

Tetracycline-mediated IgE isotype-specific suppression of ongoing human and murine IgE responses *in vivo* and murine memory IgE responses induced *in vitro*

Rauno Joks^{1,2}, Tamar Smith-Norowitz^{2,3}, Maja Nowakowski^{1,2,4}, Martin H. Bluth^{2,4,5,6} and Helen G. Durkin^{1,2,4}

¹Department of Medicine, ²Center for Allergy and Asthma Research, ³Department of Pediatrics, ⁴Department of Pathology, and ⁵Department of Surgery, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, USA

⁶Present address: Department of Pathology, Wayne State University, Detroit, MI, USA

Correspondence to: R. Joks; E-mail: rauno.joks@downstate.edu

Received 25 April 2009, accepted 8 January 2010

Abstract

We previously reported that minocycline treatment of allergic asthmatic patients had oral steroid sparing effects and improved their clinical status and that minocycline suppressed *in vitro* induction of IgE responses by their PBMC. The effect of minocycline on human or animal IgE responses *in vivo* has not been studied. Allergic asthmatics (serum IgE: 505 ± 535 IU ml⁻¹) were given minocycline (150 mg po to 250 mg po BID) as add-on therapy to standard care for up to 10 months; control subjects (IgE: 405 ± 472 IU ml⁻¹) received standard care ($n = 6$ per group). Serum immunoglobulin (IgM, IgG, IgE and IgA) levels were determined monthly (Nephelometry, Unicap Total IgE Fluoroenzyme immunoassay). BALB/c mice ($n = 6$ per group) were injected intraperitoneally with benzylpenicilloyl₁₄-Keyhole limpet hemocyanin (BPO₁₄-KLH) in alum on days 0, 21 and 42, fed with minocycline or doxycycline (10–100 mg kg⁻¹) on day 44 and numbers of BPO-specific IgG₁, IgE and IgA antibody-forming cell (AFC) in mesenteric LN and spleen and serum immunoglobulin levels were determined on days 46–70 (enzyme-linked immunosorbent spot assay, ELISA). The ability of minocycline or doxycycline to suppress *in vitro* induction of murine memory IgE responses also was investigated.

Minocycline strongly suppressed serum IgE levels of allergic asthmatics (9% per month) ($P = 0.012$). Minocycline (and doxycycline) also strongly suppressed peak murine IgE AFC and serum IgE responses (>95, ~75%, respectively) and *in vitro* induction of memory IgE responses by murine mesenteric LN and spleen cells (>95%). Tetracycline suppression of all human and murine IgE responses was IgE isotype specific. Suppression of murine IgE responses *in vivo* was dose dependent and lasted 5–7 days.

Keywords: allergy, asthma, human, IgE, mice, tetracycline

Introduction

We previously demonstrated that oral treatment of allergic asthmatic humans with minocycline, a well established antibiotic (1), improved their clinical status and had oral steroid sparing effects (2, 3). Lee *et al.* (4) reported that another tetracycline, doxycycline, reduced airway hyperresponsiveness and inflammation in mice with toluene diisothiocyanate-induced asthma (4). The mechanism(s) by which tetracyclines achieved these effects has not been studied, but could involve suppression of their IgE responses. In this

regard, Kuzin *et al.* (5) demonstrated that doxycycline suppressed anti-CD40/IL-4-mediated *in vitro* induction of IgE production by murine spleen cells. We subsequently demonstrated that minocycline or doxycycline suppressed anti-CD40/IL-4-mediated *in vitro* induction of IgE production by PBMC obtained from allergic asthmatic humans (6). In preliminary studies, we reported that minocycline treatment of allergic asthmatic patients suppressed their ongoing IgE responses *in vivo* (7–10).

2 Tetracyclines suppress IgE responses

The mechanisms by which minocycline suppresses IgE responses *in vivo* (7–10) and *in vitro* (5, 6) are unknown. It is well recognized that tetracyclines have non-antibiotic pleiotropic anti-inflammatory properties, including ability to inhibit production of IL-1 and tumor necrosis factor alpha and development of septic shock (11), inflammatory cell trafficking (12), lymphocyte proliferative responses (13), generation of reactive oxygen species (14), iNOS protein expression (15) and matrix metalloproteases (16), any of which might play a role. Nevertheless, it is also possible that antibiotic activities of tetracycline, which is bacteriostatic (17), may play a role in suppression *in vivo* by mediating release of bacterial cell wall components (BCWC), which can mediate switching of T cell subsets in rodents (18) and suppress rodent IgE responses (19–22). Further, Durkin *et al.* (22) demonstrated that certain BCWC (peptidoglycan) suppress IgE responses, findings that antedate the hygiene hypothesis (23). Studies of Gerhold *et al.* (24) demonstrated that LPS suppresses IgE responses.

The present studies investigate the ability of tetracyclines to regulate ongoing human and murine IgE responses *in vivo* and murine memory IgE responses *in vitro*. We found that tetracyclines strongly suppressed these responses and that tetracycline-mediated suppression of IgE responses was IgE isotype specific. Suppression of murine IgE responses *in vivo* was dose dependent and transient, lasting 5–7 days.

Methods

Human studies

Subjects. This open-label study was approved by the Institutional Review Board at SUNY Downstate Medical Center. The procedures followed were in accordance with institutional guidelines involving human subjects. The protocol is registered at ClinicalTrials.gov as No. NCT00536042 (www.clinicaltrial.gov). Each subject gave written informed consent.

Adult asthmatic subjects (ages 18–75 years) with a history of allergic asthma (25), aeroallergen sensitization by epicutaneous skin testing (26) and/or *in vitro* allergen-specific IgE (Pharmacia Unicap 100, Pharmacia Diagnostics, Uppsala, Sweden) were eligible to enroll. Excluded from the study were pregnant women, patients with chronic obstructive pulmonary disease or chronic liver diseases and those with a history of hypersensitivity to tetracyclines.

Procedures. Adult asthmatic subjects were given minocycline capsules for treatment of asthma as add-on therapy to standard therapy (25) for up to 1 year. Treatment regimens were not altered during the 1-year period, excluding use of oral steroid rescue therapy. Each subject received a prescription for minocycline 150 mg po twice daily. These doses were increased by 50 mg BID every 8 weeks to a maximum dose of 250 mg twice daily. This dosing regimen was selected because it was previously used to treat patients with rheumatoid arthritis (27). It is well recognized that minocycline can cause nausea or dizziness (17). If a subject had either nausea or dizziness, she/he was advised to decrease the dose by discontinuing first the 50 mg capsule. If this was insufficient to diminish side effects, the

patient was advised to discontinue the 100 mg capsule and continue with the 50 mg capsule dose. The subject would then continue with this dose until completion of the study. This approach optimized dosing and assured potential for maximum benefit with minimal adverse effects.

Total serum IgE and IgM, IgG and IgA levels were determined monthly [total IgE Fluoroenzymeimmunoassay (Pharmacia Diagnostics), Nephelometry, respectively] in the Clinical Immunology Laboratory, SUNY Downstate UH, Brooklyn, NY, USA. Additional data (not reported here) were obtained, including spirometry, change in quality of life and oral steroid requirements (see ref 3). All subjects underwent routine hematologic and hepatic blood toxicity screens every 2 months.

Statistical analysis. Mixed linear models were applied, with month, treatment status and their interaction as fixed factors. Dependent variables were IgM, IgG, IgE, IgA transformed where necessary to correct skew and heteroskedasticity. Appropriate structures for covariance over time were fitted empirically using the Akaike information criterion. Satterthwaite corrections to denominator degrees of freedom were applied.

Mouse studies

Animals and immunization. BALB/c mice, purchased from Jackson Laboratory, Bar Harbor, ME, USA, were randomly bred for up to three generations in the Animal Center at SUNY Downstate. Experimental and control mice, males or females, 6–8 weeks old, were age and sex matched in individual studies. Mice were injected intraperitoneally with benzylpenicilloyl₁₄-keyhole limpet hemocyanin (BPO₁₄-KLH) (10 µg), prepared according to published methods (28), in aluminum hydroxide gel (alum, 0.2 ml) on days 0, 21 and 42 to induce peak BPO-specific IgE responses on days 46–60 (*n* = 6 per group), as reported in our previous studies (21, 22, 28). Mice were fed (gavage) with either minocycline or doxycycline (10–100 mg kg⁻¹) on day 44 and killed on days 46–70, at which times the numbers of BPO-specific IgG₁, IgE and IgA antibody-forming cells (AFC) in mesenteric LN and spleen were determined *ex vivo* in enzyme-linked immunosorbent spot (ELISPOT) assay. Data represent the mean of triplicate wells and are reported as either <30 IgG₁, <6 IgA or <1 IgE AFC 10⁻⁷ cells.

Detection of AFC responses (ELISPOT assay). Antibodies. Rat mAb directed against mouse IgG₁ (H143-225-8.1), IgE (641-46-48) and IgA (L22-8.1) were provided by Novartis, Basel, Switzerland; these antibodies are commercially available. The antibodies were selected for the absence of isotype cross-reactivity using a panel of hybridoma cells that secrete anti-phosphorylcholine murine mAb of the various isotypes. Horseradish peroxidase-conjugated sheep anti-rat Ig was purchased from Amersham Corp., Arlington Heights, IL, USA. All antibodies were titrated to determine optimal concentration for use.

AFC. The numbers of BPO-specific IgG₁, IgE and IgA AFC were detected *ex vivo* and on days 0–10 of culture in ELISPOT assay performed according to the method of Smith

et al. (28), and as in our previous studies (21, 22). To induce hapten-specific memory responses *in vitro*, mesenteric LN or spleen cells ($5 \times 10^6 \text{ ml}^{-1}$; total volume of 8 ml) were cultured for 0–10 days in six-well flat-bottom tissue culture plates (Costar, Cambridge, MA, USA) $\pm$ BPO₂₅-KLH (12.5 – 3200 ng ml^{-1}) $\pm$ varying concentrations of minocycline or doxycycline (results for BPO₂₅-KLH at 50 ng ml^{-1} and tetracyclines at 100 ng ml^{-1} are reported) in complete medium (21, 22, 28) at 37°C in a humidified atmosphere of 7% CO_2 in air, after which cells were recovered, and the numbers of BPO-specific memory IgG₁, IgE and IgA AFC were determined in ELISPOT assay. In this system, residual *in vivo* AFC responses of mesenteric LN and spleen are detected until day 2 of culture (see Table 3). On day 3, BPO-specific memory IgE (and other) antibody responses induced *in vitro* first appear, and usually peak on day 5 (see also 23–25). Therefore, the effects of minocycline or doxycycline added to cultures on day 0 were measured on day 5. Data are expressed as AFC 10^{-7} cells: either 10^7 cells plated in ELISPOT assay for *ex vivo* studies or 10^7 cells for mesenteric LN and spleen cell cultures. Because dilutions are made before addition of cells in the ELISPOT assay, one IgG₁ spot represents 30 AFC 10^{-7} cells, one IgA spot represents 6 AFC 10^{-7} cells and one IgE spot represents 1 AFC 10^{-7} cells. Therefore, when no spots are detected, data are expressed as <30, <6 and <1 AFC, respectively.

Detection of serum IgE responses (ELISA). Levels of anti-BPO antibodies of various isotypes in pooled serum samples obtained from six mice per group on day 46 were analyzed at four different dilutions by ELISA using BPO-BSA-coated plates (96-well flat-bottom ELISA PLATES; Corning No. 25802, Corning, Inc., Corning, NY, USA). Serum IgG, IgE and IgA levels were obtained by comparing serum titrations with standard curves constructed using PC-BSA-coated

plates and a panel of mouse anti-PC mAb of various isotypes. Data represent the mean of triplicate wells and are expressed as ng ml^{-1} .

Results

Human studies

All participants in this study ($n = 14$), whether treated with minocycline as add-on therapy or not, received standard care before and throughout the study period (Table 1). All had either severe persistent (subject nos 1–8), mild persistent (nos. 9–12) or mild intermittent (nos. 13, 14) asthma. The duration of their asthma was either lifelong, i.e. since early childhood (nos. 1–4, 8, 9, 12) or 2–55 years (nos. 5–7, 10, 11, 13, 14).

Of the 14 allergic asthmatic subjects who participated in this study, most were African-American (nos. 1–7, 9–11, 13), two were Hispanic (nos. 8, 12) and one was Caucasian (no. 14). Twelve subjects were female and two were male (nos. 7, 14).

The allergic asthmatic subjects on standard care ($n = 14$) described in Table 1 were divided into two groups according to their willingness to participate in the minocycline open trial: those who would receive oral minocycline in addition to standard therapy for up to 1 year (nos. 1, 2, 6–8, 12, 14) (minocycline group) and those who would continue to receive standard therapy but did not receive oral minocycline (nos. 3–5, 9–11, 13) (control group).

Serum immunoglobulin (IgE and IgM, IgG and IgA) responses of allergic asthmatic subjects before commencement of study. IgE. All allergic asthmatic subjects in the minocycline group (subject nos. 1, 2, 6–8, 12, 14) had elevated levels of serum IgE (137 – 1367 IU ml^{-1}) (normal serum IgE levels: $<100 \text{ IU ml}^{-1}$, where $1 \text{ IU} = 2.4 \text{ ng ml}^{-1}$) (29) (Table 1 and Fig. 1A). Levels of serum IgE in the control

Table 1. Serum immunoglobulin (IgM, IgG, IgA and IgE) levels of allergic asthmatic subjects receiving standard care^a

Patient no.	Asthma severity	Gender (age in years)	Duration	Serum immunoglobulin levels ^b			
				IgM (mg dl^{-1})	IgG (mg dl^{-1})	IgA (mg dl^{-1})	IgE (IU ml^{-1})
1	SP	F (46)	LL	154	1440	313	274
2	SP	F (45)	LL	58	997	154	137
3	SP	F (51)	LL	141	1110	120	56
4	SP	F (66)	LL	nt	962	365	65
5	SP	F (55)	25	172	1180	767	nt
6	SP	F (44)	27	nt	nt	nt	238
7	SP	M (72)	5	150	1120	264	342
8	SP	F (28)	LL	nt	nt	nt	974
9	MP	F (43)	LL	nt	nt	nt	15
10	MP	F (63)	55	102	1280	322	987
11	MP	F (21)	12	120	1890	247	1146
12	MP	F (55)	LL	111	1510	324	1367
13	MI	F (47)	2	105	1360	205	815
14	MI	M (46)	3	86	1010	185	146

F, female; LL, lifelong (since early childhood); M, male; MI, mild intermittent; MP, moderate persistent; nt, not tested; SP, severe persistent.

^aDiagnosis and management of asthma in accordance with National Institutes of Health Guidelines, July, 1997 (see Methods).

^bNormal ranges for serum immunoglobulin levels at SUNY Downstate Medical Center (range): IgM 60–263, IgG 694–1618, IgA 69–378 mg dl^{-1} , IgE $< 100 \text{ IU ml}^{-1}$.

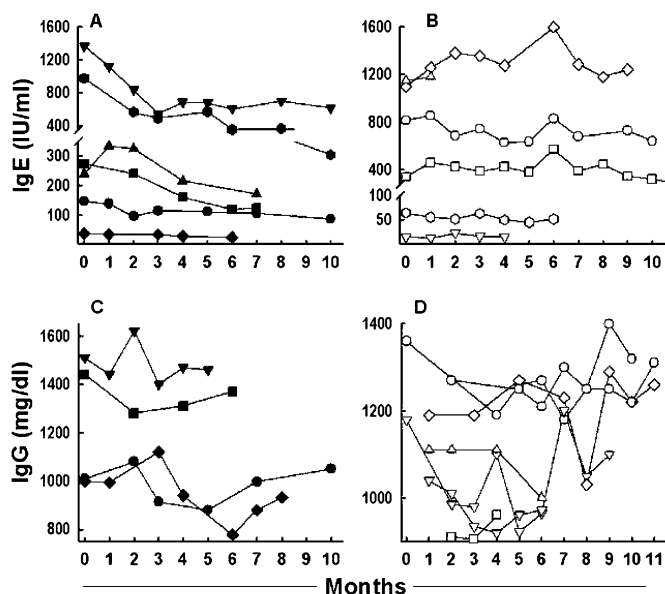


Fig. 1. Effect of oral minocycline treatment on serum immunoglobulin (IgM, IgG, IgE and IgA) levels of allergic asthmatic humans. Adult allergic asthmatic subjects ($n = 6$) were given minocycline (150–250 mg po* BID**) as add on to standard therapy for up to 1 year. Control allergic asthmatic subjects ($n = 8$) received standard therapy (see Table 1 for description of subjects). Serum IgE and IgM, IgG and IgA levels of all subjects were measured before and monthly after initiation of minocycline treatment (total IgE fluoroenzymeimmunoassay and nephelometry, respectively). IgE levels of minocycline-treated subjects are shown in panel (A) and controls in panel (B); IgG levels of minocycline treated subjects are shown in panel (C) and controls in panel (D). IgM and IgA levels, like IgG levels, did not significantly change in minocycline-treated or control groups ($P = ns$) (data not shown). Data for IgE are expressed as IU per milliliter and for IgG milligram per deciliter; * per os (by mouth) and ** bis in die (twice daily).

allergic asthmatic group (subject nos. 3–5, 9, 10, 11, 13) ranged from 15 to 1146 IU ml⁻¹ (Table 1 and Fig. 1B).

IgM, IgG and IgA. Serum immunoglobulin levels in the minocycline and control groups ranged from IgM 58 to 154, IgG 997 to 1510, IgA 154 to 324 mg dl⁻¹, IgM 102 to 172, IgG 962 to 1510 and IgA 120 to 767 mg dl⁻¹, respectively. All were within normal ranges (IgM 60–263, IgG 694–1618 and IgA 69–378 mg dl⁻¹) (30), excluding control subject no. 5 who had elevated IgA (767 mg dl⁻¹) (data for serum IgE shown in Table 1 and Fig. 1A and B; data for IgG shown in Fig. 1C and D and data for IgM and IgA not shown).

Effect of oral minocycline treatment on serum immunoglobulin levels of allergic asthmatic subjects. IgE. Allergic asthmatic subjects (nos. 1, 2, 6–8, 12, 14) who received oral minocycline treatment for up to 1 year had dramatic decreases in their serum IgE levels at the end of their treatment periods ($47.6 \pm 14.7\%$) (Figs 1A and 2, top panel). The decreases in serum IgE levels of these subjects were first observed at 2 months ($17 \pm 29.4\%$), decreased further, then leveled off at 4–6 months ($41.2 \pm 21.2\%$) and remained decreased at the end of the treatment period ($47.6 \pm 14.7\%$). In contrast to the minocycline group, serum IgE levels of the untreated control group were not suppressed (Fig. 1B).

IgM, IgG and IgA. Unlike the decreases in serum IgE observed in the minocycline-treated allergic asthmatic group (Figs 1A and 2), minocycline treatment had virtually no effect on their serum IgM, IgG and IgA levels, which, at the end of the minocycline clinical trial resembled those detected before minocy-

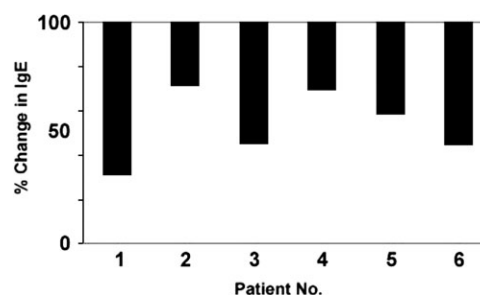


Fig. 2. Serum IgE levels of allergic asthmatic subjects at termination of minocycline treatment. Subjects are the same adult allergic asthmatics ($n = 6$) described in Figure 1 and Table 1, who were given minocycline (150–250 mg po BID) as add on to standard therapy for up to 1 year. Serum IgE levels were measured before and monthly after initiation of minocycline treatment, until treatment terminated or continued to 1 year (total IgE fluoroenzymeimmunoassay). Data are expressed as percent change at 1 year compared with baseline levels at initiation of minocycline treatment.

cline treatment and those of normal subjects (data for IgG shown in Fig. 1C and D and data for IgM and IgA not shown).

Mouse studies

Minocycline- and doxycycline-mediated suppression of peak murine-specific IgE AFC responses. Peak BPO-specific IgE and IgA AFC responses were detected in mesenteric LN and spleen on day 46 (mesenteric LN and spleen of six mice assayed individually) (data for IgE and IgA shown in Fig. 3 and data for IgG1, not shown). When mice were fed (gavage) with varying doses of either minocycline or

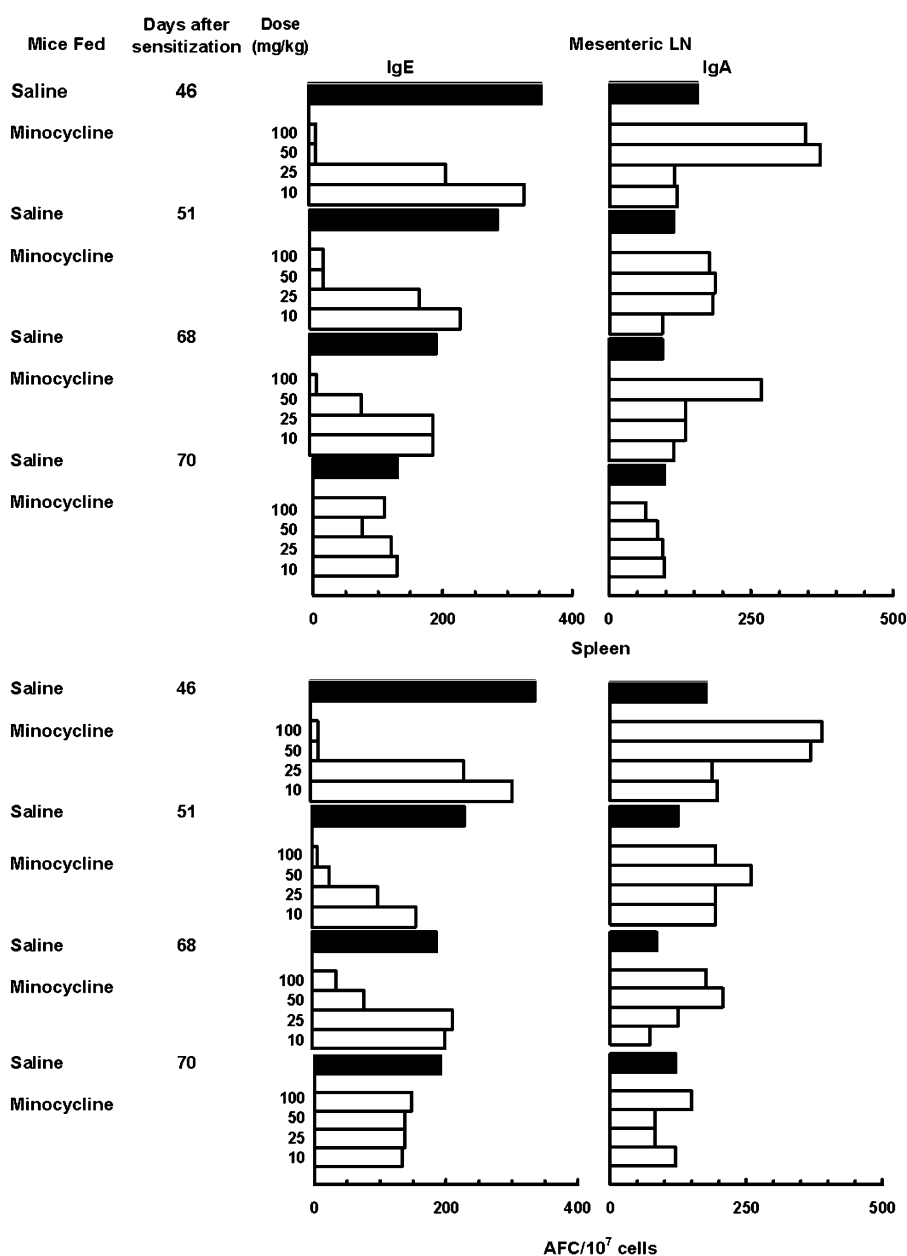


Fig. 3. Effect of oral minocycline and doxycycline given at the peak of the BPO-specific IgE response *in vivo* on AFC (IgG1, IgE and IgA) responses in mesenteric lymph node and spleen of BPO-KLH-sensitized mice (ELISPOT assay). BALB/c mice ($n = 6$ per group) were injected intraperitoneally with BPO₁₄-KLH (10 μ g) in alum (0.2 ml) on days 0, 21 and 43, fed (gavage) with minocycline or doxycycline (10–100 mg kg⁻¹) or with saline on day 44 and killed on days 46–70, at which times the numbers of BPO-specific IgG₁ (data not shown), IgE and IgA AFC in mesenteric lymph node and spleen were determined *ex vivo* in ELISPOT assay using BPO-BSA-coated plates. Representative experiment 1 of 3 with similar results. Data are from six mice per group, and represent mean values obtained with triplicate wells. Data are expressed as AFC 10^{-7} cells. Similar results obtained with doxycycline are not shown. With minocycline (and doxycycline) treatment, IgG₁, like IgA, AFC numbers did not decrease. In all experiments, peak suppression of IgE responses (>95% on day 46) was obtained with minocycline (or doxycycline) at 100 and 50 mg kg⁻¹.

doxycycline on day 44 (10–100 mg kg⁻¹) ($n = 6$ per group), with either minocycline or doxycycline at 50 and 100 mg kg⁻¹. BPO-specific IgE AFC responses in mesenteric LN and spleen were strongly suppressed on day 46, compared with immunized mice fed with saline on day 44 (95–99%) (data for minocycline shown in Fig. 3). Similar levels of suppression were obtained with doxycycline treatment at 50

and 100 mg kg⁻¹ (95–99%) (data not shown). Suppression obtained with minocycline (and doxycycline) at 50 and 100 mg kg⁻¹ lasted ~12 days, after which BPO-specific IgE AFC responses increased. In contrast to IgE responses that were suppressed, minocycline (and doxycycline) treatment did not result in decreased IgA (Fig. 3) or IgG₁ (data not shown) responses.

6 Tetracyclines suppress IgE responses

As was true of minocycline-mediated suppression of human IgE responses, minocycline-mediated suppression of murine responses was dose dependent and IgE isotype specific.

Minocycline-mediated suppression of serum IgE (ELISA). When BPO-KLH-sensitized mice were fed with either minocycline or doxycycline, there were statistically significant decreases ($P < 0.01$) in levels of serum IgE but not IgG or IgA. IgE levels were suppressed ~75% on day 46 (Table 2). Minocycline- and doxycycline-mediated suppression of murine IgE responses was IgE isotype specific in that IgG and IgA levels did not decrease.

Minocycline/doxycycline-mediated suppression of *in vitro* induction of BPO-specific memory IgE responses. Peak hapten-specific memory IgE responses are induced *in vitro* when mesenteric LN or spleen cells obtained from BPO-KLH sensitized mice at the peak of the hapten-specific IgE response *in vivo* (day 46) are cultured for 5 days in the presence of specific antigen (Table 3). Inclusion of either minocycline or doxycycline in culture prevented *in vitro* induction of BPO-specific memory IgE responses but had no effect on induction of BPO-specific memory IgG₁ or IgA responses (data for minocycline shown in Table 3; similar data obtained for doxycycline are not shown).

Discussion

This is the first report that (i) minocycline treatment of allergic asthmatic patients strongly suppresses their ongoing IgE responses, (ii) minocycline or doxycycline treatment of BPO-KLH-sensitized mice at the peak of the hapten-specific IgE response virtually abrogates this IgE response, (iii) minocycline and doxycycline suppress *in vitro* induction of murine memory IgE responses and (iv) there is a statistically significant minocycline- or doxycycline-mediated suppression of human IgE responses *in vivo* and murine IgE responses *in vivo* and *in vitro* that is IgE isotype specific.

Our finding that oral minocycline treatment strongly suppressed ongoing IgE responses of allergic asthmatic patients may explain our earlier observations in these patients that such treatment improved their asthma symptoms, had significant oral steroid sparing effects and improved their spirometric outcomes (2, 3), indicating the potential usefulness of minocycline in anti-allergy/asthma therapy. The dose of oral minocycline required to initiate suppression of IgE responses in the present studies was ~150 mg, given twice daily for two months, with prolonged treatment of the allergic asthmatic patients resulting in suppression of IgE responses of about 50%. The doses used in this study exceeded the standard antibiotic dose of 100 mg po twice daily, which can cause side effects including dyspepsia, nausea and dizziness (31). Similar side effects were observed in the present study, requiring dose adjustments. Doses of minocycline used in our study were similar to those previously used by others to improve symptoms of patients with rheumatoid arthritis, with similar side effects (27).

The decreases in patient IgE levels observed in the present studies were substantial (~50%). Nevertheless, serum

Table 2. BPO-specific antibodies in sera of BPO₁₄-KLH-sensitized mice fed with minocycline or with doxycycline

Mice	Mice fed	IgG	IgE	IgA
Sensitized	Minocycline	71.3 ± 2.4	0.26 ± 0.05	67.1 ± 3.2
	Doxycycline	70.3 ± 2.1	0.28 ± 0.05	61.5 ± 5.5
	Saline	71.4 ± 4.1	0.87 ± 0.05	64.7 ± 5.8
Unsensitized	—	neg	neg	neg

neg, negative.

Levels of anti-BPO antibodies of various isotypes in pooled serum samples obtained on day 46 were analyzed, at four different dilutions, by ELISA, using BPO-BSA-coated plates. Serum Ig levels were obtained by comparing serum titrations with standard curves constructed using PC-BSA-coated plates and a panel of mouse anti-PC mAb of various isotypes. Results obtained after feeding with 10 mg kg⁻¹ tetracyclines (peak) are shown. Data represent the mean percent of triplicate wells in three experiments performed using sera obtained from freshly bled mice (4–6 per group) and are expressed as nanogram per milliliter ± SD. Student's *t*-test (two-tailed, samples of equal variance): IgE levels of minocycline versus saline-fed mice $P < 0.01$; IgE levels of doxycycline versus saline-fed mice $P < 0.01$. Levels of IgG and IgA for both minocycline or doxycycline versus saline-fed mice $P = \text{ns}$.

IgE levels of most patients at the end of the minocycline treatment period remained above normal levels. This raises the interesting question of whether optimization of minocycline treatment doses/regimens might further improve patient symptoms, and this is currently being investigated. The use of single minocycline (or doxycycline) doses in our murine studies to virtually abrogate peak IgE responses for >1 week suggests that optimal doses may be identified for human studies.

The mechanism(s) by which minocycline suppressed human and murine IgE responses *in vivo* and *in vitro* and improved human asthma symptoms (2, 3) is unknown. It could be that minocycline-mediated suppression of ongoing human and murine IgE responses *in vivo* is attributable to antibiotic activity of minocycline, which is bacteriostatic (17), presumably resulting in lysis of bacteria and release of BCWC. Earlier studies of Durkin *et al.* (22) demonstrated that certain bacteria and BCWC (peptidoglycan and its synthetic derivatives suitable for use in man) strongly suppress rodent IgE responses. Subsequent studies by Braun-Fahrlander *et al.* (32) reported that increased levels of endotoxin were inversely associated with atopic wheezing. The results of these studies are consistent with the hygiene hypothesis that suggests that bacteria play important roles in regulation of IgE/allergic responses (23). Although the cell/cytokine pathways involved in suppression of these responses remain to be defined, preliminary studies of H. G. Durkin (unpublished data) have shown that bacterial peptidoglycan interacts with its toll-like receptors on cells of gut to initiate suppression of rodent IgE responses *in vivo*. Nevertheless, bacterial activity cannot account for the ability of minocycline or doxycycline to suppress *in vitro* induction of murine memory IgE responses in the present studies or anti-CD40/IL-4-mediated induction of IgE responses by PBMC of allergic asthmatic humans in our previous studies (6) because no bacteria were present in culture. Therefore, at least *in vitro*, other (nonbacterial), mechanisms are involved in suppression of IgE responses. It is well recognized that pleiotropic

Table 3. Minocycline-mediated suppression of *in vitro* induction of BPO-specific memory AFC responses by spleen cells obtained from BPO₁₄-KLH-sensitized mice at the peak of the *in vivo* IgE response

Days of culture	Agent in culture		AFC per 10 ⁷ cells		
	BPO ₂₅ -KLH (50 ng ml ⁻¹)	Minocycline (100 ng ml ⁻¹)	IgG1	IgE	IgA
Sensitized mice					
—	—	—	121 ± 6	113 ± 6	33 ± 7
1	+	—	32 ± 14	25 ± 7	<6
2	+	—	<30	<1	<6
3	+	—	820 ± 142	<1	59 ± 11
4	+	—	1646 ± 193	45 ± 9	316 ± 43
5	+	—	4403 ± 545	54 ± 7	933 ± 99
5	—	—	<30	<1	<6
6	+	—	5189 ± 598	<1	561 ± 52
1	—	+	42 ± 11	37 ± 3	<6
2	+	+	<30	<1	<6
3	+	+	467 ± 92	<1	48 ± 14
4	+	+	1364 ± 125	<1	336 ± 62
5	+	+	4401 ± 560	<1	642 ± 84
5	—	+	<30	<1	<6
6	+	+	5189 ± 594	<1	561 ± 53
Unsensitized mice					
—	—	—	<30	<1	<6
5	+	—	<30	<1	<6
5	—	+	<30	<1	<6
5	+	+	<30	<1	<6

Similar results were obtained with doxycycline in culture; similar results obtained with minocycline and doxycycline for mesenteric lymph nodes. BALB/c mice were injected intraperitoneally with BPO₁₄-KLH in alum on days 0 and 21 and killed on day 46. The numbers of BPO-specific AFC (IgG₁, IgE and IgA) in spleen were measured in ELISPOT assay, either *ex vivo* or after cells were cultured for 1–6 days ± BPO₂₅-KLH (50 ng ml⁻¹) ± minocycline. Data represent the mean percent of triplicate wells in three experiments (4–6 mice per group) and are expressed as AFC 10⁻⁷ cells ± SD. <30, <1, <6 IgG₁, IgE, IgA AFC, respectively, indicate no ELISPOTs of these isotypes were detected.

anti-inflammatory effects are associated with tetracyclines (11–16, 33), and it is, therefore, possible that these or other mechanisms may play a role in suppression of IgE responses *in vitro*, as well as *in vivo*.

Whatever the mechanism(s) *in vivo* or *in vitro*, the fact that tetracyclines suppressed both human and murine IgE responses *in vivo* and murine IgE responses *in vitro* without decreasing IgM, IgG or IgA responses indicates that they specifically target IgE responses and appears to rule out generalized cytotoxicity of T and B cells. However, it remains possible that there was selective toxicity of cells involved in IgE responses; if so, this may be a way in which IgE responses are regulated *in vivo*. Other evidence that IgE responses are specifically targeted is provided by recent studies of Erstein *et al.* (34) in our laboratory, which demonstrated that minocycline treatment of allergic humans suppresses allergen-induced mast cell-mediated late phase responses but does not interfere with responses to recall antigen (classic delayed-type hypersensitivity skin testing). Taken together, these observations strongly indicate the suitability of minocycline/doxycycline as anti-allergy drugs. The present studies in which minocycline and doxycycline suppressed *in vitro* induction of memory IgE responses suggests that memory T or B cells are targeted by non-antibiotic activities of these tetracyclines. This indicates that antibiotic activity of these tetracyclines is not required for suppression of memory IgE responses *in vitro* and further indicates that chemically modified tetracyclines that lack antibiotic activity would be ideal candidates for anti-allergy drugs. An ideal

chemically modified tetracycline for anti-allergy activity would continue to suppress IgE but not other immune responses, would be devoid of antibiotic activity and would not produce adverse effects associated with the parent molecules, including graying of skin (35) and development of autoimmunity (36) and deposition in growing bones and teeth (17).

To date, there is no commercially available drug that decreases human IgE responses at the level of IgE production. All currently available therapies, including omalizumab (anti-IgE monoclonal antibody) target the cellular and clinical sequelae of IgE-triggered immune responses either after IgE is produced or after it binds to its cellular receptors (37). However, the fact that tetracyclines suppress *in vitro* induction of memory IgE responses by mechanisms that do not involve antibiotic activity indicates the potential usefulness of chemically modified tetracyclines lacking antibiotic activity as anti-allergy/asthma agents. Development of such agents would markedly enhance our ability to treat diseases with altered IgE regulation.

Acknowledgements

We are grateful for the statistical support provided by Jeremy Weedon, PhD, Associate Director of the Scientific/Academic Computing Center at SUNY Downstate Medical Center. We also wish to thank Jonathan I. Silverberg, MD, PhD, MPH at SUNY Downstate for making the graphs for this paper.

These data were presented in preliminary form at the following annual meetings of the American Academy of Allergy Asthma & Immunology San Antonio, TX, USA, 2005; and Philadelphia, PA, USA, 2008; and at

the Keystone Symposium, Allergy, Allergic Inflammation and Asthma, Breckenridge, CO, USA, 2006.

References

- Saivin, S. and Houin, G. 1988. Clinical pharmacokinetics of doxycycline and minocycline. *Clin. Pharmacokinet.* 15:355.
- Joks, R., Daoud, A., Orloff, K. *et al.* 1998. Minocycline as an anti-inflammatory agent in the treatment of asthma (Abstract). *J. Allergy Clin. Immunol.* 101:12.
- Daoud, A., Gloria, C. J., Tanningco, G. *et al.* 2008. Minocycline treatment results in reduced oral steroid requirements in adult asthma. *Allergy Asthma Proc.* 29:286.
- Lee, K. S., Jin, S. M., Kim, S. S. and Lee, Y. C. 2004. Doxycycline reduces airway inflammation and hyperresponsiveness in a murine model of toluene diisocyanate-induced asthma. *J. Allergy Clin. Immunol.* 113:902.
- Kuzin, I. I., Snyder, J. E., Ugine, G. D. *et al.* 2001. Tetracycline inhibit activated B cell function. *Intern. Immunol.* 12:921.
- Smith-Norowitz, T. A., Bluth, M. H., Drew, H. *et al.* 2002. Effect of minocycline and doxycycline on IgE responses. *Ann. Allergy Asthma Immunol.* 89:172.
- Joks, R., Daoud, A., Tanningco, G. *et al.* 2005. Minocycline treatment results in reduced oral steroid requirements and suppresses IgE responses in adult asthma. (Abstract). *J. Allergy Clin. Immunol.* 115:10.
- Durkin, H. G., Bluth, M., Pincus, M., Smith-Norowitz, T., Nowakowski, M. and Joks, R. 2005. Minocycline mediated suppression of ongoing IgE responses *in vivo* and *in vitro* (Abstract). In NIH Keystone Symposium—Allergy, Allergic Inflammation and Asthma, Beaver Run Resort, Breckenridge, Colorado April 6–April 11, 2006.
- Durkin, H. G., Smith-Norowitz, T. A., Pincus, M., Nowakowski, M., Bluth, M. H. and Joks, R. 2007. New treatment for suppression of ongoing human and murine IgE responses (Abstract). *J. Allergy Clin. Immunol.* 119(1 Suppl.):12.
- Joks, R., Smith-Norowitz, T. A., Bluth, M. H., Nowakowski, M. and Durkin, H. G. 2008. Minocycline treatment of allergic asthmatic patients and BPO-KLH sensitized mice at the peak of the hapten specific IgE response suppresses IgE, but not IgM, IgG or IgA responses (Abstract). *J. Allergy Clin. Immunol.* 121:23.
- Shapira, L., Soskolne, W. A., Houri, Y., Barak, V., Halabi, A. and Stabholz, A. 1996. Protection against endotoxic shock and lipopolysaccharide-induced local inflammation by tetracycline: correlation with inhibition of cytokine secretion. *Infect. Immun.* 64:825.
- Akamatsu, H., Niwa, Y., Kurokawa, I., Masuda, R., Nishijima, S. and Asada, Y. 1991. Effects of subminimal inhibitory concentrations of minocycline on neutrophil chemotactic factor production in comedonal bacteria, neutrophil phagocytosis and oxygen metabolism. *Arch. Dermatol. Res.* 283:524.
- Thong, Y. H. and Ferrante, A. 1979. Inhibition of mitogen induced human lymphocyte proliferative responses by tetracycline analogue. *Clin. Exp. Immunol.* 35:443.
- Miyachi, Y., Yoshioka, A., Imamura, S. and Niwa, Y. 1986. Effect of antibiotics on the generation of reactive oxygen species. *J. Invest. Dermatol.* 86:449.
- Amin, A. R., Patel, R. N., Thakker, G. D., Lowenstein, C. J., Attur, M. G. and Abramson, S. B. 1997. Post-transcriptional regulation of inducible nitric oxide synthase mRNA in murine macrophages by doxycycline and chemically modified tetracyclines. *FEBS Lett.* 410:259.
- Paemen, L., Martens, E., Norga, K. *et al.* 1996. The gelatinase inhibitory activity of tetracyclines and chemically modified tetracycline analogues as measured by a novel microtiter assay for inhibitors. *Biochem. Pharmacol.* 52:105.
- Hardman, J. G. and Limbird, L. E. 2001. *Tetracyclines. Goodman and Gilman's The Pharmacological Basis of Therapeutics.* 10th edn. p. 124. McGraw-Hill, New York.
- Durkin, H. G., Bazin, H. and Chice, S. M. 1985. Bacterial regulation of IgE isotype expression by Peyer's patch B lymphocytes. *J. Leukoc. Biol.* 23:772.
- Durkin, H. G. and Chice, S. M. 1985. Modulation of IgE isotype expression on B lymphocytes in Peyer's patches of germfree rats. *Prog. Clin. Biol. Res.* 181:387.
- Durkin, H. G., Chice, S. M., Gaetjens, E., Bazin, H., Tarcsay, L. and Dukor, P. 1989. Origin and fate of IgE bearing lymphocytes. II. Modulation of IgE expression by Peyer's patch B cells by feeding with certain bacteria and bacterial cell wall components, or by thymectomy. *J. Immunol.* 143:1777.
- Auci, D. L., Chice, S. M., Dukor, P. and Durkin, H. G. 1993. Control of IgE responses. IV. Isotype specific suppression of peak BPO-specific IgE antibody forming cell responses and of BPO specific IgE in serum of BPO-KLH sensitized mice. *Int. Arch. Allergy* 101:167.
- Auci, D. L., Kleiner, G. I., Chice, S. M., Athanassiades, T. J., Dukor, P. and Durkin, H. G. 1998. Control of IgE responses. V. Oral administration of a synthetic derivative of the inner bacterial cell wall (SDZ 280.636) to sensitized mice induces isotype specific suppression of peak hapten specific antibody forming cell responses, serum IgE levels and immediate hypersensitivity responses. *Immunol. Invest.* 27:105.
- Strachan, D. P. 1989. Hay fever, hygiene and household size. *BMJ.* 299:1259.
- Gerhold, K., Avagyan, A., Beier, K., Ahrens, B., Quarcio, D. and Hamelmann, E. 2005. Prenatal exposure with lipopolysaccharide prevents allergen sensitization and eosinophilic airway inflammation in a murine model. (Abstract). *J. Allergy Clin. Immunol.* 115:262.
- National Asthma Education and Prevention Program. Expert Panel Report 2: Guideline for the Diagnosis and Management of Asthma. 1997. National Institutes of Health, Bethesda, MD: Publication No. 97: 4051.
- Rosenstreich, D. L., Eggleston, P., Kattan, M. *et al.* 1997. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N. Engl. J. Med.* 336:1356.
- Breedveld, F. C., Dijkmans, B. A. and Mattie, H. 1990. Minocycline treatment for rheumatoid arthritis: an open dose finding study. *J. Rheumatol.* 17:43.
- Smith, M. C., Heusser, C., Wagner, K., Blasier, K. and Durkin, H. G. 1990. An *in vitro* murine model of a penicillin specific IgE, anamnestic response. *J. Immunol. Methods* 131:55.
- Seagrott, V. and Anderson, S. G. 1981. The second international reference preparation for human serum immunoglobulin E and the first British standard for human serum immunoglobulins. I. *J. Biol. Stand.* 9:431.
- Rose, N. R., Hamilton, R. G. and Detrick, B. 2002. *Manual of Clinical Laboratory Immunology*, 6th edn. p. 179. ASM Press, Washington, DC.
- Tilley, B. C., Alarcon, G. S., Heyse, S. P. *et al.* 1995. Minocycline in rheumatoid arthritis. *Ann. Intern. Med.* 122:81.
- Braun-Fahrlander, C., Riedler, J., Herz, U. *et al.* 2002. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N. Engl. J. Med.* 347:869.
- Liu, J., Kuszynski, C. A. and Baxter, B. T. 1999. Doxycycline induces Fas/Fas Ligand-mediated apoptosis in Jurkat T lymphocytes. *Biochem. Biophys. Res. Commun.* 260:562.
- Erstein, D. P., Stanek, A., Dapul, G. *et al.* 2009. Minocycline therapy inhibits development of mast cell mediated cutaneous late phase responses. (Abstract). *J. Allergy Clin. Immunol.* 123:180.
- Fay, B. T., Whiddon, A. P., Puumala, S., Black, N. A., O'Dell, J. R. and Mikuls, T. R. 2008. Minocycline-induced hyperpigmentation in rheumatoid arthritis. *J. Clin. Rheumatol.* 14:17.
- Sturkenboom, M. C. J. M., Meier, C. R., Jick, H. and Stricker, B. H. C. 1999. Minocycline and lupuslike syndrome in acne patients. *Arch. Intern. Med.* 159:493.
- Expert Panel Report 3: Guidelines for the Diagnosis and Management of Asthma. Available at: www.nhlbi.nih.gov/guidelines/asthma/epr3/index.htm; last accessed August 31, 2007.