The Natural Immune Response to Inhaled Soluble Protein Antigens Involves Major Histocompatibility Complex (MHC) Class I-restricted CD8+ T Cell-mediated but MHC Class II-restricted CD4+ T Cell-dependent Immune Deviation Resulting in Selective Suppression of Immunoglobulin E Production

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Summary

The immunological basis for atopy is currently ascribed to an inherent bias in the CD4+ T cell response to nonreplicating antigens presented at mucosal surfaces, resulting in dominance of the T helper 2 (Th2) interleukin 4 (IL-4)-producing phenotype, which favors IgE production. In contrast, the "normal" response to such antigens involves a predominance of interferon γ (IFN-γ)-producing Th1 clones. This difference has been suggested to be the result of active selection in atopics for Th2 (and hence against Th1) clones at the time of initial antigen presentation. In the study below, we demonstrate that the natural immune response to inhaled protein antigens, particularly in animals expressing the low immunoglobulin E (IgE) responder phenotype, includes a major histocompatibility complex (MHC) class I-restricted CD8+ T cell component, the appearance of which is associated with active suppression of IgE antibody production. Thus, continued exposure of rats to aerosolized ovalbumin (OVA) antigen elicits a transient IgE response, that is terminated by the onset of a state of apparent "tolerance" to further challenge, and this tolerant state is transferable to naive animals with CD8+ T cells. Kinetic studies on in vitro T cell reactivity in these aerosol-exposed rats demonstrated biphasic CD4+ Th2 responses which terminated, together with IgE antibody production, and coincident with the appearance of MHC class I-restricted OVA-specific IFN-γ-producing CD8+ T cells. However, the latter were not autonomous in vitro and required a source of exogenous IL-2 for initial activation, which in CD8+-enriched splenocyte cultures could be provided by small numbers of contaminating OVA-specific CD4+ T cells. This represents the first formal evidence for the induction of an MHC class I-restricted T cell response to natural mucosal exposure to an inert protein antigen, and is consistent with a growing literature demonstrating sensitization of MHC class I-restricted CD8+ T cells by deliberate immunization with soluble proteins. We suggest that crossregulation of MHC class II-restricted CD4+ T cells via cytokine signals generated in parallel CD8+ T cell responses represents a covert and potentially important selection pressure that can shape the nature of host responses to nonreplicating antigens presented at mucosal surfaces.

Our current perception of how the allergic phenotype is expressed in humans is based largely upon data implicating IL-4 and IFN-γ from different CD4+ T cell subsets in the regulation of IgE synthesis in murine systems (1, 2). A key feature of these models has been the use of active immunization regimes comprising relatively high levels of antigen administered with powerful adjuvants, which elicit rapid high titer primary IgE responses. The success of this approach is undoubted, and the essential predictions from the animal model, viz. the existence of compartmentalized CD4+ T cell responses to ubiquitous environmental allergens which differ between atopics and normals, have already been borne out by the published human data (3–5).

However, the factors that determine the nature of the compartmentalization of the CD4+ T cell response in different individuals, in particular the selection for potentially pathogenic CD4+ Th2 clones in atopics, remain to be defined.

The approach taken by our laboratory to this complex ques-

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tion has followed a different route. We have reasoned that whereas maximal stimulation of the T cell system via antigen plus IgE-selective adjuvants would be expected to mobilize the major cellular mechanisms that normally promote IgE production, hyperstimulation of this nature may also mask more subtle control mechanisms that function under conditions of "physiological" antigen exposure. This is particularly the case in relation to immune responses to inhaled antigens, which in humans is associated with ostensibly adjuvant-free stimulation of the airway mucosa with nanogram levels of soluble proteins.

Accordingly, we have developed an experimental model for eliciting immune responses to low levels of inhaled protein antigen administered by aerosol. Our general findings (for reviews see references 6 and 7) indicate that this form of exposure elicits variable immune responses, depending on the genetics of the animals employed, ranging from primary IgE responses accompanied by the expression of allergic reactivity through to the development of profound immunological tolerance.

In the present study, we focus on T cell cytokine response in aerosol-exposed rats, particularly during the early sensitization phase, selecting two strains at the extremes of the IgE response range. We report that whereas high and low IgE responder phenotype indeed segregate, respectively, with IL-4 and IFN-γ reactivity in the T cell response, the initial source of T cell–derived IFNγ are MHC class I–restricted CD8$^+$ T cells, which in this model respond to soluble OVA antigen.

Materials and Methods

Animals. Inbred adult (10–16 wk) Brown Norway (BN)[1] and Wistar Albino Glaxo (WAG) rats were specific pathogen free and barrier housed.

Antigen Exposure and Immunization. The animals were exposed to an aerosol derived from 1% OVA (wt/vol) in saline (Grade V; Sigma Chemical Co., St. Louis, MO) via the Airborne Infection Apparatus (Tri-R Instruments, New York, NY) for 30 min daily, 5 d per week for up to 2 wk, or once per week for 6 wk, as previously described (8). Other groups of animals were chronically exposed to 1% OVA aerosol once weekly for 3–6 mo, after an initial 1–2-wk daily exposure protocol. In one series of experiments, animals of the WAG strain were administered 100 mg of OVA by gavage on two consecutive days. Intraperitoneal immunization employed 100 μg OVA in 10 mg aluminium hydroxide gel (AH).

Antibody Determinations. Anti-OVA IgE were measured by a modification of the radio-allergosorbent test (RAST) RIA, employing iodinated affinity-purified goat anti-rat IgE (9). IgG levels were determined as haemagglutinating antibody (HA) titers as previously described (8).

Antibodies. mAbs used were provided gratis by Professor A. Williams and Dr. D. Mason (University of Oxford, Oxford, UK), as follows: OX19 (anti-rat CD5), W3/25 and OX35 (anti-rat CD4), OX8 (anti-rat CD8), OX6 (anti-MHC class II) OX18 (anti-MHC class I), and OX21 (anti-human C3b) (10, 11). NDS62 (anti-IL-2 R) (12) was a gift from Dr. M. Dallman (John Radcliffe Hospital, Oxford, UK). Anti-rat CD3 (13) was from Pharmingen (San Diego, CA). Fluorescent staining was carried out using FITC-conjugated Abs, and sheep F(ab')$_2$ anti-mouse Ig-PE as a secondary Ab.

Cell Preparation. Cell preparation from spleen, respiratory tract lymph nodes (RTLN), mesenteric nodes (MLN), and PBL employed standard procedures (14).

Isolation of Lymphocyte Subsets. Except where specified, CD4$^-$ (CD8 enriched) or CD8$^-$ (CD4 enriched) lymphocyte subsets were purified by negative selection, gating out cells staining with either anti-CD4-FITC or anti-CD8-FITC, using a cytometer (Epics Elite; Coulter Electronics, Hialeah, FL). Depleted cell preparations contained $\leq$2% stained cells (see figure legends). Splenic B cells were isolated by depletion of T cells utilizing a rosette depletion technique (15).

Cell Cultures. Spleen and LN cells were cultured at 4×10^5 per microplate well in RPMI containing 10^{-5} M 2-ME plus antibiotics supplemented with 5% normal rat serum and stimulated with 100 μg/ml OVA. Supernatants were harvested after 24 h and frozen at -20°C until assayed for cytokines, or cell proliferation was measured after 96 h as incorporation of [^{3}H]thymidine into DNA (14).

Ab Blocking Experiments. Sodium sulphate precipitated preparations of antibodies OX21, OX18, OX6, W3/25, and NDS62 (5 μg/ml final concentrations) were added to cells at the initiation of culture (conditions as above).

IL-2 Assay. The standard bioassay employing the CTLL-2 cell line was used to quantify IL-2 in culture supernatants. The standard curve was generated from dilutions of human recombinant IL-2 (Cetus Corp., Emeryville, CA).

IFN-γ Assay. IFN-γ levels were determined by an ELISA method as detailed (16), and standardized against recombinant rat IFN-γ (all reagents were kindly provided by Dr. P. Van der Meide, TNO Primate Center, Rijswijk, The Netherlands).

IL-4 Assay. A semi-quantitative assay for rat IL-4 was employed (17) based on the striking increased expression of MHC class II molecules on B cells mediated by IL-4. Splenic B cells were cultured for 24 h in rIL-4 (a kind gift from Dr. A. McKnight, University of Oxford) as a positive control, test supernatants, or medium alone. After culture, the cells were stained with OX6-FITC and surface MHC class II expression assessed by flow cytometry. The mean fluorescence intensity (MFI) was measured and after subtraction of background (cells cultured in medium) the MFI of the test samples was expressed as a percentage of the MFI of the positive control. IL-10 has also been shown to stimulate MHC class II expression on B cells (18). However, the contribution of IL-10 could not be assessed here because of the lack of relevant blocking Abs. Since IL-10 is also a product of activated Th2 (as opposed to Th1) cells (19), this did not potentially confound the interpretation of our data.

Results

Primary Immune Responses to OVA Antigen Administered by Parenteral Immunization Versus Passive Inhalation. Fig. 1 contrasts the IgE and IgG responses of BN and WAG rats to OVA. Parenteral stimulation with antigen plus the IgE-selective adjuvant AH elicited a high-titer 1° IgE response in the high IgE responder BN strain (Fig. 1 *a*) which was in the order of 30-fold above that seen in the low IgE re-

[1] *Abbreviations used in this paper:* AH, aluminium hydroxide; BN, Brown Norway; HA, haemaggluting antibody; MFI, mean fluorescence intensity; MLN, mesenteric lymph node; RAST, radio-allergosorbent test; RTLN, respiratory lymph node; WAG, Wistar Albino Glaxo.

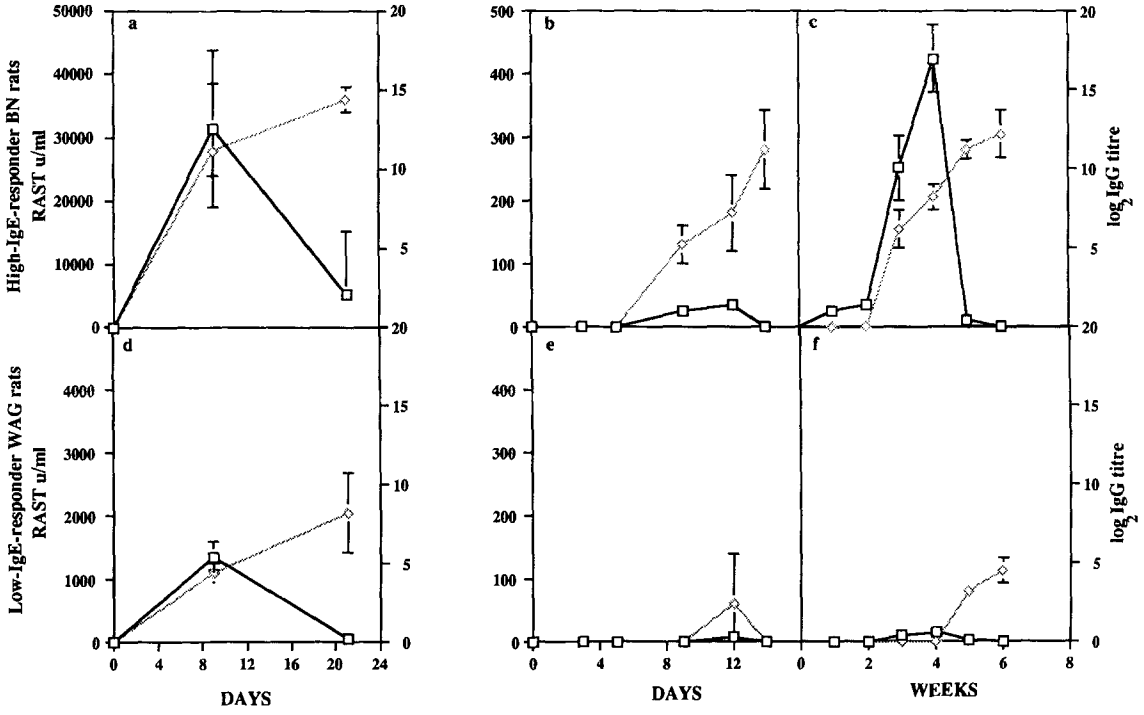

Figure 1. IgE and IgG anti-OVA production in high (BN) and low (WAG) IgE responder rats after OVA exposure. Groups of rats (n = 10) were immunized intraperitoneally with 10 mg OVA in 100 mg AH adjuvant (*a* and *d*) and bled days 9 and 21 after immunization. Further groups of animals (n = 10) were exposed to 1% OVA in saline by aerosol for 30 min daily for 10 d and bled on days 0, 3, 5, 9, and 12 during this period (*b* and *e*). Additional animals were exposed to 1% OVA by aerosol for 30 min, once weekly (*c* and *f*) for 6 wk and bled on a weekly basis as indicated. Anti-OVA IgE (□) and IgG (◇) titers were estimated by RAST and HA, respectively, and expressed as $\bar{X}$ ± SD. Parallel challenge of OVA-exposed animals with BSA in AH, revealed normal anti-BSA IgE responses (data not shown).

sponder WAG (Fig. 1 *d*). IgE production terminated completely by day 21 in the WAG, whereas the response in the BNs typically included a low level "persistent" component that continued for several weeks (data not shown). Corresponding IgG responses in the strains varied only over a twofold range.

Repeated exposure of BN rats to aerosolized OVA either daily (Fig. 1 *b*) or weekly (Fig. 1 *c*) also induced significant IgE Ab production. However, the peak responses were ~100-fold below those achieved by parenteral immunization, and terminated after a few weeks despite continued exposure. IgG responses in the aerosol-exposed BNs, in contrast, attained levels comparable with those observed with active immunization, and remained elevated. Aerosol exposure of low IgE responder WAG animals (Fig. 1, *e* and *f*) elicited smaller (barely detectable) IgE responses that were again transient, with accompanying variable low titers of IgG.

Parenteral Antigenic Challenge of Rats Preexposed to Antigen

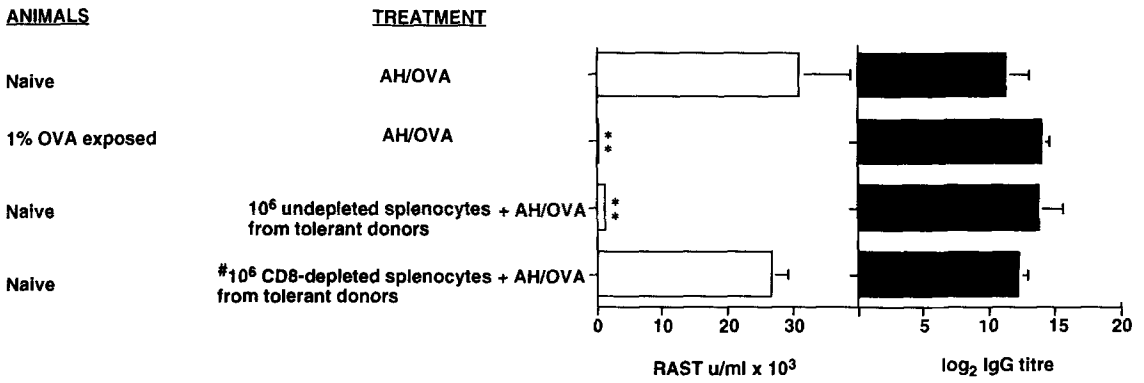

Figure 2. IgE and IgG anti-OVA response in aerosol-exposed BN rats after intraperitoneal challenge with OVA in AH adjuvant. Data shown are peak titers as $\bar{X}$ ± SD Rast U/ml (IgE) on day 9 and $\log_2$ IgG (as HA) on day 21 after challenge. (*) <controls by Student's t test, p <0.001. (#) Previous experiments indicate that depletion for CD3⁺ or CD5⁺ but not CD4⁺ cells abrogates adoptive transfer (9, 14).

via Aerosol. The experiments in Fig. 2 contrast the OVA-specific IgE and IgG responses of naive BN rats with those preexposed to aerosolized OVA once daily for 10 d. It can be seen that OVA-specific IgE production in response to parenteral challenge was markedly reduced in aerosol-exposed animals. Comparable results (data not shown) were obtained with the WAG strain. Additionally, adoptive transfer of unfractionated or CD8-enriched splenocytes from aerosol-exposed to naive animals, conferred a comparable state of hyporesponsiveness in the IgE Ab class, whereas depletion of T cells expressing CD8 or CD5 abrogated this suppression (14 and Fig. 2). In contrast, adoptive transfer of suppression was not affected by the removal of $CD4^+$ T cells from the donor inoculum (14).

In Vitro Activation and Cytokine Production by T Cells from Low and High IgE Responder Rats after In Vivo Antigen Challenge. Fig. 3 shows the kinetics of IL-4 and IFN-γ production after active immunization of low versus high IgE responder rats, and clearly illustrates the reciprocal role of these cytokines in IgE regulation. Thus, the low-titer 1° IgE response of WAG rats was associated with an early IL-2/IL-4 response in the spleen and lymph nodes draining the site of antigenic challenge, the IL-4 component being rapidly terminated with the onset of a wave of IFN-γ production. In contrast, BN rats demonstrated a sustained IL-2/IL-4 response, with no evidence of IFN-γ production during the first 14 d.

The experiments of Fig. 4 examine in vitro T cell cytokine responses after repeated inhalation of low levels of antigen. Transient IgE production in the BNs (Fig. 1) was associated with the presence of antigen-specific IL-4–secreting T cells (and low-level IL-2 activity) in the LNs draining the upper respiratory tract. In contrast, the low responder WAGs displayed little or no specific IgE Ab, and cytokine production by OVA-activated T cells from these animals was restricted to IL-2/IFN-γ.

After daily aerosol exposures for 1–2 wk followed by a 2-d "rest" period, the animals were subsequently reexposed to the aerosol once weekly for up to 6 mo (designated chronic exposure in Fig. 4). Parenteral rechallenge with AH/OVA at any time during this period failed to elicit an IgE response in either strain, as per Fig. 2. This state of selective "tolerance" in the IgE Ab isotype was associated with the presence of OVA-specific IFN-γ secreting and IL-2–secreting T cells, the former restricted to the CD8-enriched population and the latter represented in both CD4 and CD8 populations. Parenteral rechallenge of tolerant animals produced variable degrees of boosting of both CD8 IL-2 and IFN-γ activity in the low IgE responder strain.

A limited series of experiments also examined the capacity of splenic T cells from parenterally immunized and chronically aerosol-exposed BN rats to respond to in vitro challenge with OVA via proliferation and IL-2 release. Splenocytes from AH/OVA immunized rats taken during the early phase of the 1° IgE response proliferated vigorously and

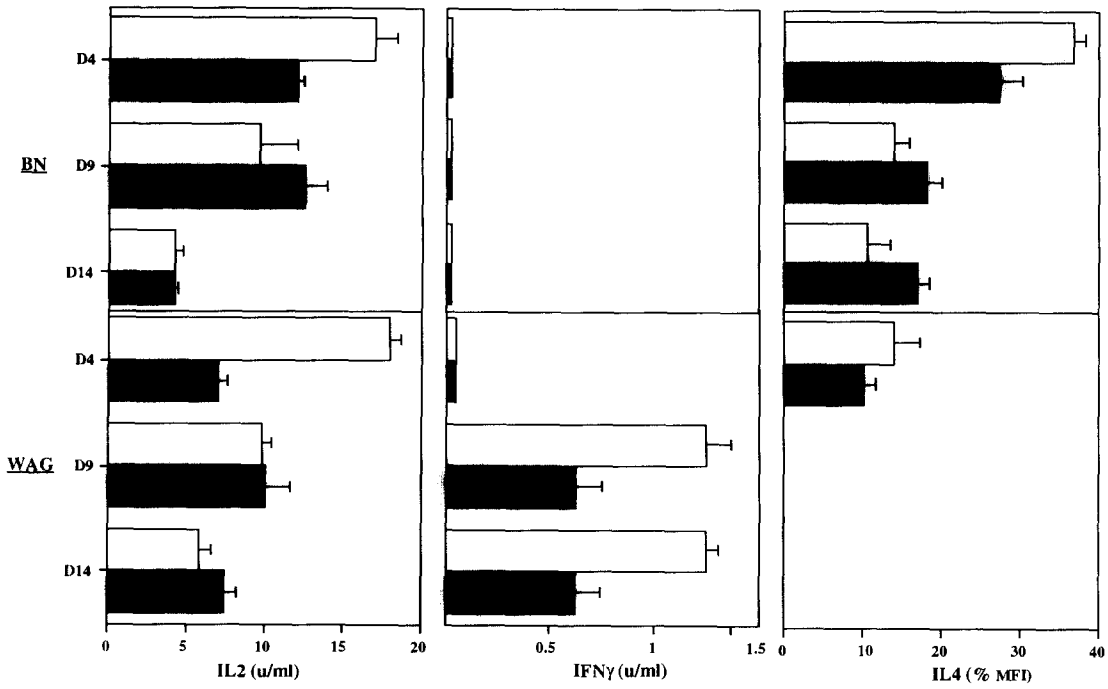

Figure 3. Cytokine production in high (*BN*) versus low (*WAG*) IgE responder animals after active immunization. Groups of rats (n = 5) were immunized intraperitoneally with AH/OVA and LN (□) and spleen (■) removed on days 4, 9, and 14 after immunization. Cells were cultured in the presence of OVA (100 μg/ml) for 24 h, and supernatants were harvested and assayed for IL-2, IL-4, and IFN-γ. Results are expressed as $\bar{X} \pm$ SD for triplicate measurements from a representative experiment (n = 3). Supernatants from control cultures (cells plus medium alone or the irrelevant Ag BSA) were negative. Subsequent examination of 48-h supernatants (data not shown) did not alter interpretation of these results.

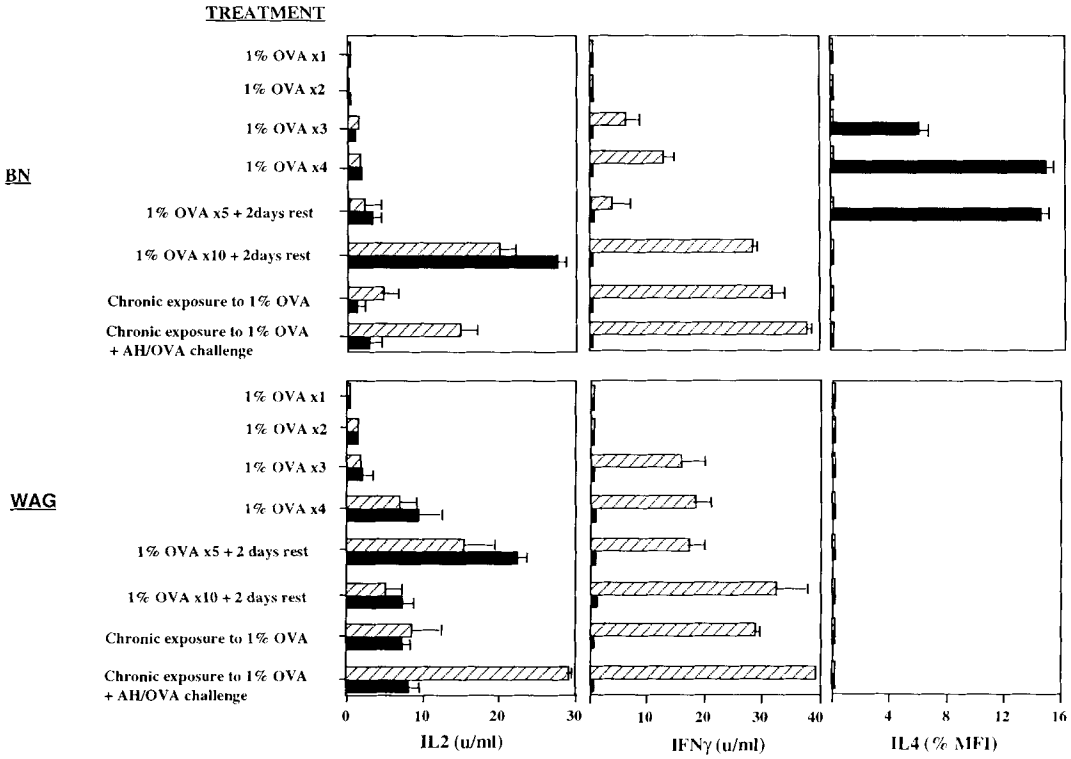

Figure 4. Cytokine production in high (*BN*) versus low (*WAG*) IgE responder animals after exposure to OVA aerosols. Groups of rats (n = 5) were exposed daily to 1% OVA via inhalation for varying periods up to 10 d. RTLN (1% OVA 1–4×) and pools of RTLN and spleen (other exposure periods) were removed 24 h after exposure indicated and sorted into CD4⁻ (CD8 enriched) (▨) and CD8⁻ (CD4 enriched) (■) populations. All separated preparations contained ≤2% negatively selected cells. The cells were cultured and assayed for cytokines as per Fig. 3. Results are expressed as $\bar{X}$ ± SD (triplicate measurements) from a representative experiment (n = 3).

released high levels of IL-2 after exposure to OVA (Table 1). OVA-specific responsiveness (albeit at a lower level) was also consistently seen in CD8⁻ splenocytes from aerosol-exposed rats, in particular after rechallenge of donor animals with AH/OVA, consistent with the presence of autonomous OVA-responsive CD4⁺ T cells. However, both CD4⁻ and undepleted splenocytes from the same animals were unresponsive to antigen, suggesting inhibition of CD4⁺ T cell proliferation and IL-2 release via a subset of the OVA-immune CD8⁺ T cell population.

Table 1. *Inhibition of OVA-specific CD4⁺ T Cell Proliferation and IL-2 Secretion In Vitro by OVA-specific CD8⁺ T Cells*

Animals	Cell population	Proliferation (^{3}H-DNA synthesis)	IL-2 (U/ml)
Normal	Undepleted	167,000 ± 6,379	12.0 ± 1.5
plus AH/OVA	CD4⁻	ND	ND
challenge	CD8⁻	ND	ND
Chronic aerosol	Undepleted	873 ± 44	0.2 ± 0
exposure	*CD4⁻	41 ± 1	4.8 ± 0.3
(tolerant)	*CD8⁻	16,916 ± 807	1.25 ± 0.1
Chronic aerosol	Undepleted	4,121 ± 69	1.2 ± 0.1
exposure	*CD4⁻	543 ± 32	7.0 ± 0.9
plus AH/OVA challenge	*CD8⁻	33,606 ± 913	2.9 ± 0.6

Data is expressed as $\bar{X}$ ± SD.
* ≤2% contaminating CD4⁺ or CD8⁺ cells.

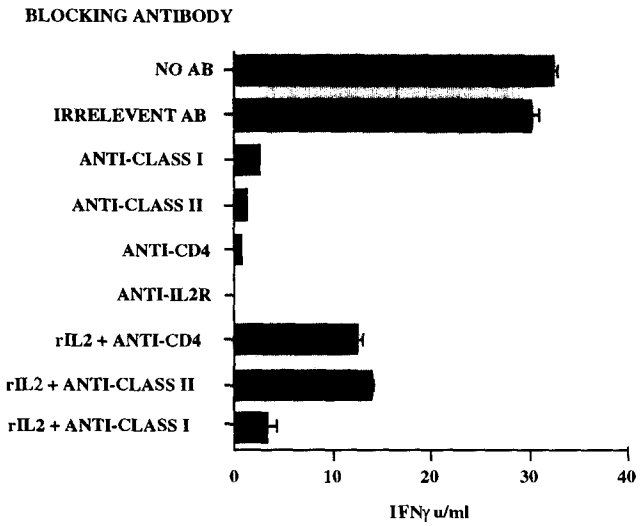

Figure 5. Blocking of antigen-specific IFN-γ production by CD4⁻ splenocyte using mAbs against function-associated surface molecules. Splenocytes from groups of BN rats (n = 3) chronically exposed to 1% OVA-aerosol were sorted to yield a CD4⁻ population (CD4⁺ contamination <1%), and stimulated in vitro with OVA as per Fig. 3. mAbs (as indicated) were added at 5 μg/ml to the culture. Supernatants were assayed for IFN-γ as per Fig. 3. Supernatants from control cultures of cells with medium alone (no Ag), IL-2 alone (no Ag), or irrelevant Ag (BSA) were all negative. Results are expressed as $\bar{X}$ ± SD (triplicate values) from a representative experiment in a series of four.

Interactions between MHC Class I- and II-restricted T Cells during the Response to OVA Antigen. In Fig. 5, in vitro activation of CD8-enriched splenocytes (containing <1% CD4⁺ T cells) from aerosol-exposed OVA-tolerant BN rats was attempted in the presence of a range of mAbs directed against T cell function-associated surface molecules, using established protocols (20). T cell activation was assessed via measurement of IFN-γ secretion into culture supernatants in the presence of 100 μg/ml OVA. IFN-γ secretion was inhibited by mouse mAb against MHC class I and II, CD4, and the high affinity IL-2R, but not by a control mAb (OX21). The blocking effects of anti-CD4 and anti-MHC class II (but not anti-MHC class I) mAb were significantly reversed by the addition of 10 U/ml rIL-2 to the cultures.

Activation of OVA-specific CD8⁺ T Cells in Adoptive Recipients. As noted in Fig. 2, adoptive transfer of splenocytes from aerosol-exposed tolerant rats into syngeneic recipients confers a state of OVA-specific hyporesponsiveness analogous to that of the original donors, as evidenced by their failure to develop IgE responses after parenteral challenge with AH/OVA. It can be seen from Fig. 6 that in vitro stimulation of T cells (in particular CD8 enriched) taken from animals 2 d after adoptive transfer and in vivo challenge, reveals high levels of IL-2 and IFN-γ secretion.

Variable Representation of CD8⁺ OVA-specific T cells in the Peripheral Blood of Low and High IgE Responder Rats after Tolerance Induction. The experiments in Fig. 7 examine the OVA reactivity of PBL from rats, after different forms of in vivo challenge. In the low IgE responder WAGs, the peripheral blood clearly contained a significant population of OVA-specific IFN-γ producing CD8⁺ T cells, both in tolerant (aerosol exposed) animals and during the early stages of the primary immune response in naive animals (Fig. 7, *d–f*). Tolerized BN rats in contrast demonstrated no detectable IFN-γ–producing T cells, even after tolerance induction and subsequent parenteral rechallenge (Fig. 7, *a* and *b*), but during the primary IgE response (Fig. 7 *c*) they demonstrated high levels of antigen-specific IL-4 reactivity in peripheral blood which was attributable to CD4⁺ T cells.

Induction of Antigen-specific IFN-γ–producing T Cells by OVA Feeding. Stimulation of the gastrointestinal tract via antigen feeding induces a form of tolerance (known as oral tolerance) equivalent to that achieved by inhalation of much lower levels of aerosolized antigen. The experiments of Fig. 8 employed a standard protocol for oral tolerance induction in the rat, 100 mg feeds of OVA administered by gavage on two consecutive days, and the tolerant status of a parallel group of fed animals was confirmed by parenteral challenge (data not shown). Culture supernatants from OVA-stimulated CD4- and CD8-enriched T cells from the MLN of these animals, prepared 4 d after the last feed, demonstrated high levels of IFN-γ activity.

Discussion

The state of antigen-specific hyporesponsiveness, which develops in experimental animals after exposure to inhaled protein antigens, was originally described as a form of immuno-

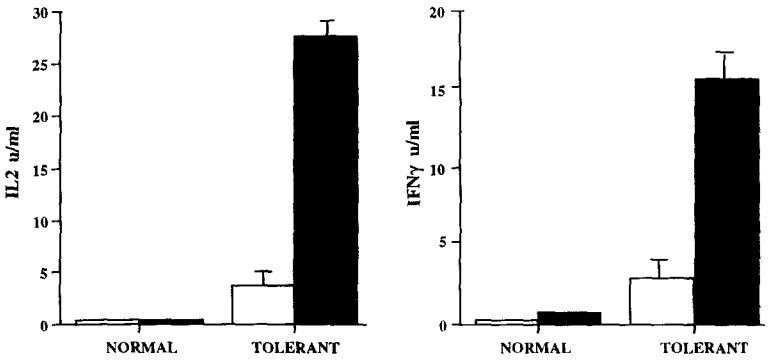

Figure 6. Cytokine production in T cells from adoptive recipients, after AH/OVA immunization. CD8⁺ splenocytes (98.8% pure) prepared from aerosol-exposed tolerant (BN) rats by positive selection employing the OX8 mAb, were transferred into naive recipients (10^6 cells/animal, n = 5), concomitant with AH/OVA challenge. Splenocytes were removed from the recipients 2 d after transfer and immunization, separated into CD8-enriched (■) and CD4-enriched (□) subsets as per Fig. 4 and stimulated with OVA as per Fig. 3. Supernatants were harvested and assayed for cytokines as per Fig. 3. Results are expressed as $\bar{X}$ ± SD from a representative experiment. The tolerant status of a parallel group of adoptive recipients was confirmed via AH/OVA challenge and assessment of serum IgE Ab, as per Fig. 2. Comparable data were also obtained employing CD4⁻ splenocytes prepared by negative selection for CD8⁺ (data not shown).

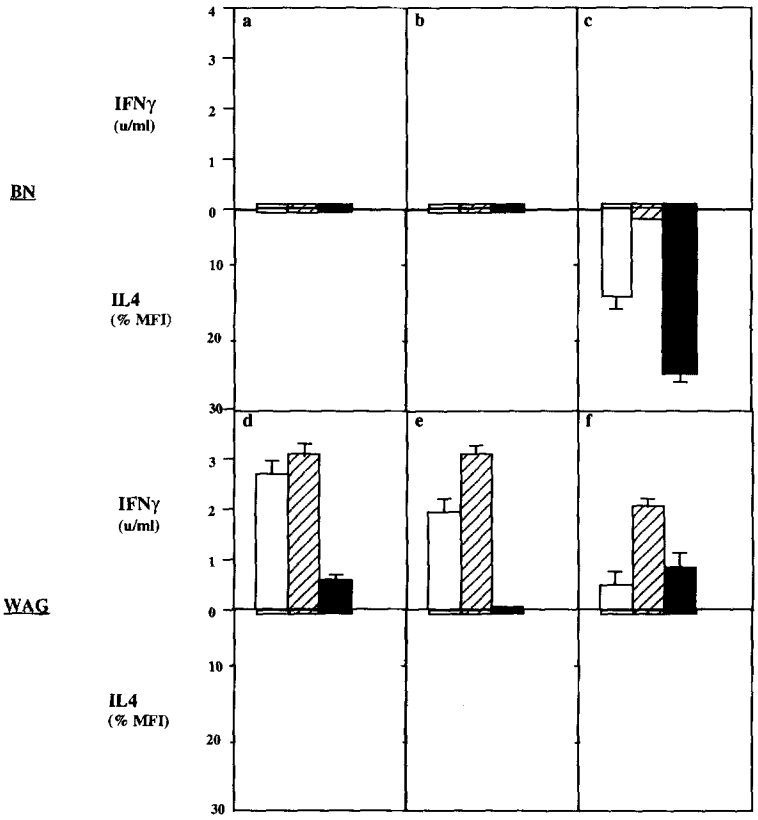

Figure 7. Cytokine production by PBL from high (*BN*) and low (*WAG*) IgE responder rats after OVA exposure. PBL were prepared from group of rats (n = 6) tolerant to OVA after chronic aerosol exposure (*a* and *d*), tolerant and challenged with AH/OVA (day 4 after challenge; *b* and *e*), and AH/OVA-challenged naive animals (*c* and *f*). Undepleted (□), CD8 enriched (▨), and CD4-enriched (■) cells were prepared as per Fig. 4 and stimulated in vitro by OVA, and 24-h supernatants were harvested and assayed for cytokines as per Fig. 3. Results are $\bar{X}$ ± SD of triplicate samples from a representative experiment.

logical tolerance (21, 22), by analogy with the process of oral tolerance to fed antigens (23). Sensitivity to tolerogenesis via the respiratory tract was shown to be genetically determined (24, 25), and was variably selective for the IgE Ab isotype, the degree of selectivity also being related to genetic background. Thus, acquired IgE hyporesponsiveness to inhaled antigen was usually accompanied by suppression of corresponding delayed type hypersensitivity (DTH) reactivity (26), but apparently tolerized animals in several rat and mouse strains manifested residual IgG and secretory IgA reactivity as well as antigen-responsive T cells in primary lymphoid organs (6, 7). This tolerant state is accordingly more accurately described as a form of immune deviation.

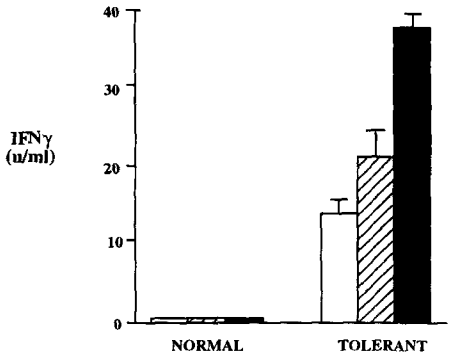

Figure 8. Cytokine production by MLN cells in normal versus OVA-fed WAG rats. Undepleted (□), CD8-enriched (▨), and CD4-enriched (■) cells were prepared from groups of three normal or OVA-fed (*tolerant*) animals, and cultured with OVA and assayed for IFN-γ as per Fig. 3. Results are expressed as $\bar{X}$ ± SD of triplicate samples from a representative experiment.

The present study sought to define the differences between the T cell responses of phenotypically high and low IgE responder rats to inhaled OVA antigen, employing two strains that demonstrate 30-fold variations in the relative magnitude of their 1° IgE responses to parenteral antigenic challenge (Fig. 1, *a* and *d*). Consistent with recent data from other species (1, 2), these differences in IgE responder phenotype are reflected by the relative intensity of IFN-γ versus IL-4 production by OVA-specific T cells (Fig. 3).

The relative magnitude of the IgE response to parenteral immunization is mirrored by the response to antigen inhalation. Thus, repeated exposure to aerosolized OVA resulted in transient IgE Ab production in the high IgE responder BNs, suggestive of an initial $CD4^+$ Th2 response (Fig. 1, *b* and *c*), and OVA-specific IL-4–secreting $CD8^-$ T cells were concomitantly observed in the RTLN during this period (Fig. 4). A corresponding IgE response did not develop in the low IgE responder WAGs (Fig. 1, *e* and *f*), but they instead demonstrated a vigorous IFN-γ response (Fig. 4) analogous to their pattern of cytokine production after parenteral immunization.

The tolerant state that developed after aerosol exposure, selective for IgE in the BNs (Fig. 2), but also encompassing IgG in WAGs (24), was associated with the presence of OVA-specific IL-2 secreting and IFN-γ–secreting T cells in lymphoid organs (Fig. 4). IL-2–secreting activity was observed

in both the $CD4^+$ and $CD8^+$ compartments, but IFN-γ activity was restricted to the enriched $CD8^+$ T cell population. Previous experiments have shown that tolerance in this model can be adoptively transferred with $CD8^+$ T cells (9, 14), as illustrated also in the data in Fig. 2. Successful adoptive transfer of tolerance was also associated with the development of this pattern of cytokine reactivity in T cells of adoptive recipients (Fig. 6), but in this case, IL-2 production was more marked in the $CD8^+$ population. Additionally, low but significant levels of IFN-γ–secreting activity were observed in $CD4^+$ T cells of the adoptive recipients after parenteral challenge (Fig. 6), suggesting a growing contribution from the $CD4^+$ Th1 T cell compartment to the overall IFN-γ response, driven by restimulation with antigen.

The experiments in Fig. 5 address the important issue of MHC restriction of the regulatory T cell response in this model, and highlight the role of exogenous IL-2 in initiation of $CD8^+$ T cell activation. MHC class II–restricted $CD8^+$ T cells have recently been implicated in other systems (27), including those that are specific for OVA antigen (28). However, the profound inhibition of OVA-induced IFN-γ secretion by CD8-enriched splenocytes from the aerosol-exposed animals by anti-class I mAb (Fig. 5) suggests the effector cells here are classical MHC class I–restricted $CD8^+$ T cells. Notwithstanding this result, the finding of equivalent levels of blocking of antigen-induced IFN-γ secretion by mAbs against MHC class II, CD4, and high-affinity IL-2R imply that additional signals are required for activation of these $CD8^+$ cells.

As noted in the figure legends, these CD8-enriched cell preparations were contaminated with up to 2% $CD4^+$ T cells, and the inhibitory activity of anti-CD4 and anti-class II mAbs, together with the demonstration of reversal of this inhibition with rIL-2, suggest an obligatory contribution from OVA-responsive IL-2–secreting $CD4^+$ T cells in the activation of the $CD8^+$ population. This is consistent with recent reports on the dependence of some MHC class I–restricted $CD8^+$ T cells on IL-2 from $CD4^+$ T cells during primary activation (29, 30), in particular under conditions of suboptimal stimulation (29). This appears to be particularly the case in rats, where $CD4^+$ contamination levels as low as 1% appear sufficient to provide the permissive IL-2 signal required during the initial phase of in vitro $CD8^+$ activation (30). However, once the OVA-specific $CD8^+$ T cell response has been initiated and appropriately boosted in this system, autocrine production of IL-2 by the $CD8^+$ population, evident in the data of both Figs. 4 and 6, obviates the requirement for the CD4 T cells.

We have not been able to demonstrate OVA-induced in vitro proliferation of $CD8^+$ T cells in this system, including with the chronically exposed animals illustrated in Fig. 4. Moreover, as shown in Table 1, chronically exposed animals contained OVA-specific T cells that were capable of proliferation and IL-2 release, but this capacity was inhibited in the presence of $CD8^+$ T cells. The same $CD8^+$ T cells were clearly capable of IL-2 release themselves, so it is unlikely that the lack of IL-2 per se was the factor limiting $CD4^+$ T cell proliferation. The underlying mechanism(s) are currently under separate investigation in our laboratory.

Implicit in our interpretation of the data presented above is the suggestion that MHC class I–restricted $CD8^+$ T cells can be primed and subsequently reactivated, in response to low levels of inhaled soluble OVA antigen. Until recently, it was believed that the exclusion of soluble antigens from the MHC class I pathway of antigen presentation was absolute. However, it is now clear that the cytoplasm of APCs can be "loaded" in vitro with soluble protein antigens by pulsing in either hypertonic or isotonic medium, effectively charging surface class I molecules with sufficient processed antigen for $CD8^+$ T cell priming (31–34).

The present model argues this process can also occur under normal conditions in vivo, in response to low levels of soluble antigen impacting upon the respiratory mucosa, and earlier dose–response data from this model (24, 25) imply that in certain strains of mice and rats the threshold levels of inhaled antigen required for initiating such $CD8^+$ responses is in the nanogram zone. An additional precedent for this thesis are recent reports indicating in vivo activation of antigen-specific $CD8^+$ T cells that mediate adoptive transfer of oral tolerance, via feeding soluble OVA (35). It is pertinent to note that the rats fed with a tolerogenic dose of OVA in the present experiments also demonstrated IFN-γ–secreting OVA-specific T cells in the LNs draining the gastrointestinal tract (GIT) (Fig. 8). In this situation, the concentration of antigen available in the microenvironment of the gastric mucosal APCs would also be extremely low, particularly in the experiments described by Miller et al. (35) where oral tolerance mediated by $CD8^+$ T cells was induced by repeated feeds of only 1.0 mg OVA.

In relation to the respiratory tract, we have recently identified dendritic cells as the principal resident professional APC population, where they form a network throughout the alveolar septa and the airway epithelium (36, 37). We have further demonstrated that OVA inhalation effectively charges their MHC class II molecules with sufficient OVA peptide to activate $CD4^+$ T cells (38–40). We do not at present have data on the capacity of the airway intraepithelial dendritic cells to present inhaled OVA antigen via the class I pathway. However, the demonstration of the unique potency of dendritic cells as APCs for MHC class I–restricted $CD8^+$ T cells in other systems (29, 41) suggests they are likely candidates for this role. The situation in the GIT may be more complex as a wider range of potential APCs appears available for T cell priming, which may account for the differences in the relative contribution of $CD4^+$ T cells to the overall T cell–mediated IFN-γ response in OVA-fed versus aerosol-exposed animals (cf. Figs. 4 and 8).

The data presented above thus suggests a novel control mechanism for protection against the development of $CD4^+$ Th2-mediated allergic reactivity to inhaled nonreplicating antigens, i.e., deviation of the host response away from the Th2 phenotype via the creation of a cytokine milieu rich in $CD8^+$ T cell–derived IFN-γ, which selects against the growth of Th2 clones during the early phase of the immune

response (42, 43). Whereas such a mechanism potentially explains overall suppression of IgE responses to inhaled antigens in these two strains, there are a series of key questions which remain unanswered, in particular concerning the basis for the genetically determined differences in threshold inhaled antigen dosage required to induce this form of immune deviation (24, 25). Additionally, the fact that DTH reactivity is suppressed along with IgE responsiveness in the WAG strain but not in the BN (26), suggests that cytokines other than IFN-γ may also play a role in the process. It is of interest to note in this regard that blocking Abs against both IFN-γ (44) and TGF-β (45) have independently been shown to abrogate oral tolerance.

The degree to which this form of acquired immune deviation modulates the human immune response to airborne environmental antigens, remains to be established. In approaching the question, it is important to note that the key element of this animal model of acquired tolerance, is its applicability to only the initial stages of the immune response in the naive host, i.e., it has not been possible to deviate ongoing IgE responses in animals with an established $CD4^+$ T-memory population.

In this context, it is now generally conceded that primary T cell sensitization to environmental antigens in humans most commonly occurs in early infancy, when the majority of such antigens are first encountered (46). Transient low-level IgE serum Ab production to both inhalant and food antigens, analogous to those described in the model above, are now recognized to occur in normal nonatopic children (46), suggesting the initial development of $CD4^+$ Th2-like responses to these antigens which (with continued exposure) are eventually suppressed. However, the published data on human T cell reactivity to environmental antigens relates exclusively to PBL from chronically stimulated adults, where the only detectable antigen-specific T cells are reportedly $CD4^+$ Th2 (in atopics) and $CD4^+$ Th1 (in nonatopic normals). The latter T cell populations would thus have undergone repeated stimulation and selection over a period of years, which may obscure more subtle mechanism(s) operative during priming.

In particular, antigen-specific IFN-γ-secreting $CD8^+$ T cells may create a milieu that favors the slow outgrowth of IFN-γ–secreting $CD4^+$ Th1 T cells which, by virtue of their greater inherent capacity for proliferation, eventually supplant the $CD8^+$ population. This possibility is consistent with the animal data of Fig. 6, demonstrating the de novo appearance of antigen-specific IFN-γ–secreting $CD4^+$ T cells in this system only after adoptive transfer and restimulation in the recipients. It is also likely that PBL provide an imperfect window on the human $CD8^+$ T cell response. The recirculation properties of $CD8^+$ T-memory cells are poorly understood, and it is evident from the data in Fig. 7 that the degree of representation of antigen-specific $CD8^+$ T cells in PBL varies markedly between animal strains. Primary lymphoid organs (such as tonsils) may provide more appropriate T cell populations for similar studies in humans, and this approach is currently being followed in our laboratory.

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References

1. Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145.
2. Finkelman, F.D., J. Holmes, I.M. Katona, J.F. Urban, Jr., M.P. Beckmann, L.S. Park, K.A. Schooley, R.L. Coffman, T.R. Mosmann, and W.E. Paul. 1990. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8:303.
3. Wierenga, E.A., M. Snoek, C. De Grast, I. Chretien, J.D. Bos, H.M. Jansen, and M.L. Kapsenberg. 1990. Evidence for compartmentalization of functional subsets of $CD4^+$ T-lymphocytes in atopic patients. *J. Immunol.* 144:4651.
4. Parronchi, P., D. Macchia, M.-P. Biswas, C. Simonelli, E. Maggi, M. Ricci, A.A. Ansari, and S. Romagnani. 1991. Allergen- and bacterial-antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. *Proc. Natl. Acad. Sci. USA.* 88:4538.
5. Robinson, D.S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A.M. Bentley, C. Corrigan, S.R. Durham, and A.B. Kay. 1992. Predominant T_{H2}-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326:298.
6. Holt, P.G., and J.D. Sedgwick. 1987. Suppression of IgE responses following antigen inhalation: a natural homeostatic mechanism which limits sensitization to aeroallergens. *Immunol. Today.* 8:14.
7. Holt, P.G., and C. McMenamin. 1989. Defence against allergic

sensitization in the healthy lung: the role of inhalation tolerance. *Clin. Exp. Allergy.* 19:255.
8. Sedgwick, J.D., and P.G. Holt. 1983. Induction of IgE-isotype specific tolerance by passive antigenic stimulation of the respiratory mucosa. *Immunology.* 50:625.
9. McMenamin, C., J. Oliver, B. Girn, B.J. Holt, U.R. Kees, W.R. Thomas, and P.G. Holt. 1991. Regulation of T-cell sensitisation at epithelial surfaces in the respiratory tract: suppression of IgE responses to inhaled antigens by $CD3^+$ TcR α^-/β^- lymphocytes. *Immunology.* 74:234.
10. Mason, D.W., R.P. Arthur, M.J. Dallman, J.R. Green, G.P. Spickett, and M.L. Thomas. 1983. Functions of rat T-lymphocyte subsets isolated by means of monoclonal antibodies. *Immunol. Rev.* 74:57.
11. Hsiung, L.M., A.N. Barclay, M.R. Brandon, E. Sim, and R.R. Porter. 1982. Purification of human C3b inactivator by monoclonal-antibody affinity chromatography. *Biochem. J.* 203:293.
12. Tellides, G., M.J. Dallman, and P.J. Morris. 1989. Mechanism of action of interleukin-2 receptor (IL-2R) monoclonal antibody (MAb) therapy: target cell depletion or inhibition of function? *Transplant. Proc.* 21:997.
13. Nicolls, M.R., G.A. Aversa, N.W. Pearce, A. Spinelli, M.F. Berger, K.E. Gurley, and B.M. Hall. 1993. Induction of long-term specific tolerance to allografts *in vivo* by therapy with a monoclonal antibody to rat CD3. *Transplantation (Baltimore).* 55:459.
14. Sedgwick, J.D., and P.G. Holt. 1985. Induction of IgE-secreting cells and IgE-isotype-specific suppressor T cells in respiratory tract lymph nodes of rats exposed to an antigen aerosol. *Cell. Immunol.* 94:182.
15. Mason, D.W., W.J. Penhale, and J.D. Sedgwick. 1987. Preparation of lymphocyte subpopulations. *In* Lymphocytes: A Practical Approach. G.G.B. Klaus, editor. IRL Press, Oxford. 35–54.
16. Van der Meide, P., T.H. Borman, J.A. Wubben, C.A. Botman, K. Vijvergberg, and H. Schellekens. 1990. A sensitive two site enzyme immunoassay for the detection of rat interferon gamma in biological fluids. *J. Interferon. Res.* 10:183.
17. McKnight, A.J., and B.J. Classon. 1992. Biochemical and immunological properties of rat recombinant interleukin-2 and interleukin-4. *Immunology.* 75:286.
18. Go, N.F., B.E. Castle, R. Barrett, R. Kastelein, W. Dang, T.R. Mosmann, K.W. Moore, and M. Howard. 1990. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J. Exp. Med.* 172:1625.
19. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann. 1989. Two types of mouse T helper cell IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170:2081.
20. Sedgwick, J.D., R. Mossner, S. Schwender, and V. ter Meulen. 1991. Major histocompatibility complex-expressing nonhematopoietic astroglial cells prime only $CD8^+$ T lymphocytes: astroglial cells as perpetuators but not initiators of $CD4^+$ T cell responses in the central nervous system. *J. Exp. Med.* 173:1235.
21. Holt, P.G., J.E. Batty, and K.J. Turner. 1981. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology.* 42:409.
22. Holt, P.G., and S. Leivers. 1982. Tolerance induction via antigen inhalation: isotype specificity, stability and involvement of suppressor T-cells. *Int. Arch. Allergy. Appl. Immunol.* 67:155.
23. Tomasi, T.B.J. 1980. Oral tolerance. *Transplantation (Baltimore).* 29:352.
24. Sedgwick, J.D., and P.G. Holt. 1984. Suppression of IgE responses in inbred rats by repeated respiratory tract exposure to antigen: responder phenotype influences isotype specificity in induced tolerance. *Eur. J. Immunol.* 14:893.
25. Holt, P.G., D. Britten, and J.D. Sedgwick. 1987. Suppression of IgE responses by antigen inhalation: studies on the role of genetic and environmental factors. *Immunology.* 60:97.
26. Sedgwick, J.D., and P.G. Holt. 1985. Downregulation of immune responses to inhaled antigen: studies on the mechanism of induced suppression. *Immunology.* 56:635.
27. Salgame, P., R. Modlin, and B.R. Bloom. 1989. On the mechanism of human T cell suppression. *Int. Immunol.* 1:121.
28. Shinohara, N., M. Watanabe, D.H. Sachs, and N. Hozumi. 1988. Killing of antigen-reactive B cells by class II–restricted, soluble antigen-specific $CD8^+$ cytolytic T lymphocytes. *Nature (Lond.).* 336:481.
29. Boog, C.J.P., J. Boes, and C.J.M. Melief. 1988. Role of dendritic cells in the regulation of class I restricted cytotoxic T lymphocyte responses. *J. Immunol.* 140:3331.
30. Salomon, D.R., D.J. Cohen, J.M. Williams, and C.B. Carpenter. 1984. T cell synergy in the primary MLR: proliferative kinetics, effector cell generation, and IL2 production. *J. Immunol.* 133:3075.
31. Moore, M.W., F.R. Carbone, and M.J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell.* 54:777.
32. Carbone, F.R., and M.J. Bevan. 1990. Class I–restricted processing and presentation of exogenous cell-associated antigen in vivo. *J. Exp. Med.* 171:377.
33. Rock, K.L., S. Gamble, and L. Rothstein. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science (Wash. DC).* 249:918.
34. Pfeifer, J.D., M.J. Wick, R.L. Roberts, K. Findlay, S.J. Normark, and C.V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature (Lond.).* 361:359.
35. Miller, A., O. Lider, and H.L. Weiner. 1991. Antigen-driven bystander suppression after oral administration of antigens. *J. Exp. Med.* 174:791.
36. Holt, P.G., M.A. Schon-Hegrad, J. Oliver, B.J. Holt, and P.G. McMenamin. 1990. A contiguous network of dendritic antigen presenting cells within the respiratory epithelium. *Int. Arch. Allergy Appl. Immunol.* 91:155.
37. Schon-Hegrad, M.A., J. Oliver, P.G. McMenamin, and P.G. Holt. 1991. Studies on the density, distribution, and surface phenotype of intraepithelial class II MHC antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J. Exp. Med.* 173:1345.
38. Holt, P.G., J. Oliver, C. McMenamin, and M.A. Schon-Hegrad. 1992. Studies on the surface phenotype and functions of dendritic cells in parenchymal lung tissue of the rat. *Immunology.* 75:582.
39. Holt, P.G., J. Oliver, C. McMenamin, N. Bilyk, G. Kraal, and T. Thepen. 1993. The antigen presentation functions of lung dendritic cells are downmodulated in situ by soluble mediators from pulmonary alveolar macrophages. *J. Exp. Med.* 177:397.
40. Holt, P.G., M.A. Schon-Hegrad, and J. Oliver. 1988. MHC class II antigen-bearing dendritic cells in pulmonary tissues of rat: regulation of antigen presentation activity by endogenous macrophage populations. *J. Exp. Med.* 167:262.
41. Nonacs, R., C. Humborg, J.P. Tam, and R.M. Steinman. 1992. Mechanisms of mouse spleen dendritic cell function in the

generation of influenza-specific, cytolytic T lymphocytes. *J. Exp. Med.* 176:519.

42. Fernandez-Botran, R., V.M. Sanders, T.R. Mosmann, J.W. Uhr, and E.S. Vitetta. 1988. Lymphokine-mediated regulation of the proliferative response of clones of TH1 and TH2 cells. *J. Exp. Med.* 168:543.
43. Gajewski, T.F., and F.W. Fitch. 1988. Anti-proliferative effect of IFNγ in immune regulation. I. IFNγ inhibits the proliferation of TH2 but not TH1 murine HTL clones. *J. Immunol.* 140:4245.
44. Zhang, Z., and J.G. Michael. 1990. Orally inducible immune unresponsiveness is abrogated by IFNγ treatment. *J. Immunol.* 144:4163.
45. Miller, A., O. Lider, A.B. Roberts, M.B. Sporn, and H.L. Weiner. 1992. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of TGF-β following antigenic specific triggering. *Proc. Natl. Acad. Sci. USA.* 89:421.
46. Holt, P.G., C. McMenamin, and D. Nelson. 1990. Primary sensitization to inhalant allergens during infancy. *Pediatr. Allergy Immunol.* 1:3.