich.). Booster injections consisted of 500 μg of IgE in incomplete Freund's adjuvant (ICFA, Difco) injected subcutaneously 2 weeks after primary immunization, and 100 μg of IgE in 4 mg of alum injected i.p. 2 weeks thereafter. The resulting RAME antibodies were treated in four different ways to obtain different levels of purification as follows:

a) NMS-adsorbed. RAME serum was adsorbed twice with NMS protein-Sepharose-4B to remove any contaminating reactivities with NMS proteins other than IgE. Eighteen milliliters of RAME antisera were passed through a column consisting of a 12-ml bed volume by using PBS as the elutant. *b) NMS-adsorbed, RAME globulin fraction.* NMS-adsorbed RAME serum was precipitated with ammonium sulfate at 50% saturation at 4°C, redissolved in PBS, and extensively dialyzed against PBS. *c) NMS-adsorbed, DEAE-fractionated RAME IgG.* NMS-adsorbed RAME globulin fraction was further fractionated into IgG by ion-exchange chromatography on DE-52 eluted with 0.0175 M phosphate buffer, pH 6.9. *d) Affinity-purified, RAME IgG.* NMS-adsorbed, DEAE-fractionated RAME IgG was further purified by affinity chromatography on IgE-Sepharose-4B, the column being eluted with 0.1 M glycine-HCl, pH 2.7, and the resulting fraction was neutralized immediately with 1 M Tris-HCl buffer, pH 8.5.

2. Goat anti-mouse IgE (GAME) antiserum. GAME was prepared by immunizing Saanen goat with 100 μg of purified IgE in CFA injected subcutaneously. Booster injections consisted of 100 μg of IgE in ICFA injected by the same route 2 and 5 weeks after primary immunization. The resulting GAME serum was NMS-adsorbed and then fractionated into globulin fraction by ammonium sulfate precipitation as described for RAME.

3. Other antisera. Class-specific rabbit antibodies of the specificities anti-mouse μ, γ_1, and γ_2b were obtained from Litton-Bionetics, Kensington, Md. and rendered highly specific by adsorption on anti-DNP-secreting hybridomas of known heavy chain class; anti-mouse κ and λ were obtained from Miles Laboratories, Inc., Elkhart, Ind. Goat anti-rabbit γ-globulin (GARG) was purified from hyperimmune goat antisera by affinity chromatography on rabbit IgG-Sepharose-4B.

Radioiodination of proteins. Purified GARG and purified IgE were labeled with [^{125}I] by the solid-phase lactoperoxidase method (25) yielding specific activities of 30 to 40 μCi/μg.

Radioimmunoassays: 1. RIA for mouse IgE. Two different types of RIA were developed for detection of mouse IgE; these are described below: *a) Solution-phase RIA.* Twenty microliter solutions of serially-diluted hybridoma IgE standard (or a test sample) were dispensed into each well of a 96-well, v-bottom microtiter plate (Linbro). To each well was added 20 μl of solution of NMS-adsorbed, DEAE-fractionated RAME IgG of appropriate dilution in 5% normal rabbit serum. After 1-hr incubation at room temperature, 20 μl of [^{125}I]-labeled hybridoma IgE (10,000 cpm, diluted in 0.5% BSA-PBS) were added and the plate was again incubated at room temperature for 1 hr. Forty microliters of hyperimmunized goat-anti-rabbit IgG serum were then added and the plate was left at 4°C. After overnight incubation, the plate was spun at 2,000 rpm for 30 min. Fifty microliters of the supernatant in each well were then counted in an automatic gamma counter.

b) Solid-phase RIA for determining DNP-specific IgE. This method followed essentially the procedure described elsewhere for the measurement of DNP-specific antibodies of other Ig isotypes (26, 27). Briefly, 96-well Flex-vinyl U-bottom microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of 100 μg/ml solution of DNP_{28}-BSA at room temperature for 2 hr or at 4°C overnight. Unattached sites in the wells were saturated with a 10% FCS-PBS solution at room temperature for 2 hr. To the washed plate were added 25 or 100 μl of IgE standard (or a test sample) and the plate was incubated at 4°C for 2 to 4 hr. After thorough washing of the plate, to each well were added 100 μl of affinity-purified RAME IgG (1 μg/ml, diluted with 10% FCS-PBS) and the plate was incubated for 2 to 4 hr at 4°C. Finally, 100 μl of [^{125}I]-labeled GARG antibody (100,000 cpm per well diluted with 10% FCS-PBS) were added to the washed wells and the plate was allowed to incubate overnight at 4°C. The wells were thoroughly washed, dried, and cut off with a hot wire and then individually counted in an automatic gamma counter.

c) Solid-phase RIA for total IgE. The same procedure as that described above for DNP-specific IgE was used except 200 μg/ml of NMS-adsorbed, GAME globulin fraction were used to coat the plate.

2. RIA for other Ig isotypes. Non-IgE Ig isotypes were analyzed by solid-phase RIA by using the method described for IgE above but with class-specific rabbit anti-mouse μ, γ_1, γ_{2b}, κ, and λ diluted 1/1,000 to 1/2,000 followed by detection with [^{125}I]-labeled GARG antibody.

Serotonin release assay: 1. Cells. The rat basophilic leukemia cell line (RBL subline 2H3) was kindly provided by Dr. Henry Metzger (National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland) and was propagated and maintained as described (28).

2. Assay of incorporated [^{3}H]-serotonin release. The assay was carried out as previously described (29). Briefly, cells (1 to 2×10^6 ml) were incubated at 37°C for 1 hr with hybridoma IgE (3 μg/ml) and 4 μCi/ml of [^{3}H]-hydroxytryptamine binoxalate (28.2 Ci/mmole, New England Nuclear, Boston, Mass.). After washing, aliquots of cell suspension were incubated with antigen (DNP_{28}-BSA, 0.001 to 1000 ng/ml) or NMS-adsorbed RAME (0.1 to 100 μg/ml) at 37°C for 1 hr. One-hundred-microliter aliquots of the supernatants were counted in glass vials containing 10 ml Scinterverse (Fisher Scientific Co., Pittsburgh, Pa.).

RESULTS

Experimental design. The overall experimental plan employed in this study is presented schematically in the flow diagram summarized in Figure 1.

Construction and characterization of anti-DNP-IgE-secreting hybridomas: 1. Hybridomas producing IgE anti-DNP antibodies. The monoclonal DNP-specific IgE antibody characterized in this study has been obtained from a hybridoma constructed by fusing hyperimmune CAF_1 spleen cells with the BALB/c tumor cell line, SP2/0. The rationale for using an immunization protocol that included an adoptive transfer as the final step stems from the observation that secondary challenge in adoptive transfer recipients markedly increases the frequency of IgE-producing (and other Ig classes) lymphocytes (12). In the present study, for example, the serum IgE titers of the adoptive transfer recipients from which spleen cells were obtained for fusion were in the range of 20,480 to 40,960.

After fusion, hybrids appeared in 58 of the 192 wells and 15 of these expressed anti-DNP antibody activity in their supernatants as determined by RIA. These were further tested to determine their Ig isotypes. Supernatant fluids from selected clones displaying positive anti-DNP activity were tested by PCA reactions in rats. Two clones were identified as positive for IgE, the PCA titers of the supernatants in both cases being 2560. One of these two clones lost its IgE-secreting ability after passage *in vivo*. The other clone was subcloned and a clone was selected. Both the parent clone and its subclone are stable cell lines. The IgE characterized in the remainder of this study was obtained from the clone denoted H1 DNP-ε-26.

The IgE-secreting hybridomas were propagated *in vivo* in pristane-primed CAF_1 mice. These mice generally developed large quantities of ascites fluid. The PCA titers of both ascites fluids and serum of such carrier mice were in the range of 1 to 2×10^6, which corresponds to 2 to 3 mg/ml of IgE antibodies.

2. Hybridomas producing DNP-specific antibodies of other Ig isotypes. Hybridomas secreting anti-DNP antibodies of the μ, γ_1 and γ_2b Ig isotypes were obtained from the same fusion. The characteristics of these hybridomas are summarized in Table I.

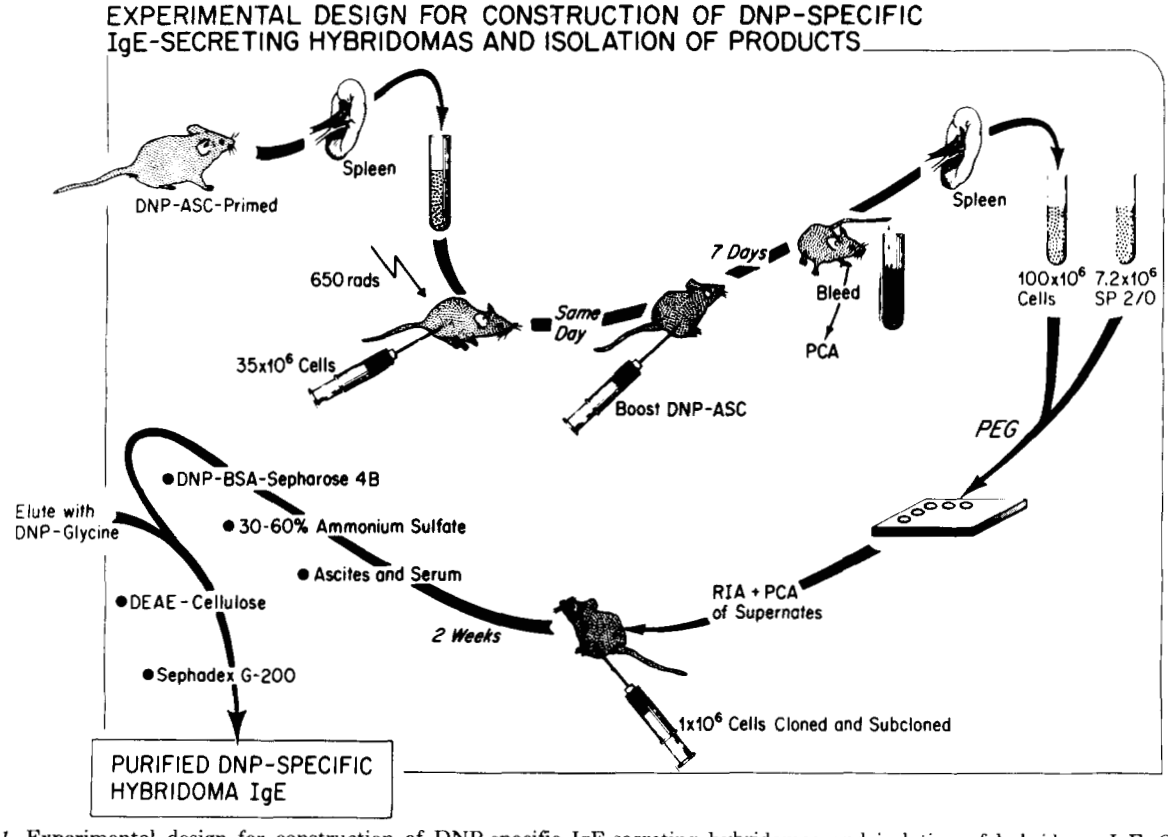

Figure 1. Experimental design for construction of DNP-specific IgE-secreting hybridomas and isolation of hybridoma IgE. See text for explanation.

TABLE I

Summary of DNP-specific murine hybridoma Ig of different isotypes obtained in this work

Identification	Heavy Chain Isotype	Light Chain Class
H1 DNP-ε-26.82	ε	κ
H1 DNP-μ-32.17	μ	κ
H1 DNP-γ-109.3	γ_1	κ
H1 DNP-γ_{2b}-10.12	γ_{2b}	λ

Purification of monoclonal IgE. Hybridoma IgE in the ascites fluids was isolated by precipitation with ammonium sulfate. At 50% saturation, only 70% of the IgE activity, as detected by PCA reactivity, was precipitated whereas approximately 90% of IgE activity was precipitated at 55% saturation. Therefore, proteins soluble at 30% and precipitable at 60% ammonium sulfate saturation at 4°C were selected.

DNP-specific IgE was then subjected to affinity chromatography on DNP-BSA-Sepharose-4B. IgE activity could be recovered by elution with either 0.02 M DNP-glycine (pH 8.0) as shown in Figure 2*A*, or with 0.1 M glycine-HCl, pH 2.7. In our experience, the former procedure clearly yielded IgE of higher purity as determined by SDS-PAGE analysis.

Affinity-purified DNP-specific IgE was then subjected to further chromatographic separation. On DEAE ion-exchange columns, IgE was eluted at 0.01 M Tris-HCl, pH 8.0, at 0.04 to 0.07 M NaCl (Fig. 2*B*). Figure 2*C* displays the chromatogram obtained when DEAE-fractionated, affinity-purified IgE was subjected to gel filtration on Sephadex G-200. Some proteins were eluted earlier than the major protein peak and are most probably aggregates of IgE.

As stated above, IgE isolated from DNP-BSA-Sepharose affinity columns by elution with DNP-glycine was already of high purity. As shown in Figure 3, no contaminating proteins could be detected in the sample of IgE isolated from H1 DNP-ε-26.82 when 30 μg of protein samples were subjected to SDS-PAGE in 5-mm tube gels.

It is worth noting that the hybridoma IgE retained biologic activity after purification by affinity chromatography, ion-exchange chromatography, and gel filtration. A solution of 300 μg/ml of IgE purified by these three steps displayed a PCA titer of 163,840.

Physical and chemical characterization of hybridoma IgE antibody. The physicochemical characteristics of DNP-specific hybridoma IgE obtained with highly purified protein sample are summarized in Table II. The amino acid and carbohydrate compositions are presented in Tables III and IV. From the high-speed sedimentation equilibrium experiments, the m.w. was calculated to be 184,000 ± 3000, which is comparable to the values of 188,000 ± 3,000 determined for human myeloma IgE (4) and 179,000 for rat myeloma IgE (6), both determined by analytical ultracentrifugation. The estimated m.w. was based on a partial specific volume ($\bar{v}$) calculated from the amino acid (Table III) and carbohydrate composition (Table IV), $\bar{v} = 0.715$.

The results of SDS-PAGE analysis of purified IgE run under reducing conditions on a 7 to 17% gradient gel is shown in Figure 4. The IgE molecule was separated into heavy and light chains under the running conditions. The heavy chain migrates slower than the heavy chain of human IgE (m.w. 72 to 76 × 10^3, 4) and mouse μ chain (m.w. 72 × 10^3) but close to that of rat IgE (68 × 10^3, 6), and the light chain has mobility similar to κ- and λ-chains. By comparing the migration of these two polypeptide chains to that of standard proteins, m.w. of 82.2 × 10^3 ± 2900 and 22.9 × 10^3 ± 500 were estimated for heavy and light chains, respectively. Thus, the IgE molecule appears to consist of two heavy plus two light chains, as expected. The total apparent m.w. of 210,000 ± 6800 daltons estimated from SDS-PAGE was slightly higher than that determined by ultracentrifugation and could be explained by the high carbohydrate content of this protein. Also included in the slab gel (Fig. 4) are

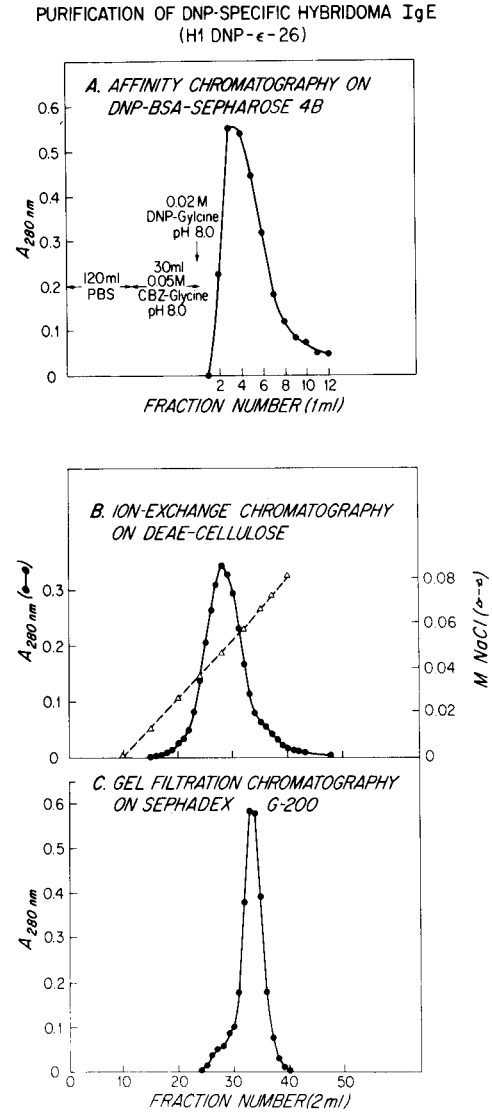

Figure 2. Purification of DNP-specific hybridoma IgE antibody. *A*, affinity chromatography on DNP-BSA-Sepharose 4B. Proteins precipitated from the hybridoma ascites fluids (7.8 ml) at 30 to 60% saturation of ammonium sulfate (pH 7.0) at 4°C was loaded on DNP-BSA-Sepharose 4B (5 ml bed volume) at a flow rate of 10 ml/hr and eluted with PBS until A_{280} was <0.01 (120 ml) that was followed by 0.05 M CBZ-glycine, pH 8.0 (30 ml). IgE was eluted with 0.02 M DNP-glycine, pH 8.0. Flow rate, 30 to 40 ml/hr. UV absorption of fractions was determined after the samples were dialyzed against PBS to remove DNP-glycine. *B*, ion exchange chromatography on DEAE-cellulose. DNP-Sepharose-purified IgE (5.5 mg in 4 ml of 0.01 M Tris-HCl, pH 8.0) was applied to DE-52 (20 ml bed volume) equilibrated in the same buffer. The material was eluted with a linear gradient of 0.01 M Tris-HCl, pH 8.0 (75 ml) to 0.01 M Tris-HCl, pH 8.0, 0.2 M NaCl (75 ml). Flow rate, 36 ml/hr. The NaCl concentration of fractions was determined from the conductivity of solutions. *C*, gel filtration chromatography on Sephadex G-200. Affinity purified, DEAE-fractionated IgE (4.4 mg in 3.5 ml PBS) was applied to a column of Sephadex G-200 (1.6 x 90 cm) equilibrated in PBS and eluted with the same buffer. Flow rate, 7 ml/hr.

Figure 3. Determination of purity of affinity-purified hybridoma IgE by SDS-polyacrylamide gel electrophoresis. DNP-specific hybridoma IgE (30 μg) purified by affinity chromatography on DNP-BSA-Sepharose (Fig. 2*A*) was run under reducing conditions in 10% polyacrylamide (5-mm tube) by using Laemmli's buffer system (20). Proteins were stained with Coomassie Blue.

TABLE II

Physicochemical characteristics of DNP-specific murine hybridoma IgE (H1 DNP-ε-26)

Physical Constant	
Molecular weight	184,000 ± 3000
$S_{20,w}$	8.2
$E^{0.1\%\ 1\ cm}_{280\ nm}$	1.62
Association constant[a]	$1.4 \times 10^8\ M^{-1}$ (25°C); $7.1 \times 10^7\ M^{-1}$ (37°C)
Carbohydrate	13.3%

[a] Affinity for *N*-ε-DNP-L-lysine.

monoclonal anti-DNP $\gamma_1\kappa$, $\gamma_2 b\lambda$ and $\mu\kappa$ obtained in this study (see Table I).

The binding affinity of the anti-DNP hybridoma IgE for DNP-lysine was assessed by equilibrium dialysis. The data are plotted in Figure 5. The plot is clearly linear indicating that the protein's binding sites are homogeneous. From the slope of the plot and ordinate intercept, an association constant of 1.4×10^8 M^{-1} at 25°C was obtained. A value of 7.1×10^7 M^{-1} at 37°C was similarly obtained.

Although not shown in Table III, we have found that purified IgE obtained from the parent clone H1 DNP-ε-26 and the subclone H1 DNP-ε-26.82 have essentially identical amino acid compositions.

Immunochemical properties of hybridoma IgE and rabbit and goat anti-mouse IgE antibodies. Rabbit and goat antibodies against mouse IgE were obtained from rabbit and goat immunized with purified IgE. The RAME and GAME antisera were made specific for IgE by adsorption on NMS protein-Sepharose-4B. The NMS-adsorbed, ammonium sulfate-precipitated RAME globulin fraction was found to be capable of completely neutralizing the reaginic activity of serum obtained

TABLE III

Amino acid composition of murine hybridoma IgE (H1 DNP-ε-26)

Amino Acid	Mean[a]	Integer
Lysine	89.16 ± 0.90	89
Histidine	32.09 ± 0.90	32
Arginine	52.63 ± 1.75	53
Aspartic acid	134.11 ± 1.43	134
Threonine[b]	131.10 ± 1.32	131
Serine[b]	172.02 ± 2.65	172
Glutamic acid	127.02 ± 1.64	127
Proline	87.04 ± 4.13	87
Glycine	89.27 ± 1.80	89
Alanine	57.02 ± 0.85	57
Cystine/2	29.86 ± 2.65	30
Valine	102.61 ± 2.54	103
Methionine	11.86 ± 0.79	12
Isoleucine	59.78 ± 1.32	60
Leucine	119.82 ± 1.32	120
Tyrosine	57.50 ± 1.91	57
Phenylalanine	53.37 ± 1.75	53
Tryptophan	30.92 ± 2.65	31
Molecular weight	159,800[c]	

[a] Mean value ± S.D. for nine analyses (three each for 24, 48, and 72-hr hydrolysis).

[b] Corrected to zero hydrolysis time.

[c] Sum of observed residue weight.

TABLE IV

Carbohydrate composition of murine hybridoma IgE (H1 DNP-ε-26)

Residue	Weight %
Hexose[a]	7.8
Fucose[b]	0.66
N-acetyl neuraminic acid[c]	0.65
N-acetyl glucosamine[d]	4.2

[a] Determined by phenol-sulfuric acid reaction (21).

[b] Determined by cysteine-sulfuric acid reaction (22).

[c] Determined by thiobarbituric acid assay (23).

[d] Determined by amino acid analyzer.

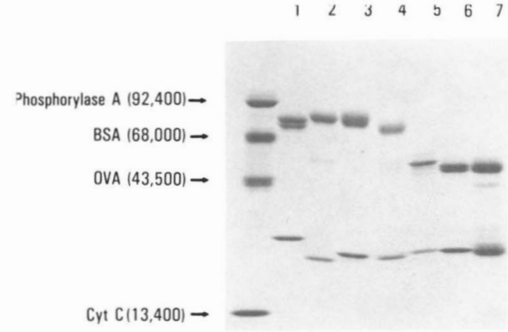

Figure 4. SDS-polyacrylamide gel electrophoretic analysis of purified mouse hybridoma IgE. 1, Human myeloma IgE (Sha); 2, rat myeloma IgE (IR162); 3, εκ; mouse hybridoma IgE (H1 DNP-ε-26); 4, μκ (H1 DNP-μ-32.17); 5, $\gamma_{2b}\lambda$ (H1 DNP-γ_{2b}-10.12); 6, $\gamma_1\kappa$ (H1 DNP-γ_1-109.3); and 7, mouse IgG were electrophoresed under reducing conditions in a 7 to 17% gradient slab of acrylamide in Laemmli's buffer system. Proteins were stained with Coomassie Blue.

from sensitized donor mice. Thus, 25 μl of high-IgE titer anti-DNP mouse serum (PCA=20,480) was mixed with 100 μl of NMS-adsorbed RAME globulin fraction or 100 μl of normal rabbit serum (NRS) as a control. The solution was incubated at room temperature for 2 hr and at 4°C overnight. The precipitates formed were spun down and the supernatant was assayed for IgE activity by PCA. The PCA titer of RAME-treated sample was <10 whereas those of both untreated and NRS-treated sample was 5120.

The existence of possible cross-reactivities of RAME with human and/or rat IgE was examined by RIA with NMS-adsorbed, ammonium sulfate-precipitated, DEAE-fractionated IgG isolated from the RAME serum. As shown in Figure 6, the RAME IgG fraction reacts with rat IgE (IR162), although the extent of cross-reactivity is very low. In contrast, the RAME IgG failed to exhibit any cross-reactivity with human IgE (Sha).

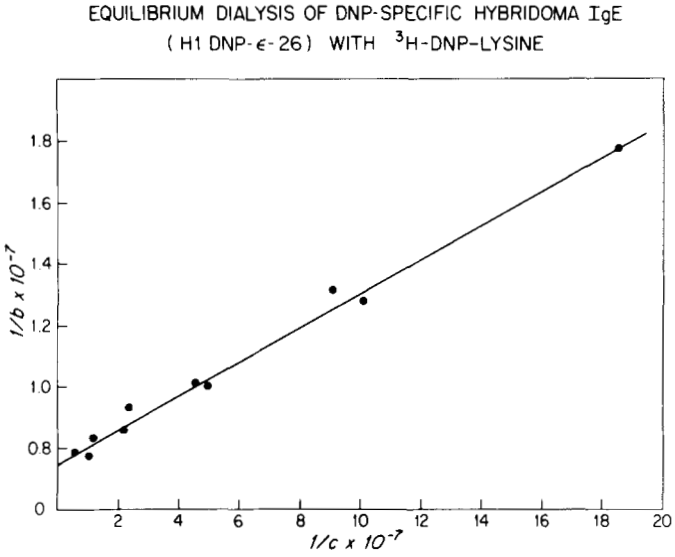

Figure 5. Equilibrium dialysis of DNP-specific hybridoma IgE with [^{3}H]-DNP-lysine. IgE solutions (25 μg/ml, 0.5 ml) were dialyzed against 120 ml of [^{3}H]-DNP-lysine in PBS ($0.625 - 10 \times 10^{-8}$ M) for 48 hr at 25°C. Duplicates were run for each hapten concentration. *b*, Concentration of bound hapten (M). *c*, Concentration of free hapten (M). Correlation coefficient, 0.993.

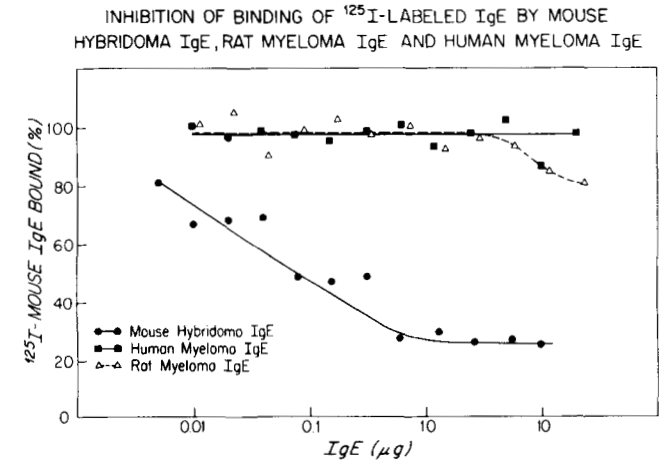

Figure 6. Inhibition of binding of ^{125}I-labeled IgE by mouse hybridoma IgE, rat myeloma IgE, and human myeloma IgE. Competition of DNP-specific hybridoma IgE, rat myeloma IgE (IR162), and human myeloma IgE (Sha) with ^{125}I-labeled mouse hybridoma IgE in binding to NMS-adsorbed, DEAE-fractionated RAME IgG was measured by solution-phase RIA. See *Materials and Methods* for details. Duplicates were run at each concentration.

TABLE V

Specificities of RIA for murine anti-DNP antibodies of the IgE, IgG2b, IgG1, and IgM isotypes

Rabbit Anti-class Specific Antibody[a]	Background	IgE[b] (H1 DNPε26.82)	IgG2b (H1 DNPγ2b10.12)	IgG1 (H1 DNPγ1109.3)	IgM (H1 DNPμ32.17)
Anti-IgE	200	3560[c]	400	400	390
Anti-γ2b	360	240	3400	360	200
Anti-γ1	230	275	200	1300	200
Anti-μ	350	340	360	300	1960
Anti-κ	200	2500	250	1700	3500
Anti-λ	300	440	1100	468	500

[a] Anti-λ: adsorbed over γ1κ; κ, μκ. Anti-γ1: adsorbed over γ2b, λ; αλ. Anti-κ: adsorbed over γ2b, λ; αλ.

[b] Ten nanograms of antibody to wells of DNP_{28}-BSA coated plates.

[c] Counts per minute.

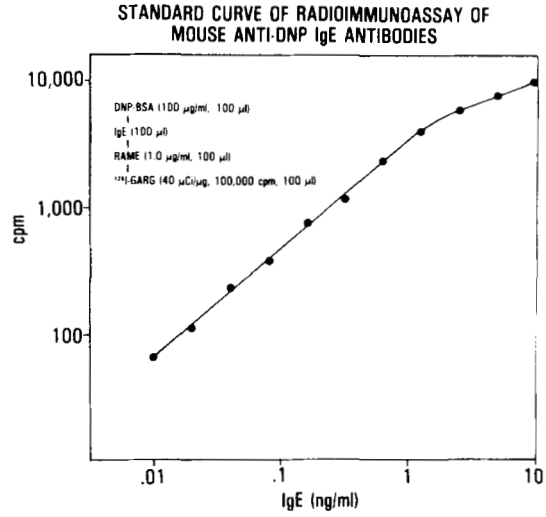

Figure 7. Standard curve of radioimmunoassay of mouse anti-DNP IgE antibodies. Solid-phase RIA (see *Materials and Methods*) of hybridoma IgE (H1 DNP-ε-26) on Flex-vinyl microtiter plates coated with DNP-BSA. Concentration of anti-DNP mouse IgE in test samples included in the same assay can be determined from cpm found in the corresponding wells by using this standard curve.

RIA for murine IgE. The availability of heterologous antibodies specific for IgE made possible the detection of murine IgE by RIA. Since the quantity of IgE in serum is usually low, even in immunized mice, especially compared to IgG, high specificity as well as high sensitivity of such an assay are absolutely required. Two assay systems were tested, one allowing measurement of antigen-specific IgE (i.e., anti-DNP), whereas the other permits quantitative determinations for total IgE. The result of solid phase RIA for anti-DNP of the IgE and other Ig class is depicted in Table V. For this experiment the binding specificity of RAME and specific antibodies to other H and L chain classes is determined by using anti-DNP hybridoma of the IgE, IgM, IgG2b, and IgG1 classes. Figure 7 depicts a standard curve for the IgE hybridoma antibodies as detected by RAME.

Under these conditions, a sensitivity of 10 pg/ml for quantitation of IgE anti-DNP was obtained. This methodology exhibits sufficient sensitivity and specificity to allow its use as a routine RIA for IgE in culture fluid or in serum as verified by the results when utilizing this assay for determining IgE responses in various test samples obtained from experiments that have been conducted in our laboratory. As shown in Table VI,

the RIA results correlated very well with results obtained by the PCA technique for several serum samples. The class specificity of this assay is evident when comparing results of samples 5 and 6. Sample 6 has more than 100-fold higher Ig of other isotypes (i.e., non-IgE) than sample 5 and yet the two samples have rather similar PCA titers. The RIA results likewise indicate that these two samples have similar contents of IgE, although sample 5 is slightly higher. It thus appears that this RIA for IgE is not appreciably affected by antibodies of the same specificity of other Ig classes.

TABLE VI

Representative radioimmunoassay of IgE and other immunoglobulin classes of test mouse serum[a]

Sample	IgM	IgG1	IgG2b	IgE	PCA
I	74	12	50	<0.04	<10
II	60	2	13	0.32	40
III	44	34	32	1.4	320
IV	240	200	120	2.4	640
V	<5	0.5	1	6.0	1280
VI	360	180	140	4.4	2560

[a] Solid-phase RIA. The Ig isotypes are expressed in micrograms per milliliter.

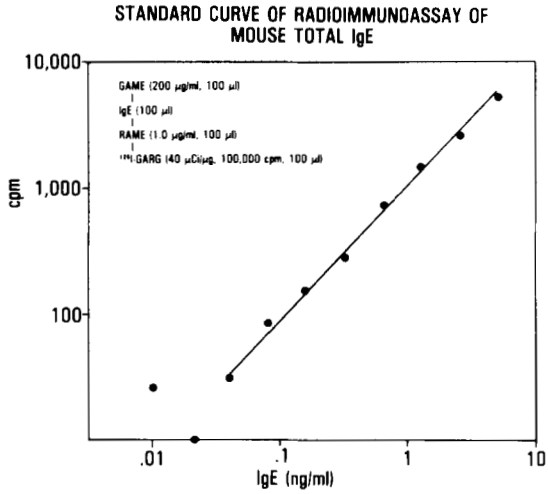

Figure 8. Standard curve of radioimmunoassay of total mouse IgE. Solid-phase RIA (see *Materials and Methods*) of hybridoma IgE (H1 DNP-ε-26) on Flex-vinyl microtiter plates coated with NMS-adsorbed, GAME-globulin fraction. Concentration of total mouse IgE in test samples included in the same assay can be determined from cpm found in the corresponding wells by using this standard curve.

TABLE VII

Mouse hybridoma IgE-mediated release of serotonin from rat basophilic leukemia cells[a]

Triggering Agent	Concentration	% Release of Serotonin[b]
	ng/ml	
DNP_{28}-BSA	0.001	8
	0.01	22
	1	27
	100	26
	1,000	23
RAME	100	3
	1,000	4
	10,000	14
	100,000	15

[a] 2.0×10^6 cells/ml were incubated for 1 hr at 37°C with 3 μg/ml of hybridoma IgE and 8 μCi/ml of ^{3}H-serotonin.

[b] Total-spontaneous release; spontaneous release 35%.

The standard curve of the RIA assay for total IgE is shown in Figure 8. With the availability of GAME in addition to RAME, the RIA for total IgE can be done with the same reagents and sequence as for antigen-specific IgE by using GAME-coated plates. A similar sensitivity of 40 pg/ml was achieved.

Hybridoma IgE-mediated release of serotonin from rat basophilic leukemia (RBL) cells. The ability of monoclonal hybridoma IgE to mediate antigen-induced triggering of mast cell-like cells to release mediators was studied with the RBL cell lines 2H3. As shown in Table VII, RBL cells sensitized with DNP-specific hybridoma IgE can be triggered by either antigen (DNP-BSA) or anti-IgE antibodies (NMS-adsorbed RAME) to release incorporated [^{3}H]-serotonin.

DISCUSSION

The present studies establish four points: 1) murine hybridomas secreting IgE antibodies of a known specificity (*i.e.*, anti-DNP) can be constructed in a manner that is easily reproducible and provides cell lines manifesting long-term stability; 2) such hybridomas secrete IgE antibodies in such plentiful amounts that it is possible to obtain substantial quantities of antigen-specific IgE molecules in highly purified form for physicochemical and immunochemical analysis; 3) the availability of such high quantities of IgE has made it possible to generate heterologous anti-IgE antibodies that are of obvious usefulness in further studies designed to isolate IgE molecules and for development of highly sensitive assay techniques for its measurement; and 4) the homogeneous hybridoma IgE can mediate antigen-induced release of serotonin from rat basophilic leukemia cells. Thus, it is possible to study the antigen-induced IgE-mediated triggering of mast cells by using this well-characterized IgE and homogeneous populations of cells.

The construction of the hybridoma described in detail herein is based on a modification of the technology originally developed by Köhler and Milstein (7, 8). Importantly, donor cells were obtained from an adoptive transfer protocol that is particularly advantageous in generating spleen cell populations with exceptionally high frequencies of DNP-specific IgE-secreting cells and their precursors (as well as cells of other Ig isotypes with anti-DNP specificity). This, in turn, presumably resulted in a much higher frequency of IgE-secreting hybridomas than other protocols might have provided. The hybridomas so produced could be propagated both *in vitro* and *in vivo*, although the *in vivo* approach resulted in considerably larger quantities of DNP-specific IgE antibodies secreted into the ascites fluids by such cells. As shown herein, the IgE anti-DNP hybridoma antibodies could be isoalted in large quantities from such ascites fluids by affinity chromatography on DNP-BSA Sepharose. For example, 30 ml of hybridoma ascites applied to a DNP-Sepharose affinity column (10-ml bed volume) yields as much as 100 mg of affinity-purified IgE.

The IgE antibody obtained by affinity chromatography on DNP-Sepharose was essentially electrophoretically homogeneous. For further physicochemical characterization, the antibody was purified by DEAE-cellulose ion-exchange chromatography and Sephadex G-200 gel filtration. Physicochemical analysis demonstrated that the IgE antibody is composed of two heavy and two light chains. The m.w. of 184,000 d, determined by equilibrium sedimentation, is comparable to that previously reported for human and rat IgE. The carbohydrate content, 13.3%, is also similar to that of human IgE.

The purified IgE was shown by SDS-PAGE and high-speed ultracentrifuge sedimentation to be homogeneous. Since IgE is considered to be a relatively labile antibody, some denaturation of the protein during its isolation was anticipated. However, when we analyzed by PCA reactivity the IgE activity of the hybridoma proteins after each purification step, the extent of denaturation appeared to be minimal. Thus, the crude hybridoma ascites fluid had PCA titers of 1,310,720 to 2,621,440, the biologic activity manifested by approximately 2 to 3 mg/ml of IgE; after all purification steps, the IgE obtained displayed a PCA titer of 320 at a concentration of 1 μg/ml, indicating a substantial retention of biologic activity. Moreover, from the PCA titer obtained with the purified IgE, we estimate that a detectably-positive PCA reaction with this particular IgE protein could be obtained with an IgE solution of 2 to 4 ng/ml (0.1 ml/site).

Heterologous rabbit and goat antisera to the murine hybridoma IgE were produced and made specific for the ε heavy chain by adsorption with NMS-conjugated Sepharose. These ε-specific antisera were found to have high capacity for neutralizing reaginic antibodies in immune mouse serum samples. With these antisera, RIA specific for IgE were developed that have sensitivities for quantitating mouse IgE at a level of 10 to 40 pg/ml. Direct comparisons between the RIA and the classical PCA reaction on identical test samples verified correlation between the two tests, thereby making it now possible to use the RIA as a more efficient procedure for measuring either total or antigen-specific IgE antibody molecules where desirable.

Heretofore, the isolation and purification of mouse and rat reaginic antibodies have been extremely difficult (30–32). The availability of large quantities of homogeneous, monoclonal murine IgE, as described herein, made the analysis of the structure of this important class of Ig possible. On the practical side, the existence of DNP-specific monoclonal IgE of relatively high binding affinity now makes it feasible to study the dynamics of mast cell triggering when surface-bound IgE antibody molecules react with their specific ligand. Such studies, which are currently underway in our laboratory, should complement and extend the previous studies of Metzger (33) and Ishizaka and Ishizaka (34) and their associates and other studies performed, by necessity, by utilizing the reaction of anti-IgE antibodies with surface-bound IgE moleules. In addition the ε class-specific heterologous antibodies can be used as a reagent to explore the development and stimulation of IgE-producing B cells. Finally, the IgE-specific RIA can, as mentioned above, replace the PCA as the assay method for mouse IgE, and will be the principal tool available for measurement of IgE in circumstances in which subnanogram levels must be detected.

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ADDENDUM

Subsequent to our submission of this manuscript for publication, we became aware of a similar study performed by Eshhar *et al.*, now published in *Journal of Immunology 124:* 775, 1980. Their studies and ours are very concordant in terms of the general properties of two respective DNP-specific IgE hybridoma products analyzed.

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