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Long-lived reciprocal regulation of antigen-specific IgE and IgG2a responses in mice treated with glutaraldehyde-polymerized ovalbumin

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SUMMARY

Previously, we discovered that administration of high M_r glutaraldehyde-polymerized ovalbumin (OA) to C57BL/6 mice prior to immunization with OA in $Al(OH)_3$ adjuvant resulted in induction of an interferon-gamma (IFN-γ) dependent, split tolerance in which maximal OA-specific IgE responses were 1–3% of those observed in saline-treated OA-[$Al(OH)_3$] immunized controls. Concomitantly, these mice exhibited up to 10^3-fold increases in OA-specific IgG2a synthesis. In this report we examine the longevity and resilience of these reciprocal effects on IgE inhibition/IgG2a enhancement over extended periods of time and following multiple re-exposures to the sensitizing allergen. The data indicate that the T-cell mediated changes in responsiveness which are induced upon exposure to glutaraldehyde-modified protein allergen, but not unmodified allergen, are (i) extremely long-lived (> 350 days); (ii) resistant to at least five re-immunizations with OA in $Al(OH)_3$ adjuvant; and (iii) antigen-specific. The results are consistent with a virtually permanent shift in the OA-specific T-cell repertoire *in vivo* from one dominated by Th2-like patterns of cytokine synthesis (IL-4) to one dominated by Th1-like (IFN-γ) cytokine production.

INTRODUCTION

Most immune responses consist of a balance of cell-mediated immunity and antibody production. Under certain circumstances, such as immune deviation[1] and split tolerance,[2,3] one component of the response dominates to the virtual exclusion of others. One current hypothesis attributes such skewing of immune responses to preferential stimulation of Th1-like or Th2-like patterns of cytokine synthesis *in vivo*. Thus, the response to *Leishmania major* infection is dominated by interferon-gamma (IFN-γ) or interleukin-4 (IL-4) production in C57BL/6 and BALB/c mice, respectively.[4,5] Similar preferential stimulation of a dominant pattern of cytokine synthesis is observed in I-A^s and non-I-A^s mice in the response to human type IV collagen.[6,7] In these systems, the immune response elicited can be predicted by the genetic background of the individual.

Studies of polyclonally stimulated, *in vitro* antibody responses indicate a central role for IFN-γ in regulation of murine IgG2a production, which is enhanced, and IgE responses, which are strongly inhibited.[8–11] IL-4, upon which IgE responses are dependent, has the opposite effect, suggesting that these cytokines act to reciprocally regulate Ig isotype production in T-cell-dependent immune responses.[12] Direct evidence of an *in vivo* role for IFN-γ and IL-4 is provided by the findings that IgE production is virtually abolished by anti-IL-4 receptor treatment[13,14] and that injection of high doses of rIFN-γ leads to suppression of polyclonal IgE responses.[15]

Because the pattern of cytokine production appears to shape the nature of the resulting immune response, an important objective would be development of methods resulting in activation of specific patterns of cytokine production. To that end, we have utilized glutaraldehyde (GA)-modified allergens in studies of antigen-specific murine IgE responses. We selected ovalbumin (OA) as model allergen because, in addition to being well defined in terms of primary, secondary, tertiary[16] and crystal structure,[17] it is a relatively common allergen in humans.[18,19]

Glutaraldehyde modification of OA at neutral pH yields highly heterogeneous polymers,[20] similar to those obtained upon GA treatment of other allergens by other investigators.[21–23] Although greatly decreased in antigenicity, these mixtures exhibit limited efficacy as tolerogens *in vivo* and, in some cases, can induce *de novo* IgE responses.[23] We have developed a reaction which yields soluble, high M_r polymers (termed OA-POL) of restricted heterogeneity, greatly increased efficacy and which exhibit highly consistent biological activity from lot to lot. Using a short-term *in vivo* assay system, we found that treatment of mice with this molecule (80 μg, i.p.) led to 90–99% inhibition of both primary and secondary IgE anti-OA re-

Abbreviations: GA, glutaraldehyde; OA, ovalbumin; OA-POL, glutaraldehyde-polymerized ovalbumin of M_r $3{\cdot}5 \times 10^7$.

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sponses. This inhibition was essentially isotype-specific for IgE, with 0–5-fold decreases in IgG1, minor changes in allergen-specific IgA, IgM and 100-fold increases in IgG2a production.[24] Its effects were antigen-specific and CD4 T-cell dependent. Collectively, the data suggest the involvement of IFN-γ, potentially via *in vivo* induction of Th1-like patterns of cytokine synthesis. Recently, we demonstrated that administration of as little as 600 μg of monoclonal anti-IFN-γ (XMG 1.2) abolished both the suppression of IgE responses and enhancement of IgG2a elicited by OA-POL treatment.[24,25] In contrast, administration of unmodified OA under the same conditions resulted in accelerated primary IgE responses. Neither primary or secondary OA-specific IgE responses were inhibited by such treatment.[24,25] These data were taken to support the view that OA-POL acts largely through preferential induction of IFN-γ. The clear implication is that cytokine synthesis patterns elicited upon antigen exposure *in vivo* are not fixed (i.e. by genetic background) but may be subject to manipulation upon administration of appropriate forms of antigen.

Most studies of IgE regulation, including our own, have focused on short-term studies of antibody production (reviewed in refs 26 and 27). At the same time, extrapolation from murine models of immunotherapy to clinical trials has often proved to be disappointing.[28] This may be due, in part, to the brief periods (4–6 weeks or less) over which most animal experiments are carried out.

In this report, we examine the longevity of isotype-specific regulation induced with this class of chemically modified allergens. To our surprise, a single course of OA-POL treatment resulted in an essentially permanent shift in the pattern of responsiveness of C57BL/6 mice to unmodified OA. The resulting isotype-specific inhibition of IgE responsiveness was maintained for at least 1 year in the absence of further treatment, despite multiple re-exposures to the sensitizing allergen. The T-cell-dependent decreases in the capacity to produce IgE responses were paralleled by 500-fold or greater increases in anti-OA IgG2a responses—a finding consistent with preferential induction of IFN-γ-producing regulatory T cells by this modified allergen. We interpret these results in terms of differential patterns of cytokine synthesis elicited by *in vivo* exposure to native and chemically modified allergens and suggest that this system is well suited to analysis of factors which govern induction of allergic responses.

MATERIALS AND METHODS

Animals

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

Preparation of chemically modified ovalbumin

Ovalbumin (OA) (Grade VI, Sigma Chemical Co, St Louis, MO; or 5× recrystallized, ICN Biomedicals, Montreal PQ, Canada) 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 (GA) (6% in 0·15 M NaCl) was added drop-wise 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 hr in order to yield high M_r OA polymers. After extensive dialysis against borate-buffered saline (0·1 M, pH 8·3), the solution was routinely applied to a Biogel A-50m (Biorad 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 (V_e/V_o of 1·4 to 1·55) eluting at an average M_r of $3{\cdot}5 \times 10^7$.[20,29] The V_o of Biogel A-50m is $5{\cdot}0 \times 10^7$. This preparation, designated OA-POL, could be stored for at least 2 months at 4° without evidence of any changes in its chemicals or immunologic properties. This method of glutaraldehyde polymerization was developed and used throughout this study, in preference to chemical modification carried out at neutral pH in phosphate-buffered saline (0·1 M) with all other reaction conditions as above, a procedure found to yield highly heterogeneous mixtures of reaction products.[20,30]

Immunization and treatment of mice

A course of OA-POL treatment consisted of three 80 μg injections given i.p. in saline. Mice were immunized from 14 to 356 days later by i.p. injection of 2 μg OA or 10 μg TNP_{22}-KLH adsorbed onto 2 mg $Al(OH)_3$ adjuvant. Age-matched, saline-treated animals were used as controls, with naive 6–8-week-old mice included as additional controls in some long-term experiments. Blood was obtained by cardiac puncture 10 days after each immunization and the sera were stored at −20° until analysed.

Determination of antigen-specific antibody levels

OA-specific murine IgG, IgG1, IgG2a, IgA and IgM levels were determined in an alkaline phosphatase-based ELISA calibrated against a murine anti-OA standard, as previously described. Briefly, ELISA plates (Corning 25805, Corning Science Products, NY) were coated overnight with antigen (OA or DNP_7-BSA) 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 hr at 37°, the plates were washed and an excess of alkaline phosphatase-conjugated rabbit anti-mouse IgG, IgG1, IgG2a, IgA or IgM (Southern Biotechnology Associates, Birmingham, AL) was added overnight at 4°. 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 405 nm. IgG concentrations are expressed in μg/ml as deduced from a standard curve obtained with a standard serum of known concentration of anti-OA IgG.[20] Results for IgG1, IgG2a, IgA and IgM are expressed as ELISA titres using the midpoint of the titration curves obtained 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 prior to use. IgE anti-OA levels were determined by 48-hr passive cutaneous anaphylaxis (PCA) in female Sprague–Dawley rats, essentially as described elsewhere.[31] Duplicate or triplicate analyses, which rarely differed by more than one twofold dilution, were conducted for each sample.

Table 1. Glutaraldehyde-polymerized, but not unmodified, OA elicits IgE selective inhibition of anti-OA responses

Treatment (Days −14, −12, −10)	Challenge (Day 0)	IgE		IgG1		IgG2a		IgA		IgM	
		Day 10	Day 35	Day 10	Day 35	Day 10	Day 35	Day 10	Day 35	Day 10	Day 35
None	OA	800	5000	2191	144,000	20	50	5	20	380	710
OA-POL	OA	10	100	19,200	124,800	2500	72,960	10	30	570	1250
OA	OA	1280	4000	325,000	$1{\cdot}65 \times 10^6$	150	ND	10	15	980	1950

C57BL/6 mice were injected i.p. with saline, OA or OA-POL (80 μg in saline) 10, 12 and 14 days before immunization with 2 μg OA in $Al(OH)_3$ adjuvant. Peak and secondary anti-OA responses are expressed as reciprocal titres. The data shown are from one experiment representative of three performed.

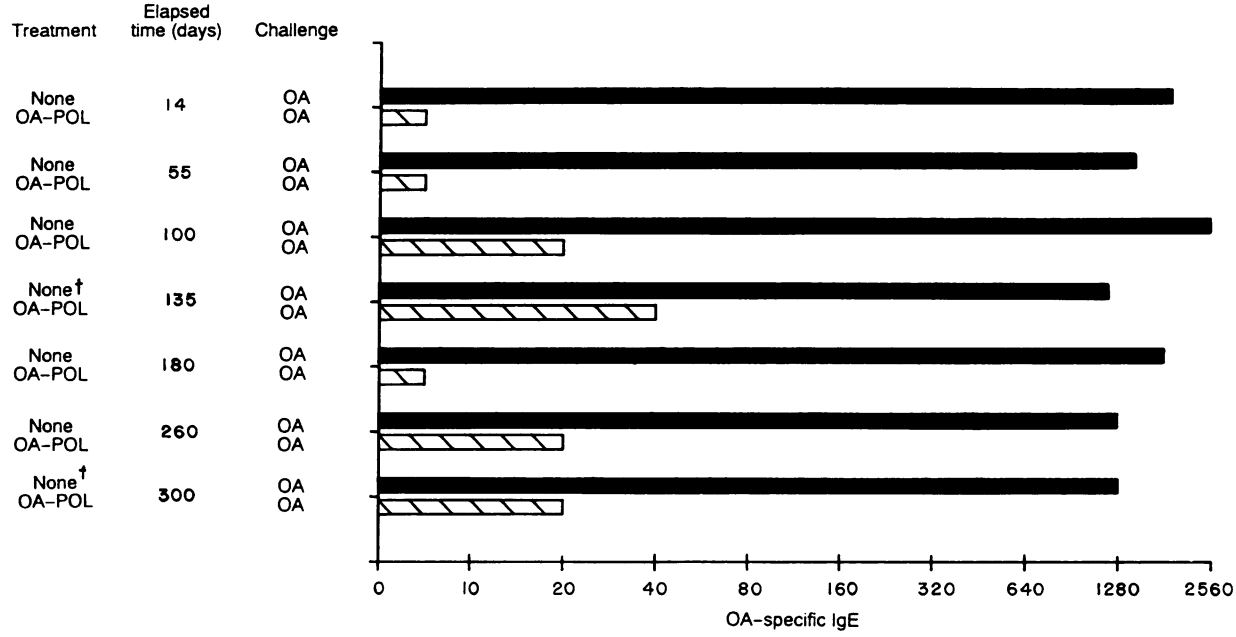

Figure 1. Persistent inhibition of anti-OA IgE responses following.a single course of treatment with glutaraldehyde-polymerized ovalbumin (OA-POL). C57BL/6 mice were treated with three 80 μg i.p. injections of OA-POL in saline or with saline alone. From 14 to 300 days later, an independent cohort of treated (▩) or age-matched normal (■) mice was challenged with 2 μg OA [$Al(OH)_3$] and their anti-OA IgE responses were assessed 10 days later. Parallel control groups of 6–8 weeks-old mice (†) were challenged in conjunction with those challenged 135 and 300 days after OA-POL treatment. IgE responses of these young, untreated mice were not different from those of age-matched, untreated controls (data not shown). Data presented are from one of two experiments.

Cell transfers

Mice pretreated with OA-POL as described above were killed 6 months (185 days) later and, where specified, T cells were depleted by treatment with anti-Thy-1.2 (undiluted tissue culture supernatant derived from the hybridoma HO 13.4, kindly provided by Dr K. Rock, Harvard Medical School, Boston, MA) and Low-tox rabbit complement (Cedarlane Laboratories Ltd, Grimsby, Ontario, Canada) prior to transfer. Under these conditions 40–45% of lymphocytes were lysed while complement-treated controls exhibited approximately 10% killing. All recipients were immunized with 2 μg OA [in $Al(OH)_3$] and bled 1–3 weeks later.

Statistical analysis

PCA and ELISA titres were log transformed, following which geometric means were compared using Student's unpaired two-tailed *t*-test.

RESULTS

Inhibition of IgE responses following treatment with glutaraldehyde polymerized OA

Polymerization of OA one half pH unit above its isoelectric point yields freely soluble molecules of M_r $3{\cdot}5 \times 10^7$ (OA-POL), as determined by gel filtration.[20,29] Treatment of mice with three 80 μg OA-POL injections (i.p.) resulted in markedly decreased primary and secondary anti-OA IgE responses in all (14/14) strains examined to date.[32] Table 1 summarizes the effects of treatment with unmodified OA or OA-POL on OA-specific antibody responses in C57BL/6 mice in conventional short-term studies. Here, as previously, responses were assessed following challenge with unmodified OA in $Al(OH)_3$ adjuvant 10 days after treatment.

Anti-OA IgE responses were decreased by 97–99% ($P < 0{\cdot}001$) following exposure to OA-POL. Concomitantly,

Table 2. Long-lived inhibition of IgE responses is resistant to multiple re-exposures to antigen*

Treatment	Elapsed time (days)	Challenge	IgE 2°	IgE 3°	IgE 4°	IgE 5°	IgG 2°	IgG 3°	IgG 4°	IgG 5°	IgM 2°	IgM 3°	IgM 4°	IgM 5°
Saline	83	OA	4000	8000	4000	ND	739	1115	560	ND	360	1205	1006	ND
OA-POL	83	OA	100	80	50		1016	975	548		632	720	510	
Saline	128	OA	4000	2500	3200	1600	1938	1251	1387	1187	230	717	1530	880
OA-POL	128	OA	40	160	80	40	1487	1356	1417	1006	945	1005	1780	1000
Saline	163	OA	4000	3200	3200	1280	933	552	488	683	1012	1000	1920	2050
OA-POL	163	OA	160	80	80	40	1000	500	460	409	970	505	1400	1680
Saline	208	OA	2560	800	1280	ND	767	623	560	ND	685	518	2028	ND
OA-POL	208	OA	20	20	10		623	718	369		800	719	1240	
Saline	328	OA	4000	2000	ND	ND	744	292	ND	ND	1920	509	ND	ND
OA-POL	328	OA	160	20			718	489			975	960		

* C57BL/6 mice were treated with OA-POL or saline on Days 0, 2, 4. Five cohorts of three mice each were challenged with OA [$Al(OH)_3$] either 83, 128, 163, 208 or 328 days later and at monthly intervals thereafter. All immunizations were given in the absence of any further treatment with OA-POL. OA-specific IgE (titre), IgG (μg/ml) and IgM (titre) responses obtained 7 days post-immunization are shown from one of three experiments. Comparison between OA-POL- and saline-treated groups using Student's two tailed t-test yields the following P values: IgE $P<0{\cdot}001$, IgG $P<0{\cdot}05$, IgM $P>0{\cdot}05$ in most cases.

Table 3. Reciprocal relationship between inhibited IgE responses and elevated IgG2a responses in OA-POL-treated C57BL/6 mice*

Treatment	Challenge	IgE 1°	IgE 2°	IgE 3°	IgG1 1°	IgG1 2°	IgG1 3°	IgG2a 1°	IgG2a 2°	IgG2a 3°
None	OA	1600	4000	8000	5750	76,800	108,800	10	20	25
OA-POL (Day −55)	OA	<10	100	80	33,280	52,800	37,200	1900	26,200	25,600
None	OA	3200	4000	2500	6800	302,780	305,971	5	10	30
OA-POL (Day −100)	OA	20	40	160	62,080	55,780	65,380	57,500	42,500	39,500
None	OA	800	2560	800	5360	360,000	176,400	15	35	55
OA-POL (Day −180)	OA	<10	20	20	52,800	61,000	29,000	48,000	39,680	28,800
None	OA	1200	4000	2000	2200	267,725	68,525	15	30	30
OA-POL (Day −300)	OA	40	160	20	87,930	90,835	49,100	36,500	19,950	17,600

* Peak OA-specific antibody responses (titre) in mice treated and challenged as described in Table 2. Significant differences exist between saline- and OA-POL-treated groups for IgE ($P<0{\cdot}001$) and IgG2a ($P<0{\cdot}001$) at all times examined.

IgG2a responses were elevated from 500- to 1500-fold as a consequence of OA-POL treatment. In marked contrast, administration of unmodified OA under the same conditions led to no inhibition of IgE production and actually resulted in an accelerated primary response (Day 7 IgE titre 1600). Primary IgG1 responses were markedly elevated in these experiments in both OA-pretreated and OA-POL pretreated groups. OA-specific IgA and IgM production was largely unaffected.

Induction of long-term regulation of IgE responses

Most *in vivo* studies of immunoregulation have examined time periods of less than 6 weeks. Caution is required to extrapolate from such studies to long-term regulation of IgE responses. To directly determine the capacity of OA-POL to induce long-lived changes in murine responsiveness to native allergen, we treated 40 mice with OA-POL or saline, and at regular intervals from 14 to 300 days later, challenged age-matched cohorts of three mice each with OA [$Al(OH)_3$]. Figure 1 demonstrates that anti-OA IgE responses in OA-POL-treated mice were decreased by more than 95% regardless of when (14–300 days later) the first allergen challenge occurred. In most cases, peak IgE responses in the OA-POL-treated groups ranged from 1% to 3% of those observed in age-matched (or in 8-week-old, data not shown) untreated controls. Tenfold increases, or decreases, in the amount of OA [$Al(OH)_3$] used for challenge had no effect on the inhibition observed (data not shown).

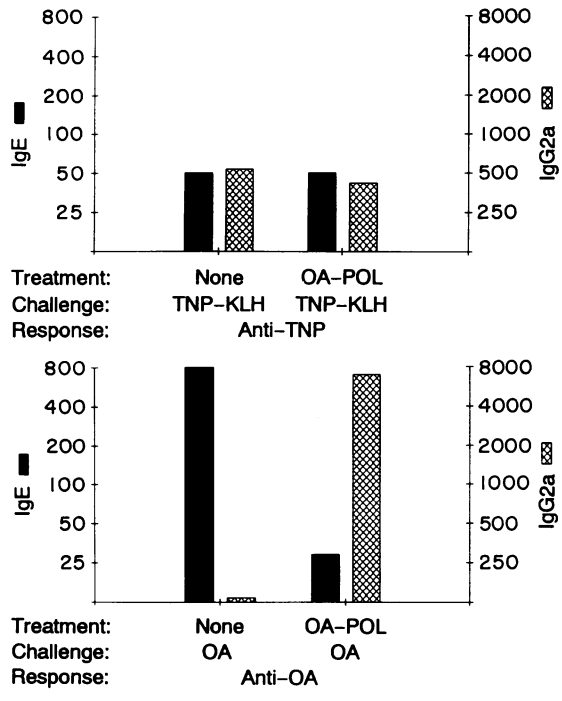

Figure 2. Antigenic specificity of OA-POL's effects on IgE and IgG2a responses. Mice were treated with OA-POL or saline and 185 days later challenged with TNP_{22}-KLH (a) or OA (b). Peak IgE (■) and IgG2a (▩) responses 10 days later are indicated.

Isotypic specificity and resilience of long-term suppression to further allergen exposure

To assess the persistence of the changes induced, mice were treated with OA-POL and, along with age-matched controls, challenged repeatedly with OA [$Al(OH)_3$] beginning 83–328 days after treatment (Table 2). Secondary IgE responses were decreased by 95% or greater in comparison to untreated controls, despite up to five immunizations. Secondary anti-OA IgG, IgM and (not shown) IgA responses exhibited minimal change in most instances.

Examination of anti-OA IgG responses by subclass revealed striking increases in anti-OA IgG2a production (500–10,000-fold) which paralleled the 95–99% inhibition of anti-OA IgE production found upon OA-POL treatment (Table 3). In this experiment, production of IgG1 was decreased in the secondary response of several groups, but to a lesser, and more variable, extent. IgA and IgM responses to OA were not significantly affected by OA-POL treatment (data not shown). Similar increases in antigen-specific IgG2a and inhibition of IgE responses were observed in each experiment.

Antigenic specificity

The specificity of this long-lived inhibition of IgE responses was demonstrated upon challenge of saline or OA-POL-treated mice 6 months after treatment. In the experiment shown (Fig. 2), IgE responses to OA were inhibited by 94% and IgG2a responses increased 350-fold, while IgE and IgG2a responses to TNP were unaffected by OA-POL treatment prior to TNP_{22}-KLH immunization.

T-cell dependency of changes in OA-specific responsiveness

To examine the means by which IgE responses were inhibited, whole or T-cell depleted spleen cell populations obtained from mice treated with OA-POL 6 months earlier were transferred to normal recipients, which were then challenged with OA [$Al(OH)_3$]. Recipients of T cells from mice treated with OA-POL 6 months previously (Fig. 3) exhibited 400–600-fold increases in anti-OA IgG2a, concomitant with marked inhibition of IgE responses, in comparison to mice receiving normal or T-cell depleted spleen cells. These increases in IgG2a production were decreased by 60–80% if donor T cells were depleted prior to transfer. Residual increases in IgG2a probably reflect the contribution of OA-specific B cells that had undergone gene rearrangement to IgG2a in the OA-POL-treated donor prior to cell transfer.

DISCUSSION

A major goal in studies of IgE regulation is the induction of long-lasting, IgE-selective unresponsiveness. Here, we report the induction of IgE-specific, T-cell dependent, split tolerance in which treated mice were capable of generating OA-specific IgE responses that, at most, were 1–3% of those obtained in untreated controls. This state of virtual unresponsiveness peristed for the duration of the study (>350 days). The mechanism by which OA-POL-induced T cells mediate these changes in responsiveness remains unresolved, but the 500–1500-fold increases in anti-OA IgG2a production which were found concomitant with the inhibition of IgE responsiveness provide support for our hypothesis that treatment with these chemically modified allergens results in preferential induction of antigen-specific IFN-γ-secreting T cells.[24] An alternative hypothesis, that exposure to OA-POL results in decreased IL-4 production by the OA-specific T-cell repertoire, is not excluded. We are currently examining the frequency of IFN-γ versus IL-4 synthesizing cells by limiting dilution analysis to directly address this issue.

The longevity and resilience of the changes in antibody production induced upon treatment with this polymerized allergen are surprising. As demonstrated in Tables 2 and 3, at no point were IgE responses in OA-POL-treated groups comparable to those elicited in untreated controls. This isotype-selective unresponsive state persisted in spite of five booster immunizations with native allergen in adjuvant given over a 1-year period from the single initial course of treatment. In contrast, production of IgG1 was invariably increased in primary responses following OA-POL (or unmodified OA) administration, while secondary IgG1 responses tended to be inhibited. This may be explicable by a stricter dependence of IgE on IL-4, or a greater sensitivity to IFN-γ, than IgG1, particularly in antigen-specific responses.[14]

Development of immunotherapeutic strategies for control of immediate hypersensitivity has long been hindered by a major challenge; persistent inhibition of IgE responsiveness is difficult to establish. Allergen immunotherapy currently involves a multiple series of injections of allergen extracts with the stated goal of decreasing the patient's sensitivity to that allergen. Although widely practised, such therapy often yields equivocal results and is the subject of increasing scrutiny in terms of safety and efficacy.[33-36]. Much attention has been paid to different forms of chemically modified allergens, the major objectives

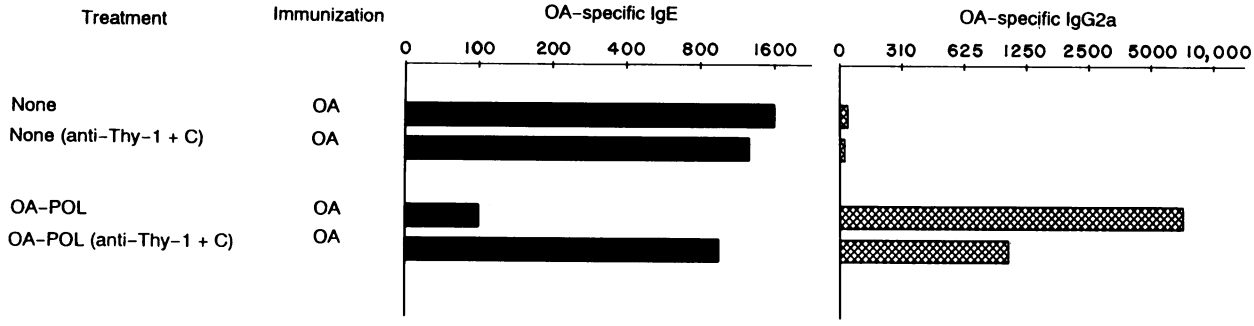

Figure 3. T-cell dependency of reciprocal changes in OA-specific IgE and IgG2a production. Responses were obtained in normal recipients following transfer of 8×10^7 spleen cells, or T-depleted spleen cells, from mice treated with OA-POL 6 months previously. All recipients were immunized with 2 μg OA in $Al(OH)_3$ immediately after cell transfer and bled 10 days later. Treatment with anti-Thy-1.2 (HO 13.4) tissue culture supernatant and complement resulted in 41% cell lysis, while complement alone killed 8% in this experiment. Data (titres) presented are from one of three experiments.

being increased efficacy and decreased likelihood of anaphylactic reactions (reviewed by refs 26 and 27), but in general the clinical results obtained have often proven disappointing.

This may be attributable in part to two limitations observed in most experimental approaches to allergen immunotherapy: (i) an inability to effectively inhibit previously established IgE responses, and (ii) difficulty in maintaining IgE inhibition in the face of repeated allergenic exposure. We have found that OA-POL treatment leads to inhibition of murine IgE responses which are (i) IgE specific, (ii) durable and (iii) resistant to multiple challenges—requirements which suggest that this approach may provide a useful system for examination of the role of differential activation of cytokine genes in IgE regulation. Moreover, even well established, ongoing IgE responses are effectively abrogated (96–99% decreased) by three courses of treatment with OA-POL.[20] To our knowledge, these studies represent the first successful exploitation of differential cytokine synthesis patterns for modulation of hypersensitivity responses.

Although this approach is successful in abrogating ongoing murine IgE responses, it should be cautioned that the amounts and conditions required far exceed the amount practical for administration to humans. Thus, at this stage, the primary value of this approach lies in its utility as a tool to examine conditions which govern the induction and regulation of *in vivo* antibody responses. Established *in vivo* IgE responses likely consist of IL-4-dependent and IL-4-independent components,[14] each of which may display different requirements for inhibition.[37]

Studies carried out in several laboratories suggested that murine Th clones can be subdivided into (at least) three major groups based on function and the pattern of lymphokines secreted, with Th1 cells secreting IL-2, lymphotoxin and IFN-γ, Th2 producing IL-4, IL-5 and IL-6, and Th0 clones displaying an intermediate pattern of cytokine synthesis upon stimulation with antigen or Con A.[38] It remains unclear if these three subsets exist as independent lineages or if the nature of the antigenic or environmental stimuli encountered determines the pattern of cytokine production. Also unresolved is the question of whether commitment to one or another pattern of cytokine synthesis by any given clone is absolute[39-41] or irreversible. Evidence has been obtained that Th1-like and Th2-like responses can be distinguished in normal cell populations,[42-47] raising the possibility that induction of distinct patterns of lymphokine production may play an important role in the regulation of humoral immune responses *in vivo*, but this remains an unresolved issue. The IFN-γ-dependent mechanism of regulation, demonstrated in short-term studies[24] and supported by the data in this report, is consistent with preferential induction of Th1-like patterns of cytokine synthesis in preference to the Th2-like pattern normally observed following OA $Al(OH)_3$ immunization—a response which leads to strong IgE and virtually undetectable IgG2a production. However, it remains to be determined if this reflects the Th1/Th2 paradigm at the single cell level.

The influences which shape the pattern of cytokine production dominating any given antigen-specific response comprise an active area of investigation. Earlier studies suggest that the genetic background of the individual is a determining factor.[4-7] However, the nature of the antigenic stimulus[48] and the type of antigen-presenting cell have also been shown to qualitatively influence cytokine production. Thus, Hauser *et al.* (49) demonstrated that Langerhans' cells play a critical role in selecting for Th2-like patterns in the derivation of antigen-specific T-cell clones, while Magilavy *et al.*[50] found that hepatic non-parenchymal cells support the proliferation of Th1 but not Th2 clones. The present study indicates that the form of the antigen plays a critical role in determining the dominant immune response *in vivo*. In this regard, the approximately 10^2-fold decreased capacity of OA-POL to be bound by B cells[29] in comparison to unmodified OA may effectively remove B-cell antigen presentation[51-53] of OA-POL and thus indirectly determine the pattern of cytokine production elicited. The role of different antigen-presenting cells in processing and presenting OA versus OA-POL to the OA specific T-cell repertoire is currently under investigation.

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