Anti-interferon γ Treatment Blocks the Ability of Glutaraldehyde-polymerized Allergens to Inhibit Specific IgE Responses

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Summary

The lymphokines interleukin 4 and interferon γ (IFN-γ) have been shown to play an important role in regulation of polyclonal immunoglobulin E (IgE) and IgG2a responses in vitro and in vivo. We demonstrate here that treatment with chemically modified ovalbumin (OA) results in long-lived, 97–99% inhibition of allergen-specific murine IgE responses and 10^3–10^4-fold increases in anti-OA IgG2a. Responses to unrelated antigens are not affected. Treatment with unmodified OA under the same conditions fails to inhibit primary or secondary IgE responses or to increase IgG2a but does lead to pronounced increases in OA-specific IgG1 production. Glutaraldehyde-polymerized ovalbumin (OA-POL)-induced changes in IgE and IgG2a responses are abrogated by in vivo treatment with purified monoclonal anti-IFN-γ antibody (XMG 1.2), a finding indicative of preferential IFN-γ production upon exposure to chemically modified, but not native, allergen. The results suggest the possibility that the pattern of cytokine synthesis elicited after exposure to protein antigens, and the resulting immune response, may be dependent upon the form of antigen to which the individual is exposed and consequently may be subject to manipulation.

The interferons are a family of proteins initially characterized on the basis of their capacity to confer antiviral resistance but are now known to exhibit highly pleiotropic function. The role of IFN-γ as a potent immunoregulatory molecule acting on a variety of immune cell types is well established (1). Studies of polyclonally stimulated, in vitro antibody responses indicate a central role for IFN-γ in the regulation of murine IgG2a production, which is enhanced, and IgE responses, which are inhibited (2–4). IL-4, upon which IgE responses are dependent, has the opposite effects suggesting that these cytokines may act to reciprocally regulate Ig isotype production in T cell–dependent immune responses as well (5). Direct evidence of a role for IFN-γ and IL-4 in the regulation of IgE-dependent hypersensitivity responses in vivo comes from the findings that IgE production is virtually abolished by anti–IL-4 or anti–IL-4 receptor treatment (6, 7) and that injection of high doses of rIFN-γ leads to suppression of polyclonal IgE responses (8).

As the pattern of cytokine production that is elicited in response to antigenic exposure appears to play an important role in shaping the resulting immune response, an important objective would be the development of methods that would allow activation of specific patterns of CK[1] synthesis. We have utilized chemically modified allergens in studies of antigen-specific murine IgE responses. Treatment with glutaraldehyde-polymerized OA (OA-POL), consisting of high molecular weight (M_r 3.5 × 10^7) soluble polymers leads to induction of IgE selective tolerance affecting both de novo and ongoing responses (9). In this report we demonstrate that treatment of C57BL/6 mice with OA-POL induces a virtually unresponsive state in which maximum IgE responses are 1–3% of these observed in control mice. Concomitantly, anti-OA IgG2a responses are elevated 1,000–10,000-fold. Our finding that these effects on the response to unmodified allergen are abrogated by in vivo administration of anti-IFN-γ mAb suggests preferential expansion of OA-specific, IFN-γ–secreting CD4 clones in vivo by modified but not native antigen.

The results indicate that preferential stimulation of distinct patterns of lymphokine synthesis may be possible and that the form of antigen encountered may play an important role in determining the nature of the resulting immune response. Development of strategies that allow preferential stimulation, or inhibition, of the synthesis of particular lymphokines would have far reaching effects on therapeutic approaches to hypersensitivity, transplantation, autoimmunity, and a variety of immunologic disorders.

[1] *Abbreviations used in this paper:* CK, cytokine; OA-POL, glutaraldehyde-polymerized ovalbumin; PCA, passive cutaneous anaphylaxis; RAG, ragweed pollen extract.

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Materials and Methods

Animals. C57BL/6 mice (6–12-wk-old) and SD rats were bred at the University of Manitoba breeding facility or were purchased from Charles River Canada (St. Constant, PQ). All animals were maintained and used in strict accordance with the guidelines issued by the Canadian Council on Animal Care.

Preparation of Chemically Modified OA. OA (Grade VI; Sigma Chemical Co., St. Louis, MO) or (5× recrystallized; ICN Biomedicals, Montreal, PQ) was treated with glutaraldehyde (Eastman Kodak Co., Rochester, NY) as follows. OA was dissolved at 25 mg/ml in sodium acetate/acetic acid buffer (0.1 M, pH 5.3), 0.5 pH unit above its isoelectric point. Glutaraldehyde (6% in 0.15 M NaCl) was added dropwise with stirring over a period of several minutes to obtain a final molar ratio of 200:1 GA/OA. The reaction was allowed to proceed for 5 h, in order to yield high molecular weight OA polymers. After extensive dialysis against borate buffered saline (0.1 M, pH 8.3), the solution was applied to a BiogelA-50m (Bio-Rad Laboratories, Mississauga, ON) gel filtration column (2.5 × 90 cm) for characterization and purification. The polymerized protein was recovered as a single sharp symmetric peak (Ve/Vo of 1.4–1.55) eluting at an average M_r of 3.5×10^7 (Vo of Biogel A-50m is 5.0×10^7). This preparation, designated OA-POL, could be stored for at least 2 mo at 4°C without evidence of any changes in its chemical or immunologic properties. This method of glutaraldehyde polymerization was developed and used throughout in preference to chemical modification carried out at neutral pH in PBS (0.1 M) with all other reaction conditions as above, a procedure found to yield highly heterogeneous mixtures of reaction products (9).

Immunization and Treatment of Mice. Mice were immunized by intraperitoneal injection of 2 μg unmodified OA, DNP_2-OA, TNP_{22}-KLH, or 100 μg of a ragweed pollen extract (RAG) (generously provided by Drs. V. Strevens, and A. Sehon, Univ. of Manitoba, Winnipeg, Canada) adsorbed onto 2 mg $Al(OH)_3$ adjuvant. OA-POL treatment consisted of a course of three 80-μg i.p. injections in saline given 10, 12, and 14 d before immunization. Mice were usually bled by cardiac puncture 10 and 14 d after primary and 7 and 14 d after secondary immunizations. Sera were collected and stored at −20°C until analyzed by ELISA or passive cutaneous anaphylaxis (PCA).

Determination of Antigen-Specific Antibody Levels. IgE anti-OA levels were determined by 48-h PCA in female SD rats essentially as described (10). Means of duplicate or triplicate analyses, rarely differing by more than one twofold dilution in repeat assays, are presented. OA-specific murine IgG1, IgG2a, IgA, and IgM levels were determined in an alkaline phosphatase–based ELISA calibrated against a murine anti-OA standard (generously provided by Dr. G. Strejan, Univ. of Western Ontario, London, Canada). ELISA plates (Corning 25805; Corning Science Products, Corning, NY) were coated overnight with antigen (OA, DNP_7-BSA or RAG) at 200 μg/ml in bicarbonate buffer (0.05 M, pH 9.6). After 90-min blocking with a 1% BSA, 0.05% Tween 20 solution, and extensive washing, serial dilutions of serum samples were incubated for 4 h at 37°C, the plates were washed, and an excess of alkaline phosphatase–conjugated rabbit anti-mouse IgG1, IgG2a, IgA, or IgM (Southern Biotechnology Associates, Birmingham, AL) was added overnight at 4°C. After washing the plates extensively, *p*-nitrophenyl phosphate (Sigma Chemical Co.) was added as directed by the manufacturer and the reaction was allowed to proceed for 100 min. Background control values for wells missing one component in turn did not exceed 0.07 absorbance units at 450 nm. Results are expressed as ELISA titers using the midpoint of the titration curves obtained as compared to a constant internal standard run in each assay. Each serum sample was assayed at least twice. The isotypic specificity of each of the antibody-enzyme conjugates used was confirmed before use.

Determination of Rat IgG Concentrations. Determination of rat IgG concentrations, necessary in the purification and quantification of the rat IgG1 mAb (XMG 1.2; generously provided by Dr. T. Mosmann, University of Alberta, Edmonton, Canada) specific for murine IFN-γ was carried out as follows. ELISA plates were coated overnight with affinity-purified goat anti-rat IgG Fc (10 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) as a capture reagent. After blocking, dilutions of tissue culture supernatant or fractions eluted from the ion exchange purification process (see below) were incubated for 3 h at 37°C followed by incubation with excess alkaline phosphatase conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at 4°C. Purified rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA.) was used at 2.5×10^{-2} to 9.8×10^{-5} μg/ml as a standard. The ELISA was completed by washing and addition of substrate as described above.

Preparation and Purification of Anti-IFN-γ mAb. Tissue culture supernatants from XMG 1.2 (11) cultures were used directly in initial experiments after quantitation of total rat IgG present (as described above). In later experiments, purified antibody was prepared by ion exchange chromatography using the Pharmacia Biopilot system (Pharmacia Fine Chemicals, Piscataway, NJ). Briefly, 1.5–2.5 l of exhausted tissue culture supernatant was dialyzed against citrate buffer (0.01 M, pH 5.3) and applied to the Biopilot S-Sepharose column. Citrate buffer was passed through the column until the absorbance at 280 nm was <0.01, followed by application of an increasing ionic strength gradient (Na Citrate 0.01 M, NaCl 0–0.8 M, pH 5.3). The eluted rat mAb is >85% pure as determined by comparison of total protein present in the purified material (as estimated by the absorbance at 280 nm) with the concentration of rat IgG determined by ELISA as described above. This material, typically at 600–1,000 μg/ml, was used for in vivo intraperitoneal injection.

Statistical Analysis. PCA and ELISA titers were log transformed, following which geometric means were compared using unpaired two-tailed Student's *t* tests.

Results

Inhibition of IgE and IgG1 Responses. The effects of treatment with saline or a course of three 80-μg injections of unmodified OA or OA-POL on OA-specific IgE and IgG1 responses was examined. All mice were challenged at day 0 and at monthly intervals thereafter with OA in $A1(OH)_3$ adjuvant. As indicated in Fig. 1, OA-specific serum IgE levels in OA-POL-treated mice were inhibited >98% ($p < 0.0001$) in both primary and secondary responses. More importantly, repeated booster immunizations with OA in $A1(OH)_3$ adjuvant failed to raise the capacity of these mice to generate anti-OA IgE responses to above 3% of those observed in untreated controls. This virtually unresponsive state persisted in spite of the administration of four OA $A1(OH)_3$ immunizations over >100 d. Treatment with unmodified OA under the same conditions failed to inhibit primary or secondary specific IgE responses and actually resulted in a marked acceleration of the primary response (Fig. 1).

OA-POL treatment resulted in increased primary IgG1 responses followed by relatively small decreases in secondary

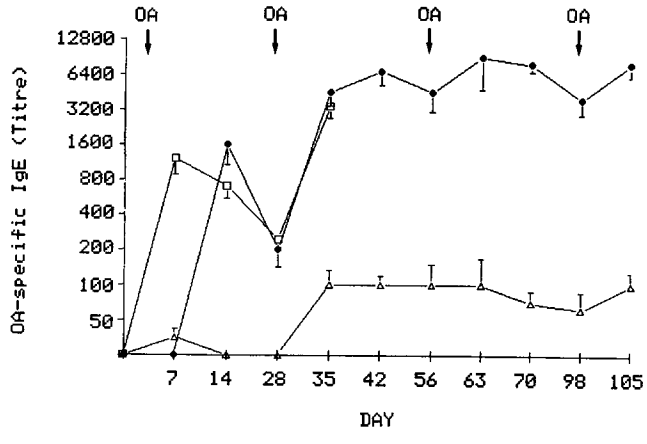

Figure 1. Inhibition of 1°, 2°, 3°, and 4° anti-OA IgE responses. C57BL/6 mice were treated with saline (●), OA-POL (Δ), or unmodified OA (□) and were immunized with 2.0 μg OA in $A1(OH)_3$ adjuvant 10 d later and at monthly intervals thereafter. OA-specific IgE responses were determined by PCA as described in Materials and Methods.

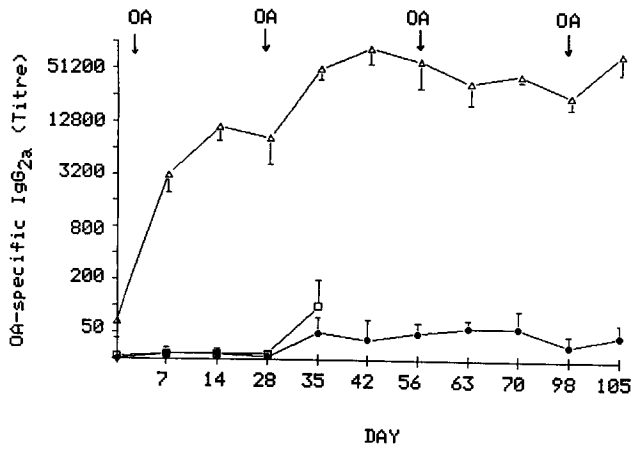

Figure 3. Enhancement of anti-OA IgG2a responses after OA-POL treatment. C57BL/6 mice were treated with saline (●), OA-POL (Δ), or OA (□), immunized, and bled as described for Fig. 1. Data are OA-specific ELISA titers.

responses (Fig. 2). Thus, in contrast to the effects on IgE responses (decreased by 30–80-fold), secondary IgG1 responses were decreased by two- to eightfold. It should be noted that unmodified OA given under the same conditions led to substantial increases in IgG1 production without inhibition of primary or secondary IgE responses.

IgA responses were very weak in all groups (ELISA titers ranged from <10 to 45) and were not affected by OA or OA-POL treatment. Similarly, IgM responses although more substantial were not significantly affected ($p > 0.05$) by treatment with either native or modified OA (data not shown).

Enhancement of OA-specific IgG2a Responses. Specific IgG2a production by OA-POL-treated mice exhibited striking increases in both primary and secondary anti-OA responses (Fig. 3). In contrast to the weak IgG2a responses of control mice, OA-POL treated animals yielded OA-specific ELISA titers 1,000–10,000-fold higher. These increases persisted for the duration of the experiments. In contrast, treatment with unmodified OA did not significantly alter anti-OA IgG2a responses.

Antigenic Specificity of OA-POL Induced Changes in Antibody Production. The changes in antibody production that were induced as a consequence of treatment with glutaraldehyde polymerized OA were antigen specific. Treatment of mice with OA-POL, though capable of inhibiting IgE responses to OA or haptens conjugated to OA by >98% (Table 1), had no impact on antibody responses to unrelated allergens (RAG) or DNP-specific antibody responses induced by this hapten on heterologous carriers (DNP_{22}-KLH) (Table 1). Interestingly, anti-DNP IgG2a production in response to DNP_2-OA, though elevated in OA-POL treated mice (3–10-fold), was markedly less enhanced than were IgG2a responses to OA in the same mice. Thus, OA-specific ELISA titers in saline or OA-POL treated, DNP_2-OA $A1(OH)_3$ immunized groups in this experiment were <5 vs. 2,400 and 35 vs. 31,400 for primary and secondary anti-OA IgG2a responses, respectively.

Sensitivity of OA-POL-induced Effects to In Vivo Treatment with Anti-IFN-γ mAb. The effect of OA-POL treatment on IgE, IgG2a, and to a lesser extent IgG1, responses suggested that exposure to this chemically modified allergen led to preferential induction of IFN-γ production by OA-specific T cells. This hypothesis was tested by in vivo administration of murine IFN-γ-specific mAb, as tissue culture supernatant (data not shown) or purified antibody (Table 2). In preliminary experiments, administration of 50–900 μg purified XMG 1.2 mAb or normal rat IgG had no effect on the development of IgE, IgG1, or IgG2a responses in normal OA ($A1(OH)_3$)-primed C57BL/6 mice (our unpublished data).

Mice were treated with a standard course of three OA-POL injections or saline 10, 12, and 14 d before immuniza-

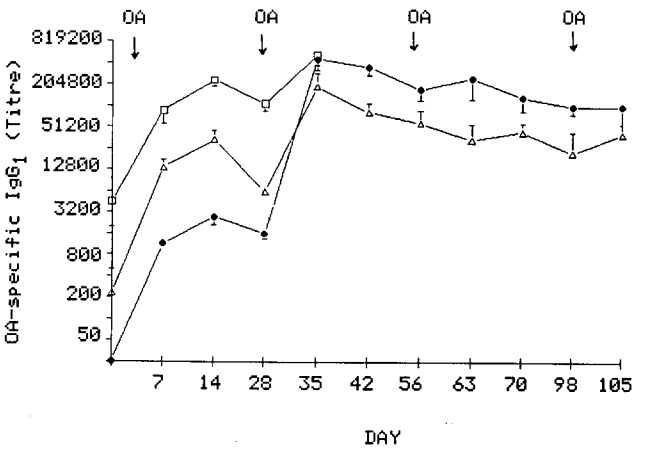

Figure 2. Effect of treatment with OA-POL on anti-OA IgG1 responses. C57BL/6 mice were treated with saline (●), OA-POL (Δ), or unmodified OA (□) and were immunized and bled as described for Fig. 1. Data are OA-specific ELISA titers.

Table 1. *Antigenic and Isotypic Specificity of OA-POL Treatment*

Treatment	Immunization	Response measured	IgE		IgG1		IgG2a	
			d10	d35	d10	d35	d10	d35
None	DNP_2-OA	Anti-DNP	200	1,600	1,500	65,300	640	1,200
OA-POL	DNP_2-OA		<10	30	1,360	61,900	1,710	12,560
None	DNP_{22}-KLH	Anti-DNP	80	200	5,900	119,050	1,310	4,350
OA-POL	DNP_{22}-KLH		80	200	6,150	125,000	1,620	6,500
None	RAG	Anti-RAG	20	80	350	34,550	10	250
OA-POL	RAG		20	100	270	25,100	20	680

C57BL/6 mice (three/group) were injected with 80 μg OA-POL or nothing 14, 12, and 10 d before immunization with DNP_2-OA [1 μg in $Al(OH)_3$], DNP_{22}-KLH [2 μg in $Al(OH)_3$] or RAG [100 μg in $Al(OH)_3$]. All mice were boosted with identical amounts of antigen in $Al(OH)_3$ on day 28. Peak primary and secondary hapten-specific or RAG-specific antibody levels were determined as described in Materials and Methods. Data are PCA titers (IgE) or ELISA titers (IgG1, and IgG2a).

Table 2. *OA-POL-induced Inhibition of IgE Responses Is Reversed by Anti-IFN-γ mAb*

Treatment	Immunization	Anti-IFN-γ	OA-specific response					
			IgE		IgG1		IgG2a	
			d10	d12	d10	d12	d10	d12
–	OA	–	1,280	800	14,510	34,000	15	15
OA-POL	OA	–	20	<10	25,700	29,100	990	1,345
OA-POL	OA	day 0, 1, 2	400	640	25,150	30,400	95	115
OA-POL	OA	day 3, 4, 5	100	80	34,000	32,100	505	525

Mice were injected with 80 μg OA-POL or nothing 14, 12, and 10 d before immunization with OA [2 μg in $Al(OH)_3$]. On the day of immunization and the two successive days (day 0, 1, 2) or day 3, 4, 5, OA-POL-treated groups were treated with 200 μg/d of purified XMG 1.2 anti-IFN-γ mAb or normal rat IgG (not shown). Mice were bled at the times indicated and OA-specific antibody production was determined by ELISA (IgG1, IgG2a) or PCA (IgE). Data shown are from one of two replicate experiments carried out with purified anti-IFN-γ.

tion with OA in $A1(OH)_3$ (day 0). On days 0, 1, and 2 or 3, 4, and 5, mice were treated with a 200-μg/injection (intraperitoneally) of purified XMG 1.2 mAb or polyclonal rat IgG as control. As previously, OA-specific IgE production was strongly inhibited after OA-POL treatment. Administration of anti-IFN-γ mAb virtually abolished this inhibition. Similarly, anti-OA IgG2a increases induced by OA-POL treatment were greatly diminished after administration of the mAb soon after immunization. Anti-IFN-γ treatment had significantly less impact on both IgE and IgG2a responses when given 3, 4, and 5 d after immunization. IgG1 responses were not significantly affected under the conditions tested. These results support a role for IFN-γ induction as the mechanism responsible for the antigen-specific changes in antibody production which occur after treatment with OA-POL.

Discussion

Our major finding is that treatment of mice with appropriately modified allergen in long-lived inhibition of their capacity to generate antigen-specific IgE, and pronounced increases in IgG2a, responses. These changes appear to result from preferential induction of IFN-γ production by OA-specific lymphocytes upon exposure to OA-POL.

The potential for IFN-γ to regulate IgE responses has been the subject of intense study. Recently, direct evidence of IFN-γ's capacity to modulate polyclonal IgE, IgG1, and IgG2a responses in vivo was obtained using anti-IFN-γ antibody treatment or high doses (75,000 U) of rIFN-γ (7, 8). In the present report we demonstrate a role for in vivo induction of IFN-γ production in the manipulation of antigen-specific responses.

Many years ago, Watanabe et al. (12) identified Ly-1^+ T cell–dependent suppression in SJL mice, which acted to inhibit IgE production in an isotype specific fashion. This constitutive inhibition was antigen nonspecific and may have reflected a similar, but polyclonal, activation of IFN-γ production in that strain. Indeed, the demonstration that SJL and SJA/9 mice give normal IgE responses when stimulated with LPS and appropriate cytokines suggests that the defects in IgE responsiveness arose as a consequence of abnormalities in T cell regulation, not B cell potential (13).

Previous studies of IgE responses using modified antigens have resulted in immunoregulatory patterns which are an-

tigen specific and isotype nonspecific; IgE specific and antigen nonspecific; or less commonly, antigen specific, and isotype specific (reviews 14, 15). In most of these studies IgG2a responses were not specifically evaluated nor was a role for IFN-γ examined. However, of particular interest is the recent finding by Hagen et al. (16) of preferential induction of IFN-γ synthesis upon administration of haptenated *Bordetella pertussis*.

Glutaraldehyde has previously been used to generate heterogeneous allergen mixtures which were examined in animal and clinical studies (17–21). Although promising in terms of enhanced safety, these preparations failed to demonstrate marked decreases in IgE production and their mechanism of action remains unknown. It is unclear if this reflects differences in the modification approaches used and thus the heterogeneity of the resultant products (22) or more basic differences in regulatory mechanisms which govern antibody formation in human and murine systems.

The means by which an antigen-nonspecific lymphokine regulates responses in an antigen-specific fashion is important. There is a growing body of evidence that antigen specificity may be mediated as a consequence of cell-cell interaction rather than specificity of the lymphokine itself. In this regard T cell receptor directed, polar release of lymphokines (23) provides an attractive hypothesis to explain the results described here.

Preliminary results (our unpublished data) indicate that changes in OA-specific IgE and IgG2a production in OA-POL-treated C57BL/6 mice are CD4 T cell dependent. Since the discovery several years ago that many murine CD4 T cell clones could be grouped into two major subsets, designated Th1 and Th2 on the basis of cytokine production and biological function (24, 25), much effort has been aimed at determining if such apparently stable patterns of differentiation exist in vivo (26–30). One would predict that stimulation of Th1-like patterns would favor DTH-like or IgG2a dominated responses while stimulation of Th2 would result in IgG1 and IgE production. Our findings are consistant with the induction of Th1-like patterns of lymphokine secretion upon OA-POL treatment in contrast to the Th2-like pattern induced by native OA, especially in the presence of $Al(OH)_3$ adjuvant.

Recent evidence suggests that the distinction between the Th1 and Th2 phenotype is not as clear cut as initially appeared. Studies in vitro with clones (31) and in vivo with spleen cells from resting or antigen-primed mice (32) support the existence of additional patterns of cytokine synthesis. Unresolved is the question of whether a given T cell in vivo can exhibit different patterns of lymphokine synthesis depending on the nature of the antigenic stimulus or the context in which it is presented. Thus OA-POL may be acting not to preferentially stimulate committed OA-specific Th1 cells in preference to Th2, but rather may steer the activation of OA-specific CD4 T cells towards IFN-γ secretion rather than IL-4/IL-5 synthesis.

The mechanism by which native or glutaraldehyde polymerized OA influences lymphokine expression remains unclear. Immune responses dominated by Th1-like or Th2-like cytokine synthesis patterns have previously been reported for certain parasitic infections (33, 34) and in the response to human basement membrane collagen (35). Recent experiments suggest an important role for the nature of APC or the costimulatory molecules produced by them in the differential activation of Th1 and Th2 clones. Thus, antigen presentation by hepatic nonparenchymal accessory cells (36) supports proliferation of long term Th1 and not Th2 clones, while T cell lines derived using hapten-modified Langerhans cells yield almost exclusively Th2-like lines (37). OA-POL differs markedly from native OA in terms of its antigenicity at the B cell level, retaining only 0.5–5% of its capacity to be bound by anti-OA antibodies (22). This suggests that the B cell may play a markedly smaller role than other APC in the presentation of OA-POL and may be responsible in part for the preferential synthesis of IFN-γ in vivo. Experiments to address this issue are underway.

Our experiments do not indicate whether the effects of IFN-γ on OA-specific IgE, IgG1, and IgG2a are direct or indirect. The increased efficacy of anti–IFN-γ treatment when administered at and soon after OA ($Al(OH)_3$) immunization suggests that a major function lies in directing the commitment of OA-specific B cells to IgG2a and away from IgE/IgG1 production. At the same time there is good evidence that IFN-γ, in addition to its well defined direct effects on B cells, specifically inhibits the generation and growth of Th2, but not Th1, clones (38) in vitro. This putative shift in the T cell repertoire away from IL-4/IL-5 secretion and towards IFN-γ production may be responsible for the long lived inability of OA-POL treated mice to generate good IgE responses, a state which persists for at least 1 yr and five booster immunizations (HayGlass, K. T., R. Gieni, and W. Stefura, manuscript submitted for publication).

It has been suggested that the relative quantities of IL-4 and IFN-γ produced during an immune response may determine the selection and magnitude of isotypes generated in response to a given antigen (5). The present results suggest that it may be possible, at the level of the repertoire, for us to manipulate the response so as to steer T cell activation towards a specific pattern of cytokine expression and consequently to control the response.

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