Antibody responses of the immunoglobulin E (IgE) class are regulated by a finely tuned network of cellular and molecular interactions. The differentiation of IgE B lymphocytes is controlled by antigen-specific and non-antigen-specific T helper and suppressor cells and their molecular products (1–3). The antigen-specific cellular interaction can be carried out by thymus-derived (T) cells which recognize immunogenic carriers through the hapten-carrier bridges (4–6). A common idiotypic determinant has been shown in antibody responses of the IgE and IgG classes to the synthetic polypeptide antigen l-glutamic acid-l-alanine-l-tyrosine (7). TEPC-15 (T-15) idiotypic determinant has been shown in serum IgE antibodies specific for phosphorylcholine (PC) (8), and surface Ig of IgE precursor cells (9). Idiotypic determinants present on IgE molecules are functionally important in regulating the production of IgE molecules, since suppression of the IgE antibody response to PC can be achieved by isologous anti-T-15 antiserum (8). Recently, the existence of the T-15 idiotypic determinant has also been demonstrated on PC-mycobacterium-primed T cells, and PC-specific T cell hybridoma (10). Likewise, IgE antibody responses to benzyl-penicilloyl coupled to carrier proteins can be suppressed by isologous antiidiotypic antibodies raised against carrier-specific antibodies (11), which suggests that regulatory T cells may be dampened through the idiotypic determinants of antigen-specific receptors on T cells.

More apparent than with other classes of Ig, IgE antibody responses have been found to be stringently controlled by non-antigen-specific IgE class-restricted regulatory cells (1, 12–17) and their molecular products (1, 2, 15–22). Much effort has been directed to elucidate the molecular basis of this class-restricted regulation in the IgE antibody system. It has been clearly established by studies conducted in the laboratories of Ishizaka and Kishimoto (10, 15–17, 23) that certain cells and molecules participating in the regulation of IgE antibody response display IgE heavy chain-
specific binding activities. Studies by Yodoi et al. (16, 23) and Hirashima et al. (17) demonstrated that IgE potentiating and suppressive factors differ in the content of carbohydrate moieties, and that both factors bind to affinity columns prepared with IgE on Sepharose 4B. Likewise, Sugimura et al. (10) and Suemara et al. (15) showed that IgE binding factors, displaying IgE class-restricted regulatory activity, can be obtained from 2,4-dinitrophenyl (DNP)-mycobacterium-primed T cells and from hybridomas prepared by fusion of DNP- or PC-mycobacterium-sensitized spleen cells with the T lymphoma cell line, BW5147.

Work conducted in this laboratory has concentrated on delineating the difference underlying the IgE response phenotype of low and high responder mice. Two IgE-restricted regulatory factors have been identified, one of which is suppressive, termed suppressive factor of allergy (SFA), and the other, which is enhancing, termed enhancing factor of allergy (EFA) (21, 22). The amount of SFA can be greatly exaggerated in serum and ascites fluids induced by repeated inoculation of complete Freund’s adjuvant (21). Recently, Chen et al. (24) showed that IgE-binding cells (FcR⁺ cells) can be induced de novo in murine lymphocytes, and that SFA can down-regulate the induction of IgE binding cells. We speculated that SFA may interact with the IgE-binding precursor cells at an early stage, functioning as anti-receptor molecules to prevent the appearance of IgE-binding cells.

Thus, it appears that regulation of the IgE antibody system is executed by a combination of antigen-specific regulation via a hapten-carrier bridge and idiotypic determinants and an IgE isotype-specific regulatory network through the linked recognition of the heavy chain of IgE molecules by IgE-binding cells, that, in turn, communicate with regulatory cells or factors that react with cell receptors for IgE. If such an isotype-specific regulatory network exists, we reasoned that the IgE antibody system might be susceptible to manipulation with molecules that interact specifically with the relevant cellular components in immature animals. We thus administered soluble IgE or cell-bound IgE, i.e., IgE covalently coupled to cell membranes of syngeneic spleen cells, to both high and low IgE responder mice beginning at the neonatal period of life. In this paper we report a novel type of immunological tolerance induced selectively and polyclonally in the class of IgE antibody responses by such treatments.

Materials and Methods

Animals. (BALB/c × A/J)F₁ (CAF₁) and (BALB/c × SJL)F₁ mice were obtained from the breeding colonies in the animal facility of Scripps Clinic and Research Foundation, La Jolla, CA. Parental strains of female BALB/c and male A/J and SJL mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Adult male Lewis rats were bought from Holtzman Co., Madison, WI.

Reagents

Proteins and Chemicals. The following materials were used: 2,4,6-trinitrobenzene sulfonic acid (TNBS) (ICN Nutritional Biochemicals, Cleveland, OH); keyhole limpet hemocyanin (KLH) (Pacific Bio-Marine Laboratories, Venice, CA); bovine serum albumin (BSA), ovalbumin (OVA), dithiothreitol (DTT), and glutaraldehyde (Sigma Chemical Co., St. Louis, MO); cyanogen bromide activated Sepharose-4B and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pharmacia Fine Chemicals, Uppsala, Sweden). DNP₉-BSA was prepared as previously described (25).

Monoclonal and Conventional Antibody Reagents. Monoclonal antibodies of various Ig
classes specific for the DNP hapten were obtained from hybridomas constructed and characterized as reported in detail previously (26); the IgE, IgG\textsubscript{i}, and IgG\textsubscript{a} DNP-specific antibodies were isolated by affinity chromatography on DNP-BSA-coupled Sepharose 4B. Rabbit anti-mouse IgA was purchased from Bionetics Laboratory Products, Litton Bionetics, Inc., Kensington, MD; other conventional antibodies were prepared in our laboratory and purified by affinity chromatography on specific immunoadsorbent columns. These included: murine anti-KLH specific antibodies, rabbit anti-mouse IgG (RAMG), goat anti-rabbit immunoglobulin (GARG), rabbit anti-KLH, and rabbit anti-BSA.

**Covalent Conjugation of Proteins to Splenocytes or Sheep Erythrocytes (SRBC)**

**Chemical Conjugation through Heterobifunctional Reagent, N-succinimidyl 3-(2-pyridylthio) propionate (SPDP) (27, 28).** 20 mM SPDP was freshly prepared by dissolving 6.24 mg of SPDP in 1.0 ml of absolute ethanol. 50 \( \mu \)l of 20 mM SPDP was added to DNP-specific monoclonal IgE, IgG\textsubscript{i}, or IgG\textsubscript{a} at 1 mg/ml phosphate-buffered saline (PBS), pH 7.5. The tube was sealed with paraffin paper and left for 30 min with occasional stirring at room temperature. The excess SPDP reagent was removed by dialysis. The resulting 3-(2-pyridylthio) propionyl protein (PDP protein) was recovered and the degree of conjugation was estimated by the amount of pyridine-2-thione released upon reduction by DTT. Approximately 60 PDP groups were conjugated to 106 daltons of protein molecules. 0.5 ml of 0.01 M DTT was added to 1 ml of PBS, or 0.5 ml of 1 M DTT was added to 1 ml of PBS. DTT-treated cells were washed thoroughly and then resuspended in PBS, after which PDP-proteins at 1 mg/ml were added in equal volume at room temperature for 1 h. Protein-coupled splenocytes or SRBC were washed twice in PBS and resuspended at appropriate concentrations in saline for injection.

**Conjugation of IgE Monoclonal Antibodies to Lymphocytes by Chemical-Reactive Hapten, TNBS.** 10 mM of TNBS was prepared by dissolving 35 mg of TNBS in 10 ml of PBS. Spleen-cell suspensions were prepared at 2 \( \times 10^8 \) cells/ml in PBS. 1.0 ml of 10 mM TNBS was added slowly to 1 \( \times 10^8 \) splenocytes in 0.5 ml PBS and the reaction proceeded with gentle stirring at room temperature for 10 min. TNBS-conjugated spleen cells were washed extensively and finally resuspended at 1% (vol/vol) in PBS to which DNP-specific monoclonal IgE antibodies were added at a final concentration of 40 \( \mu \)g/ml. After incubation at 37\textdegree C in a water bath at room temperature for 30 min, the antibody-coated splenocytes at 1% (vol/vol) in PBS were mixed with an equal volume of 4% glutaraldehyde at room temperature for 30 min. Cells were washed three times in PBS and then resuspended at appropriate concentrations in saline for injection.

**Hemagglutination Assay.** Serial twofold dilutions of antisera (25 \( \mu \)l) were made in 1% newborn calf serum (NCS)-PBS in a round-bottomed 96-well plate to which indicator cells at 0.5% (vol/vol) in 1% NCS-PBS were added in 25 \( \mu \)l. The plate was covered with paraffin and the end point of agglutination was determined after the plate was incubated at room temperature for 2 h.

**Assessment of Immune Responses**

**Measurement of Anti-KLH IgE Antibodies.** The level of KLH-specific IgE antibodies in pools of sera from each group was determined by passive cutaneous anaphylaxis reactions (PCA) using shaven male Holtzman rats (4). Serial twofold dilutions of sera were made in saline containing 1% normal rat serum. 100 \( \mu \)l of each dilution was injected into the dorsal skin area of the test rats. The PCA reactions were elicited 4 h or overnight after skin sensitization by intravenous injection of 1.0 mg of KLH in 2.0 ml of 0.05% Evan blue dye. The titer was recorded as the reciprocal of the highest dilution of serum evoking threshold PCA reactivity.

**Radioimmunoassay (RIA) for Anti-KLH IgG Responses.** A solid-phase RIA system using RAMG and GARG antibodies was used to measure the amount of KLH-specific IgG antibodies in sera of hyperimmune mice. 96-well Flex-vinyl U-bottomed microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, VA) were coated with 100 ng/ml solution of KLH at room temperature for 2 h. The plates were subsequently saturated with 10% NCS-PBS solution at room temperature for 2 h and then washed. To the washed plates
were added 100 μl of either affinity-purified KLH-specific antibodies ranging from 0.05 ng to 100 ng/ml of 1% NCS-PBS (to obtain a standard curve), or the test serum samples which were diluted 10⁴ to 10⁷-fold in 1% NCS-PBS. After 2 h incubation at room temperature, the plates were washed and to each well was added 100 μl of affinity-purified RAMG at 1 μg/ml. The plates were incubated at room temperature for 2 h. Finally, 50,000 cpm of 125I-labeled affinity-purified GARG in 100 μl was added. The plates were incubated overnight at 4°C and the wells were washed, dried, cut off with a hot wire, and counted in an automatic gamma counter. The concentration of KLH-specific IgG antibody in the appropriately diluted sample was interpolated from the linear portion of the standard curve. Statistical analysis of geometric means and standard errors of the means were obtained from at least four individual values in each group. The P values of Student’s t test between treated and control groups were determined. All the computation and statistical analyses were performed by programs stored in a TRS-80 model II (Radio Shack, Div. of Tandy Corp., Ft Worth, TX) microcomputer.

Results

Coupling of Monoclonal Antibodies and Proteins to Splenocytes. A general method for the coupling of proteins to splenocytes through disulfide-bond formation is summarized in Fig. 1. The procedure involves three steps. First, the PDP residues are introduced into the protein by the reaction of a portion of the amino groups of the protein with a heterobifunctional reagent, SPDP. The reagent contains one N-hydroxy succinimide ester moiety and one 2-pyridyl disulfide moiety. The hydroxy succinimide ester reacts with primary amino groups to give stable bonds, resulting in PDP protein. The degree of substitution can be controlled by using different amounts of reagent and lengths of reaction time to preserve the integrity of the protein molecules. Second, the addition

COUPLING OF MONOCLONAL ANTIBODY (mAB) TO CELL SURFACE OF SPLENOCYTES BY THE SPDP METHOD

A. \[
\text{mAB} - \text{NH}_2 + \text{PDP} \rightarrow \text{mAB} - \text{NH} - \text{C} - \text{CH}_2 - \text{CH}_2 - \text{S} - \text{S} - \text{PDP} \]

Aminolysis
25°C, 30 minutes

B. \[
\text{Splenocytes} + \text{Dithiothreitol} \rightarrow \text{Reduction} \rightarrow \text{Splenicote} \]

25°C, 1 hour

C. \[
\text{PDP-mAB} + \text{HS} \rightarrow \text{Thiolsulfide Exchange} \rightarrow \text{Disulfide-linked monoclonal antibody-splenocyte conjugate} \]

Fig. 1. Coupling of monoclonal antibody to cell surface of splenocytes by the SPDP method. (A) Introduction of PDP groups into monoclonal antibody by aminolysis. (B) Thiolation of splenocytes. (C) Thiolsulfide exchange reaction to form the disulfide-linked monoclonal antibody-splenocyte conjugate.
of reducing reagent, DTT, converts the disulfide bonds of membrane proteins into thio groups. Thus, in the third reaction, the PDP protein conjugates are covalently coupled to DTT-treated cells.

Using this approach, we have been able to couple monoclonal antibodies of different classes and varieties of proteins to SRBC and splenocytes, and assess the efficiency of coupling by agglutination reaction with the respective antiserum. As shown in Table I, six proteins thus far tested, BSA, KLH, monoclonal IgE, IgG1, IgG2b, and IgA can be coupled to 1 M DTT-treated SRBC. All these indicator cells are agglutinated by appropriate antisera. In exploratory experiments, we determined that 0.01 M DTT can reduce enough disulfide bonds on splenocyte membranes such that PDP-proteins were coupled with equal efficiency. Thus, as shown in Table I, 0.01 M DTT-treated splenocytes coupled with BSA and IgE via the SPDP method are agglutinated effectively and specifically by affinity-pure rabbit anti-BSA and rabbit anti-mouse IgE antisera, respectively. These indicator cells, as shown here, can be used to detect from 0.01 to 0.15 μg of specific antibody molecules in the agglutination assay.

Radiolabeling tracer studies using 125I-labeled PDP-IgE demonstrated that there are ~10 ng of IgE molecules on 25–50 × 10^6 cells, whereas chemical conjugation of splenocytes with TNBS, using 125I-labeled IgE as the radioactive tracer, resulted in the attachment of ~100 ng of DNP-specific IgE molecules on 25–50 × 10^6 cells (data not shown).

General Experimental Design. Because inbred mice vary in IgE-response phenotype according to strain (29), we first confirmed in pilot studies that the mice used in these
studies displayed dominant inheritance of high IgE-responder phenotype in both (BALB/c × SJL)F1 and (BALB/c × A/J)F1 as previously shown by Watanabe et al. (13) and Tung et al. (21).

In the experiments that follow, F1 hybrid mice were treated with the materials being tested at least three times during the first 3 wk of life, with the initial treatment always administered within the first 12–15 h after birth (day 0). In two of the experiments presented, two or three additional treatments were given after the 3rd wk of life (Figs. 5 and 2, respectively). In all experiments, the materials tested included control samples consisting of saline and/or 25–50 × 10⁶ unconjugated syngeneic spleen cells, and experimental samples consisting of 250 μg of soluble monoclonal DNP-specific IgE and/or 25–50 × 10⁶ antibody-conjugated syngeneic spleen cells. Cell numbers are expressed as a range of 25–50 × 10⁶ because the actual number injected at a given treatment point varied within this range depending on the extent of cell recovery of the treated populations after chemical conjugation procedures. In any case, the numbers were kept equivalent among the various groups injected at a given point in time. In all experiments, the volumes in which test materials were injected were 50 μl per recipient mouse in each of the first two injections, 100 μl in the third injection, and 500 μl in all injections thereafter (all administered intraperitoneally). With the exception of one experiment (Table II), immunizations of experimental and control mice were initiated when they reached 2.5 mo of age. Finally, all mice surviving through the neonatal period manifested good general health states irrespective of the nature of the test material to which they had been exposed.

**Induction of IgE Class-restricted Tolerance by Neonatal Exposure of (BALB/c × SJL)F1 Mice to Soluble IgE or IgE-conjugated Syngeneic Spleen Cells.** Groups of four (BALB/c × SJL)F1 mice were treated on six occasions (days 0, 7, 17, 24, 34, and 59) after birth with saline, soluble IgE, or syngeneic spleen cells, either unconjugated or conjugated with IgE. The IgE-conjugated cells were prepared by the direct SPDP or the indirect TNBS methods. At 2.5 mo of age, all mice were immunized with 10 μg of KLH in alum, followed 2 wk later by a booster injection of 1 μg of KLH in alum. 10 d thereafter, all mice were bled and their sera analyzed for KLH-specific IgE and IgG antibody levels.

The results of this experiment are graphically summarized in Fig. 2 and make three very obvious points. First, administration of soluble IgE, according to this treatment regimen, abolishes the capacity of such treated mice to develop subsequent IgE responses. Second, mice treated with cell-bound IgE were similarly refractory to the development of IgE antibody responses, irrespective of the method of coupling used to prepare the IgE-conjugated syngeneic spleen cells. Furthermore, it is noteworthy that the depth of unresponsiveness in the IgE class was comparable to cell-bound IgE or soluble IgE, despite the fact that the absolute amount of IgE administered via cells was 2,500-fold (or more) less than the amount of soluble IgE administered. (It should be noted, however, that practical considerations prevented us from performing a thorough titration to determine the minimum amount of soluble IgE needed to obtain the unresponsive state.) Finally, the inability of treated mice to develop antibody responses was absolutely restricted to the IgE class, since, as shown in Fig. 2, IgG anti-KLH antibody responses of all groups were comparable in magnitude.

To rule out the possibility that the effects observed by treatment with DNP-specific monoclonal IgE might be peculiar to IgE responses against KLH, two experiments
INDUCTION OF IgE CLASS-RESTRICTED TOLERANCE BY NEONATAL EXPOSURE OF (BALB/c × SJL)F1 MICE TO SOLUBLE IgE OR IgE-CONJUGATED SYNGENEIC SPLEEN CELLS

Age 2.5 mos-10 µg KLH (alum) i.v. 250 µg KLH (alum) 14 days Bleed

| Group | Treatment | IgE ANTI-KLH ANTIBODY RESPONSE (PCA Titer) |
|-------|-----------|-------------------------------------------|
| I     | Saline (50-500 µl) | 0 20 80 320 1280 |
| II    | Soluble IgE (250 µg) | <10 | |
| III   | 25-50 x 10⁶ Cells | Unconjugated Spleen Cells | |
| IV    | IgE-SPDP-Conjugated Spleen Cells | SPDP | |
| V     | TNBS | IgE ANTI-KLH ANTIBODY RESPONSE (µg/ml) |

Fig. 2. Induction of IgE class-restricted tolerance by neonatal exposure of (BALB/c × SJL)F1 mice to soluble IgE or IgE-conjugated syngeneic spleen cells. (BALB/c × SJL)F1 hybrid mice were injected intraperitoneally with 250 µg of soluble IgE, IgE bound to 25-50 x 10⁶ splenocytes, or control material in 50 µl saline on days 0 and 7 after birth and in 100 µl saline on day 17. Mice in each group were later given the same material in 500 µl saline intraperitoneally on days 24, 34, and 59. At ~2.5 mo of age, the mice were immunized intraperitoneally with 10 µg of KLH in 2 mg alum, and 14 d later they were boosted with 1 µg of KLH in 2 mg alum. Sera were collected 10 d later. The IgE anti-KLH antibody responses were measured by PCA reactions and the IgG anti-KLH antibody responses were measured by RIA. Concentration of IgG anti-KLH antibody response is expressed as geometric means ± SE of the means of at least four individual values from each group.

were performed in which mice treated in similar ways, but on only three occasions, were doubly immunized, with both KLH and OVA. The results of these experiments, summarized in Table II, clearly demonstrated that such treatments with IgE-conjugated syngeneic spleen cells or soluble IgE induced IgE class-restricted tolerance irrespective of whether KLH or OVA was used for antigenic challenge. Significant reduction of total serum IgE was also detected in mice treated with IgE-conjugated spleen cells (data not shown), which corroborated the notion of polyclonal induction of IgE class-restricted tolerance in the system described here.

Persistence of IgE Class-restricted Tolerance and Failure to Induce Such Tolerance with Syngeneic Spleen Cells Coupled with Monoclonal Antibodies of Other Immunoglobulin Classes. (BALB/c × SJL)F1 hybrid mice were treated three times within the first 3 wk after birth (days 0, 7, and 20) with saline, soluble IgE, or syngeneic spleen cells. The syngeneic spleen cells were either unconjugated or conjugated (by SPDP) with IgE or a mixture of DNP-specific monoclonal antibodies of the IgG1 and IgG2b classes. Immunization with 1 µg of KLH in alum was initiated at 2.5 mo of age and administered on three occasions (days 0, 10, and 24). The mice were bled on multiple
TABLE II
Treatment of (BALB/c X SJL)F1 Mice with Monoclonal IgE Results in Induction of Polyclonal IgE Class-restricted Tolerance to Either KLH or OVA Challenge

| Experiment | Groups | Treatment | IgE antibody responses (PCA titer) |
|------------|--------|-----------|-----------------------------------|
|            |        |           | Anti-KLH response | Anti-OVA response |
| 1          | I      | Unconjugated spleen cells | 1,280             | 1,280             |
|            | II     | IgE-conjugated spleen cells-(SPDP) | <10               | 160               |
|            | I      | Saline | 1,280             | 320               |
|            | II     | Soluble IgE | <10             | <10               |
|            | III    | Unconjugated spleen cells | 1,280             | 1,280             |
|            | IV     | IgE-conjugated spleen cells-(SPDP) | 40               | <10               |

Experiment 1: (BALB/c X SJL)F1 mice were treated with unconjugated spleen cells or IgE coupled spleen cells on days 0, 10, and 22 after birth. Immunization started when they were 1.5 mo old. Mice received three injections of 1 μg KLH in 2 mg alum and one injection of 1 μg OVA in 2 mg alum, and were boosted with a mixture of 1 μg each of KLH and OVA in 2 mg alum. The sera from four mice per group were collected 10 d after the last booster injection, and assessed for IgE anti-KLH and anti-OVA responses by PCA. Experiment 2: (BALB/c X SJL)F1 mice were treated with the materials indicated on days 0, 7, and 20 after birth. Immunization started when they were 2.5 mo old. Mice received three injections of 1 μg KLH in 2 mg alum and one injection of 1 μg OVA in 2 mg alum intraperitoneally, and were boosted with a mixture of 1 μg each of KLH and OVA in 2 mg alum. The sera were collected and assessed for IgE antibody responses as above.

occasions throughout and after the immunization period for analysis of IgE and IgG anti-KLH antibody responses.

The results of this study, depicted in Fig. 3, illustrate two major points. First, as before, administration of either soluble IgE or cell-bound IgE resulted in profound unresponsiveness of treated mice with regard to their abilities to develop antibody responses in the IgE class. No diminution in responses of such mice in the IgG class were observed. This IgE class-restricted unresponsive state resisted repeated antigenic challenges over a relatively prolonged period of time. Mice failed to display IgE responses even after three KLH immunizations and as long as 30 d after the third KLH challenge.

Second, Fig. 3 illustrates quite clearly that the induction of isotype-restricted unresponsiveness is (a) highly specific, since syngeneic spleen cells coupled with a mixture of IgG1 and IgG2b monoclonal antibodies fail to exert any detectable effect on IgE antibody responsiveness; and (b) may be unique to the IgE antibody system, since mice treated with cell-bound IgG1 plus IgG2b antibodies did not display any appreciable difference in their capacity to mount anti-KLH antibody responses of the IgG class. Because responses in these two subclasses represent a significant component of the total IgG response to immunization in this manner, one might have expected to see some diminution in total IgG anti-KLH antibody levels if treatment with these cell-bound antibodies had had any appreciable effect on the antibody-producing mechanisms of treated mice.

The form of IgE administered was important in determining the length of IgE class-restricted tolerance. In the experiment that followed, (BALB/c X SJL)F1 mice were treated three times (days 0, 7, and 20) with saline, soluble IgE, unconjugated
POLYCLONAL IgE CLASS-RESTRICTED TOLERANCE

IgE CLASS-RESTRICTED TOLERANCE OF (BALB/c × SJL)F1 MICE TREATED WITH SOLUBLE OR CELL-BOUND IgE RESISTS REPEATED ANTIGENIC CHALLENGES FOR LONG PERIODS OF TIME.

Fig. 3. IgE class-restricted tolerance of (BALB/c × SJL)F1 mice treated with soluble or cell-bound IgE resists repeated antigenic challenges for long periods of time. (BALB/c × SJL)F1 hybrid mice were injected intraperitoneally with 250 μg of IgE, IgE bound to 25–50 × 10⁴ splenocytes, or control material in 50 μl saline on days 0 and 7 after birth and in 100 μl saline on day 20. At 2.5 mo of age, they were immunized intraperitoneally with 1 μg of KLH in 2 mg alum and boosted on days 10 and 24 thereafter. Sera of these mice were collected on days 10, 24, 31, 41, and 54 after initial immunization and analyzed for IgE (panel A) and IgG (panel B) anti-KLH antibody responses.

spleen cells, or IgE coupled to syngeneic spleen cells via SPDP or TNBS. Mice were primed with 1 μg of KLH in 2 mg alum when they were 2.5 mo old and then boosted with same four more times over a period of 7 mo. Sera were collected 10–16 d after each antigenic challenge. As shown in Fig. 4, long-term IgE class-restricted tolerance was induced in mice treated with both soluble IgE and IgE-coupled syngeneic spleen cells. However, IgE-coupled syngeneic cells served as better tolerogen than soluble IgE, since tolerance persisted for 9 mo after five sequential KLH challenges in mice treated with IgE-coupled syngeneic cells by SPDP or TNBS, whereas tolerance was lost in mice treated with soluble IgE. IgG anti-KLH responses among all groups of mice were not significantly different (data not shown).

Induction of IgE Class-restricted Tolerance by Neonatal Exposure of (BALB/c × A/J)F1, (CAF1) Mice to Soluble IgE but Not to IgE Coupled to Syngeneic Spleen Cells. BALB/c and A/J strains of mice both display the high IgE-responder phenotype. Their F1 hybrid,
Fig. 4. Differential loss of IgE class-restricted tolerance in aged mice according to the method of induction of tolerance. (BALB/c × SJL)F₁ mice were treated three times (days 0, 7, and 20) with saline, soluble IgE, unconjugated spleen cells, or IgE coupled to syngeneic spleen cells via SPDP or TNBS. At 2.5 mo of age, the mice were primed with 1 μg of KLH in 2 mg alum, and four sequential boosts of the same were given over a period of 7 mo. Sera were collected 10 to 16 d after each antigenic challenge. IgE antibody responses to KLH of four mice in each group are depicted.

Fig. 5. Induction of IgE class-restricted tolerance by neonatal exposure of (BALB/c × A/J)F₁ mice to soluble IgE or IgE-conjugated syngeneic spleen cells. (BALB/C × A/J)F₁ hybrid mice were injected intraperitoneally with 250 μg of soluble IgE, IgE bound to 25-50 × 10⁶ lymphocytes or control material in 50 μl saline on days 0 and 7 after birth and in 100 μl saline on day 17. Mice were later given the same material in 500 μl saline intraperitoneally on days 34 and 42. The immunization protocol and IgE and IgG anti-KLH antibody assays were performed as those described in the legend to Fig. 2.
POLYCLONAL IgE CLASS-RESTRICTED TOLERANCE

(BALB/c × A/J)F1, CAF1, is homologous with respect to the genetic determinants for IgE responsiveness, and has been demonstrated to be a high IgE-responder mouse as well (30). We proceeded to determine whether class-restricted tolerance can be induced as well in CAF1 mice with soluble and/or cell-bound monoclonal IgE. Thus, CAF1 mice were given five repeated treatments (days 0, 7, 17, 34, and 42) with saline, soluble IgE, unconjugated spleen cells, or IgE coupled to syngeneic spleen cells via SPDP or TNBS. They were immunized with KLH in alum at 2.5 mo of age. As shown in Fig. 5, profound IgE class-restricted tolerance was induced in the group of mice that received repeated injections of high doses of soluble IgE as compared with the control group given repeated injections of saline (groups I and II). But in distinct contrast with the IgE class-restricted tolerance observed in (BALB/c × SJL)F1 mice, this isotype-restricted tolerance could not be induced by monoclonal IgE coupled to syngeneic CAF1 spleen cells irrespective of the conjugation method used. These results indicated the importance of the products specified by genetic determinants of IgE low-responder mice in association with the IgE molecules covalently coupled to the membrane of spleen cells and, furthermore, suggested that IgE class-restricted tolerance may be induced by two different mechanisms: (a) exposure to high concentrations of IgE molecules directly, and (b) exposure to low concentration of IgE in association with relevant products on cell surfaces of lymphoid cells.

Discussion

The experiments presented here demonstrate the following four points: (a) polyclonal induction of tolerance of the IgE antibody system can be induced by neonatal administration of monoclonal IgE to mice; (b) (high × low) IgE-responder F1 mice can be rendered tolerant by exposure to two forms of tolerogens, i.e., soluble IgE and IgE coupled to syngeneic cells, whereas (high × high) IgE-responder mice are rendered tolerant only by exposure to soluble IgE; (c) the tolerance induced is restricted to the IgE class since it cannot be induced by monoclonal antibodies of other Ig classes, and the tolerance, once induced, is manifested only in the IgE, and not in the IgG, antibody class response; and (d) the IgE class-restricted tolerance resists repeated antigenic challenges, and IgE coupled to syngeneic cells is more effective than soluble IgE in inducing prolonged IgE class-restricted tolerance.

Previously, other investigators (31–34) have demonstrated the capacity to modulate immune responses in an idiotype-, allotype-, and possibly isotype-specific manner by perturbing regulatory helper and/or suppressor T cells, and perhaps B cells, after treatment of experimental animals with various anti-immunoglobulin antibody reagents. The system presented in this report differs from such previous observations, however, in that an isotype-specific lesion of the IgE class was induced by perinatal exposure of experimental animals to the specific immunoglobulin in question, namely IgE. To our knowledge, this is the first demonstration of the induction of such an isotypic lesion which results in substantial diminution in the ability of such treated animals to produce IgE antibodies for prolonged periods of time after treatments of this type.

The mechanism of this phenomenon has not yet been delineated. However, the observations made thus far in this system do permit several educated interpretations and speculations. First, it is unlikely that some form of idiotype-specific suppression mechanism (i.e., antibody or cells) was induced by administration of DNP-specific
monoclonal IgE molecules that would, in turn, cross-inhibit immune responses to complex protein antigens such as KLH and OVA. By the same reasoning, carrier-specific suppression mechanisms are unlikely to be involved since KLH and OVA are non-cross-reactive carrier antigens. Second, it is not probable that allotypic determinants on the administered IgE molecules induced allotype-specific antigen-nonspecific suppression mechanisms that would regulate both anti-KLH and anti-OVA responses, because one would expect such suppressive mechanisms to be similarly exerted on antibody responses of the IgG class. Third, it is unlikely that the administered monoclonal IgE antibodies specific for the DNP hapten cross-reacted with membrane immunoglobulins of any other isotype or other cell-surface components against which responses might be manifested as IgE class-restricted tolerance. Fourth, the possibility that the simple introduction of syngeneic cells that have been modified by chemical alteration of cell-surface proteins via the heterobifunctional reagent, SPDP, could, in turn, selectively modulate the IgE antibody system was ruled out by the experiment presented in Fig. 3 in which cells coupled with IgG molecules by the same chemical procedure failed to exert any detectable effect on the subsequent IgE antibody responses.

The fact that there were obvious differences between the IgE class-restricted tolerance induced by soluble IgE on the one hand, and cell-bound IgE on the other, may provide significant clues as to the nature and type(s) of mechanism(s) underlying this phenomenon. First, the absolute quantity of monoclonal IgE administered in the form of soluble IgE vs. IgE-conjugated syngeneic cells differed at least 2,500-fold; yet IgE-conjugated spleen cells appeared to be more effective in inducing IgE class-restricted tolerance for considerably longer periods of time (Fig. 4). We speculate that IgE-conjugated spleen cells, processed in vivo, may be more stable and thus persistent in tissues, as compared with soluble IgE.

Second, and more striking, was the finding that although soluble IgE was effective in inducing tolerance in both (BALB/c × SJL)F1 and (BALB/c × A/J)F1 mice, IgE-conjugated syngeneic spleen cells, at the doses used, were effective in inducing such tolerance only in the (BALB/c × SJL)F1 hybrids (Figs. 2 and 5). This difference implies, but does not prove, that products contributed to by SJL genetic elements, probably the cell-surface determinants, are important in the induction of this tolerance phenomenon when IgE-conjugated splenocytes are used. This could mean that such determinants are involved either in presentation of the relevant IgE determinant(s) important in this mechanism or in the recognition of the IgE-conjugated spleen cells used. Syngeneic lymphocytes modified with hapten or particular idiotypes have been demonstrated to exert a potent negative regulatory effect on hapten- or idiotype-specific responses in other experimental systems (35-37). Determinants encoded by the major histocompatibility complex (MHC) played critical roles in presenting haptens to trigger precursor suppressor T cells, and the suppressor T cells thus induced were shown to be restricted to the hapten-MHC complex (35). Although the MHC appeared to play no role in presenting idiotype to precursor suppressor T cells (36-37), nevertheless, idiotype-specific T suppressor cells must be Igh congenic to the idiootypic molecules' present syngeneic lymphocytes. In the system presented herein, the importance of MHC gene products, Igh gene products, or other cell-surface determinants on both presenting and responder cells must be assessed in future experiments. Furthermore, whether the necessary gene product(s) that results in
successful induction of tolerance by IgE-conjugated cells must necessarily derive from, or be related to, the low IgE-responder haplotype must be further delineated. Finally, in this regard, the ability to induce tolerance with both soluble and cell-bound IgE in (high × low)F₁ animals, but only with soluble IgE in (high × high)F₁ mice, may imply two distinct mechanisms of tolerance induction and will require further analysis. For example, such differences may only imply that when lower doses of IgE are used, such as the quantities presented on IgE-conjugated spleen cells, this pathway may require the participation of an additional cell-surface determinant provided by genetic elements contributed by the low responder. When soluble IgE is used in doses that are many orders of magnitude higher, perhaps the requirement for participation of such cell-surface determinants is obviated even though the same mechanistic pathway is involved in the ultimate manifestation of tolerance.

Recently, several laboratories have demonstrated the existence of lymphocytes bearing Fc receptors (FcR) specific for IgE (FcRe⁺ lymphocytes). Indeed, the increasing direct and indirect evidence implicating important regulatory roles for FcRe⁺ lymphocytes in the physiological functioning of the IgE antibody system prompted us to initiate the studies presented herein. We reasoned that if FcRe⁺ lymphocytes were important regulatory constituents in the development of the IgE antibody system, then perhaps perturbation of such cells by neonatal exposure to sufficient quantities of IgE might induce abnormalities in the system upon challenge during adult life, such as those observed in these studies.

Pertinent to this reasoning is the fact that FcRe⁺ lymphocytes can be induced by monoclonal IgE and have been shown to be expressed by both B and T cells (24, 38). The functional role of FcRe⁺ T cells and factors released by such cells in regulating IgE antibody responses in vitro has been demonstrated quite clearly by Ishizaka and his colleagues (16, 17). A comparably isotype-specific regulatory function of FcRa⁺ T lymphocytes has been demonstrated to exist in the IgA antibody system by Hoover and colleagues (39), and regulatory effects of FcRγ⁺ T cells and their secreted products have been previously recorded by Fridman et al. (40).

The functions of FcR for IgG on B cells have been implicated recently as important molecular sites for relaying differentiation signals. Thus Fc fragments of IgG triggered mitogenic responses of B cells and induced polyclonal antibodies synthesis in the presence of a T cell factor (41). Moreover, monoclonal antibodies against Fc receptors for IgG trigger both mitogenic responses and polyclonal antibody synthesis (42).

In the IgE antibody system, the recent studies of our own using mice (24) demonstrated that SFA can down-regulate the induction by monoclonal IgE of FcRe⁺ lymphocytes. Such findings, together with those cited above, may be pertinent to those of the present study. Thus, one can envisage the existence of an isotype-specific regulatory network that regulates the size of the IgE antibody response capacity in both neonatal and adult animals. Such a network may operate through the linked recognition of the ε heavy chain by FcRe⁺ cells which, in turn, communicate with regulatory cells reactive with IgE precursor cells, thereby giving rise to antigen-nonspecific, class-restricted regulation of IgE antibody responses. After exposure to either large quantities of soluble IgE or IgE presented appropriately on the surface of syngeneic cell membrane, an effective damping mechanism may be generated through either enhanced production of IgE class-restricted suppressor cells, or a decrease of the efficiency of the development of an IgE-selective helper mechanism, with the
resulting impairment of the maturation and differentiation of IgE precursor B cells. Studies currently underway are designed to define more precisely the mechanism of the IgE class-restricted tolerance reported here and the relative roles of FcR~ T and/or B lymphocytes. The further elucidation of the intricate cellular network controlling IgE antibody production forthcoming from such analyses will be important in our understanding of the pathogenesis of allergic diseases and, hopefully, will provide us with an additional rationale for designing appropriate therapeutic regimens for patients with such disorders. Moreover, the understanding of isotype regulation of the IgE antibody system should shed light on isotype-specific regulation of other Ig classes as well.

Summary

Induction of IgE class-restricted tolerance was studied in high IgE-responder (BALB/c × SJL)F1 mice, of which the parental BALB/c and SJL mice are high and low IgE-responder mice, respectively. 2,4-Dinitrophenyl (DNP)-specific monoclonal IgE was administered to (BALB/c × SJL)F1 mice neonatally in two forms: soluble IgE at 250 μg per injection, or 10–100 ng of IgE coupled to 25–50 × 10^6 syngeneic splenocytes by binding to the chemically reactive hapten trinitrobenzene sulfonate (TNBS) or directly conjugated via a heterobifunctional reagent, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). Polyclonal induction of IgE class-restricted tolerance was observed in (BALB/c × SJL)F1 mice, neonatally treated with soluble IgE or IgE-conjugated syngeneic splenocytes. Thus these mice failed to mount IgE antibody responses to either keyhole limpet hemocyanin or ovalbumin challenge, assessed by the passive cutaneous anaphylaxis reaction. The IgG antibody responses to these same antigens, however, were not affected by this treatment. The IgE class-restricted tolerance induced by both forms of IgE persisted up to at least 6 mo with repeated antigenic challenges. IgE coupled to syngeneic cells by TNBS or the SPDP method induced prolonged tolerance up to 9 mo. The induction of polyclonal IgE class-restricted tolerance was achieved only by monoclonal IgE, whereas DNP-specific monoclonal IgG1 plus IgG2b coupled to syngeneic splenocytes by the SPDP method failed to modulate either IgE or IgG antibody responses. In contrast, (BALB/c × A/J)F1 mice, of which both parental strains are high IgE responders, developed IgE class-restricted tolerance upon repeated neonatal injection of soluble IgE, but not IgE-conjugated syngeneic splenocytes, indicating that gene products of the low IgE-responder mice contributed to the effective presentation and/or recognition of ε heavy chain of the IgE molecules. Taken collectively, these results demonstrated that non-antigen-specific, isotype-restricted tolerance can be induced in the IgE antibody system. The differential induction of IgE class-restricted tolerance by different forms of tolerogen in the strains studied perhaps distinguishes two underlying cellular mechanisms for IgE class-restricted tolerance.

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