

## LABORATORY SCIENCE

# Exertion of the suppressive effects of IFN- $\gamma$ on experimental immune mediated blepharoconjunctivitis in Brown Norway rats during the induction phase but not the effector phase

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**Background/aims:** Interferon gamma (IFN- $\gamma$ ) knockout mice exhibit severe allergic conjunctivitis (AC), indicating that IFN- $\gamma$  regulates the development of AC. The authors examined whether this inhibitory effect of IFN- $\gamma$  is exerted during the induction or effector phase of experimental AC.

**Methods:** Experimental immune mediated blepharoconjunctivitis (EC) was induced in Brown Norway (BN) rats, using ovalbumin (OVA) as the antigen. To investigate the role of IFN- $\gamma$  in the induction phase, EC was induced by active immunisation and IFN- $\gamma$  (10  $\mu$ g/time, total 70  $\mu$ g), or phosphate buffered saline (PBS) as a control, was injected intraperitoneally every other day from the day of immunisation. The rats were challenged with OVA eye drops 13 days after immunisation, and 24 hours later, the eyes were harvested for histology. To examine the effects of IFN- $\gamma$  in the effector phase, OVA specific T cells were transferred into syngeneic rats and IFN- $\gamma$  (10  $\mu$ g/time, total 50  $\mu$ g) or PBS was injected each day after the transfer until induction of EC 4 days later with an OVA challenge. To investigate the role of endogenous IFN- $\gamma$  during the effector phase, an anti-IFN- $\gamma$  monoclonal antibody (3 mg/time) was injected on days 3 and 4.

**Results:** Injection of IFN- $\gamma$  into actively immunised rats suppressed eosinophilic infiltration but not infiltration of mononuclear cells. In contrast, neither IFN- $\gamma$  nor anti-IFN- $\gamma$  affected EC in passively immunised rats.

**Conclusion:** IFN- $\gamma$  is a suppressive cytokine for the development of EC and exerts this suppressive effect during the induction phase.

Patients with severe cases of allergic conjunctivitis (AC) exhibit both eosinophilic and lymphocytic infiltration into the conjunctiva.<sup>1,2</sup> To understand the mechanism that leads to the development of severe AC, we established a model of AC in Brown Norway (BN) rats termed experimental immune mediated blepharoconjunctivitis (EC).<sup>3,4</sup> A series of experiments realised within the context of this model identified an important role for T helper 2 (Th2) immunity in the development of EC.<sup>3-5</sup> As clearly demonstrated in many other experimental models, Th1 cells counteract Th2 cells,<sup>6,7</sup> so that the development of diseases can be inhibited by augmenting Th1 immunity.<sup>8,9</sup> Our previous report demonstrated that IL-12, a Th1 differentiating cytokine, was able to suppress Th2 function in vitro.<sup>10</sup> However, systemic injection of IL-12 during the induction phase of EC augmented lymphocytic infiltration,<sup>10</sup> suggesting that the Th1/Th2 balance in vivo is unlikely to be modulated by the injection of a single cytokine.

Using cytokine knockout mice, it was demonstrated that interferon gamma (IFN- $\gamma$ ) is an inhibitory cytokine for the development of AC.<sup>11</sup> Whether this inhibitory effect is exerted during the induction phase or effector phase remains unclear, because the assessment was performed using only active immunisation systems. To understand the inhibitory mechanism of IFN- $\gamma$ , we used both active and passive immunisation systems<sup>12</sup> to study the roles of IFN- $\gamma$  in the induction and effector phases, respectively. Here we demonstrate that this inhibitory effect was exerted in the induction phase, but not in the effector phase.

## MATERIALS AND METHODS

### Rats

Eight to 12 week old male BN rats (Clea Japan, Tokyo, Japan) were maintained in a pathogen-free animal facility at Kochi

Medical School. All animal procedures conformed to the ARVO resolution on use of animals in research.

### Reagents

Recombinant rat IFN- $\gamma$  was purchased from Peprotech EC Ltd, UK. An anti-IFN- $\gamma$  monoclonal antibody<sup>13</sup> was purchased from U-CyTech by, Utrecht, Netherlands. Normal mouse IgG was obtained from sera of Balb/c mice by precipitation at 40% saturation with ammonium sulphate, then purified by protein G-Sepharose 4B fast flow chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). Monoclonal antibodies (Abs) used here for staining were specific for rat cells. Anti-RT1B (OX-6) and anti-RT1D (OX-17) were labelled with FITC (Pharmingen, San Diego, CA, USA). Anti-CD3 (G4.18, Pharmingen), anti-CD4 (OX-38), anti-CD8 (OX-8), anti-CD25 (OX-39, Pharmingen), and anti-CD45RA (OX-33, Seortec, Oxford, UK) were labelled with biotin. Anti-CD11b (X42, Antigenix America, NY) was PE labelled. Streptavidin-PE was purchased from Pharmingen.

### Active immunisation and treatment protocol

BN rats received a subcutaneous injection in the left hind footpad of 100  $\mu$ l containing 100  $\mu$ g of ovalbumin (OVA; grade V, Sigma Immunochemicals, St Louis, MO, USA) emulsified with CFA (Yatron, Tokyo, Japan). The day of immunisation was designated day 0. The rats were then assigned to either an IFN- $\gamma$  treated or a phosphate buffered saline (PBS) treated group. IFN- $\gamma$  (0.1, 1, or 10  $\mu$ g per injection) and PBS treatments were given by intraperitoneal injection from day 0 to day 13, every other day. On day 13, all rats were challenged with OVA eye drops (250  $\mu$ g in 50  $\mu$ l of PBS). Twenty four hours after challenge, they were killed and their eyes, blood, and lymph nodes were harvested for histological studies,

measurement of antibody production, and proliferative responses or flow cytometric analysis.

### Passive immunisation by adoptive transfer of OVA specific T cells and treatment protocol

The establishment of OVA specific T cell lines has been described in detail elsewhere.<sup>14</sup> In brief, OVA primed lymph node cells ( $2.5 \times 10^6$ /ml) were cultured in a 75 cm<sup>2</sup> culture flask (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a volume of 20 ml RPMI 1640 medium (Sigma Aldrich Co, St Louis, MO, USA) supplemented with 10% FCS (ICN Biomedical, Japan Co, Tokyo, Japan), 2-ME ( $5 \times 10^{-5}$  M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) in the presence of OVA (50  $\mu$ g/ml). Three days later, stimulated cells were expanded for 4 days in the above medium supplemented with 100 IU/ml of recombinant human IL-2 (rhIL-2, Takeda, Osaka, Japan) in the absence of OVA. After the expansion period, the cells ( $15 \times 10^6$  cells/flask) were restimulated with OVA (50  $\mu$ g/ml) in the presence of irradiated (30 Gy) syngeneic thymocytes ( $300 \times 10^6$  cells/flask) as antigen presenting cells (APC) in 10% FCS-RPMI 1640 medium at a volume of 50 ml. Three days later, live lymphocytes were separated by gradient centrifugation (Lymphocyte Separation Medium, Organon Teknika Corporation, West Chester, PA, USA) and expanded with rhIL-2 as above. Then, these cells were stimulated with OVA in the presence of irradiated thymocytes. At the same time, antigen specificity was assessed by proliferation assay. Three days after the third stimulation, live cells were collected by gradient centrifugation. Phenotypes of these cells were examined by flow cytometric analysis. Fifteen million of these T cells were injected intravenously into syngeneic male BN rats (recipients). The day of cell transfer was designated day 0. The rats were assigned to either an IFN- $\gamma$  treated or a PBS treated group. IFN- $\gamma$  (10  $\mu$ g per injection) and PBS treatments were given daily by intraperitoneal injection from day 0 to day 4. For the anti-IFN- $\gamma$  treatment, either anti-IFN- $\gamma$  or normal mouse IgG (3 mg per injection) was injected intraperitoneally on day 3 (24 hours before challenge) and day 4 (at the time of challenge). EC was induced on day 4 and was evaluated in a manner similar to that used for active immunisation.

### Counts of infiltrating cells in the conjunctiva

The eyes, including the lids, were fixed in 10% buffered formalin and embedded in paraffin. Central vertical plane sections of 4  $\mu$ m thickness were stained with haematoxylin-eosin and May-Giemsa. Two masked observers counted the numbers of infiltrating mononuclear cells, eosinophils, and neutrophils in the conjunctiva stained by these different methods. Counting of mononuclear cells was performed using an eyepiece grid of 400 $\times$  magnification.<sup>3-5 10 12 14</sup> Because cellular infiltration was not always uniform throughout the sections, three different portions (conjunctival fornix and two portions of palpebral conjunctivas) were selected for counting and an average was calculated. Eosinophils were counted throughout the section.

### Flow cytometric analysis

OVA primed lymph node cells and OVA specific T cells were incubated for 30 minutes on ice with Abs at optimal concentrations (0.02–0.5  $\mu$ g/ $5 \times 10^5$  cells). After the incubation, cells were washed with cold 2% FCS-PBS. These cells were analysed on a FACScan (Becton Dickinson, Mountain View, CA, USA) and acquisition and analysis were performed using CellQuest software.

### Assessment of cellular immune responses

Lymphocyte proliferation assays were done in quadruplicate in 96 well flat bottom plates. Lymph node cells ( $3 \times 10^5$  cells/well) from individual rats were cultured in a final volume

of 0.2 ml RPMI 1640 medium supplemented with 2-ME and 5% FCS. Cells were stimulated with OVA at final concentrations of 0.1, 1, 10, and 100  $\mu$ g/ml. After incubation for 72 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, cultures were pulsed for 16 hours with 0.5  $\mu$ Ci/well of [<sup>3</sup>H] thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). In the case of OVA specific cell lines, line cells ( $3 \times 10^4$ /well) were stimulated with OVA in the presence of 30 Gy irradiated thymocytes ( $6 \times 10^5$ /well) in the same condition as above. Forty eight hours later, [<sup>3</sup>H] thymidine was pulsed for 16 hours. Cultures were then harvested and the radioactivity was measured by standard techniques. Data were expressed as stimulation indices (mean count per minute (cpm) in stimulated cultures/mean cpm in unstimulated cultures).

### RT-PCR analysis of cytokines

RNA from OVA specific T cells prepared as above was isolated by acid/guanidine isothiocyanate/phenol/chloroform extraction and transcribed into cDNA using AMV reverse transcriptase XL (Takara Biomedicals, Tokyo, Japan) in a 100  $\mu$ l reaction mixture containing 5  $\mu$ g of total RNA. A cDNA equivalent to 0.25  $\mu$ g of total RNA was amplified in a 20  $\mu$ l reaction mixture containing 250  $\mu$ M of dNTP, 0.2  $\mu$ M of the primer pair, 2  $\mu$ l of a 10-fold dilution of PCR buffer, and 2.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus, Emeryville, CA, USA). PCR was performed on a DNA thermal cycler (Perkin Elmer, Foster City, CA, USA) for a cycle of 94°C for 10 minutes, 35 ( $\beta$ -actin, IL-10 and IFN- $\gamma$ ) or 40 (IL-2 and IL-4) cycles 94°C for 30 seconds, 55°C ( $\beta$ -actin, IL-2, IL-10 and IFN- $\gamma$ ) or 60°C (IL-4) for 30 seconds, and 72°C for 90 seconds. The molecular size marker used was X174/Hae III digest (Wako, Osaka, Japan). PCR products were electrophoresed in a 2% agarose gel with ethidium bromide staining. The predicted sizes of PCR-product and primer pairs<sup>13</sup> were as follows:  $\beta$ -actin: product size = 759 bp, 5'TTGTAACCAACTGGGACGATATGG3' and 5'GATCTTGATCTCATGGTGCTAGG3'; IL-2: product size = 342 bp, 5'CAAAGGAAACACAGCAGCACCTGG3' and 5'TCCTCAGAAATTCACCACAGTGTG3', IL-10: product size = 512 bp, 5'TCAGCACTGCTATGTTGCCTGCTC3' and 5' GAGTGTCACGTAGGCTTCTATGC3', IFN- $\gamma$ : product size = 288 bp, 5'ATCTGGAGGAAGTGGCAAAGGACG3' and 5'CCTTAGGCTAGATCTGGTGACAGC3'. IL-4 primer pair (GenBank Accession No X16058) was purchased from BioSource International, Camarillo, CA, USA and the predicted size was 177 bp.

### Detection of IgE in serum by ELISA

Total IgE in serum was measured using an ELISA kit (Morinaga, Yokohama, Japan) following the manufacturer's recommendations.

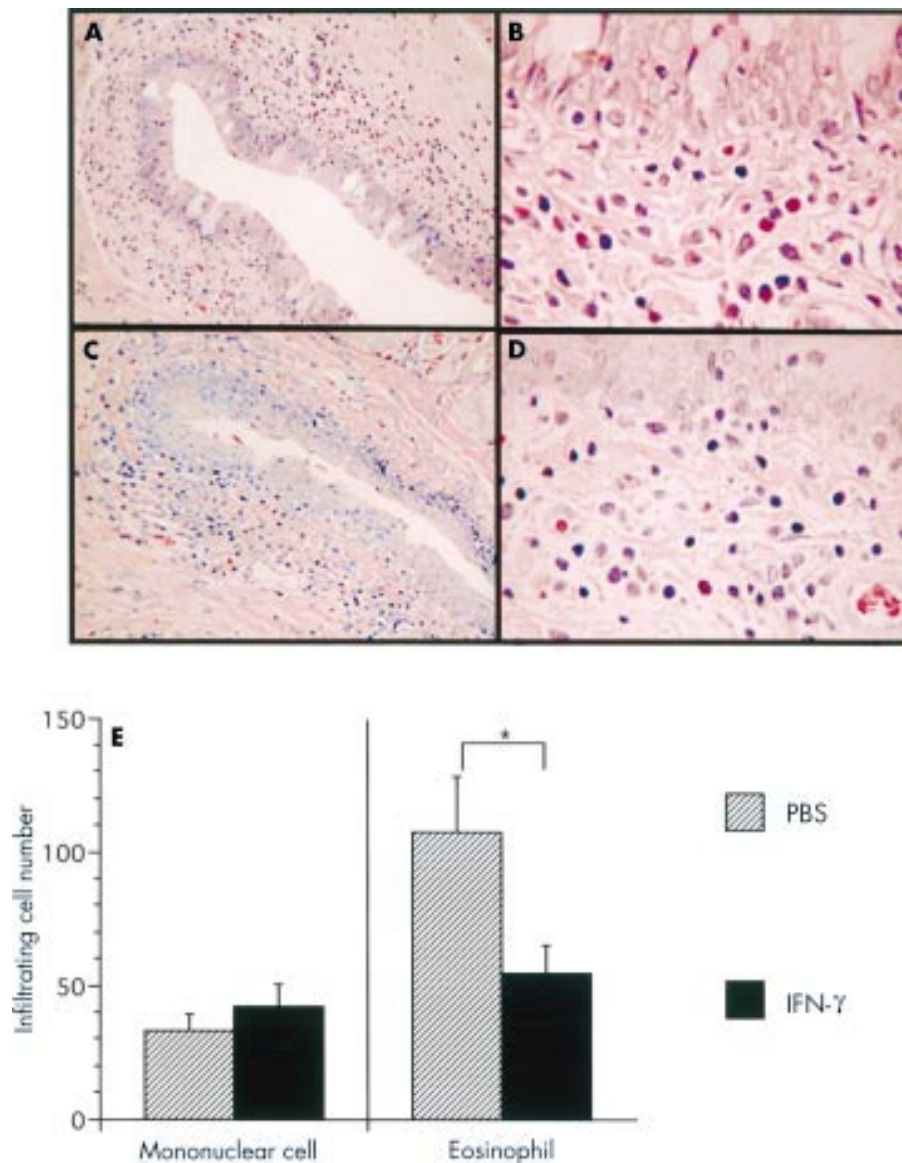
### Statistical analysis

Statistical comparisons of the number of infiltrating cells and the percentage of a cell surface molecule positive cells were performed with the Mann-Whitney U test.

## RESULTS AND DISCUSSION

A previous report using knockout mice demonstrated that IFN- $\gamma$  is an inhibitory molecule for the development of experimental AC.<sup>11</sup> However, it is still unclear whether IFN- $\gamma$  exerts this inhibitory effect during the induction or effector phase. Therefore, we examined the role of IFN- $\gamma$  in the development of EC using both active and passive immunisation systems,<sup>12</sup> which are useful for investigating the induction and effector phases, respectively.

To investigate the systemic effects of IFN- $\gamma$  on the induction phase in the development of EC, we induced EC in BN rats by active immunisation. Injection of IFN- $\gamma$  (10  $\mu$ g per injection) inhibited infiltration of eosinophils in rats with EC induced by active immunisation ( $p = 0.0265$ ), whereas other inflammatory cells were not affected by this treatment (Fig 1). Lower

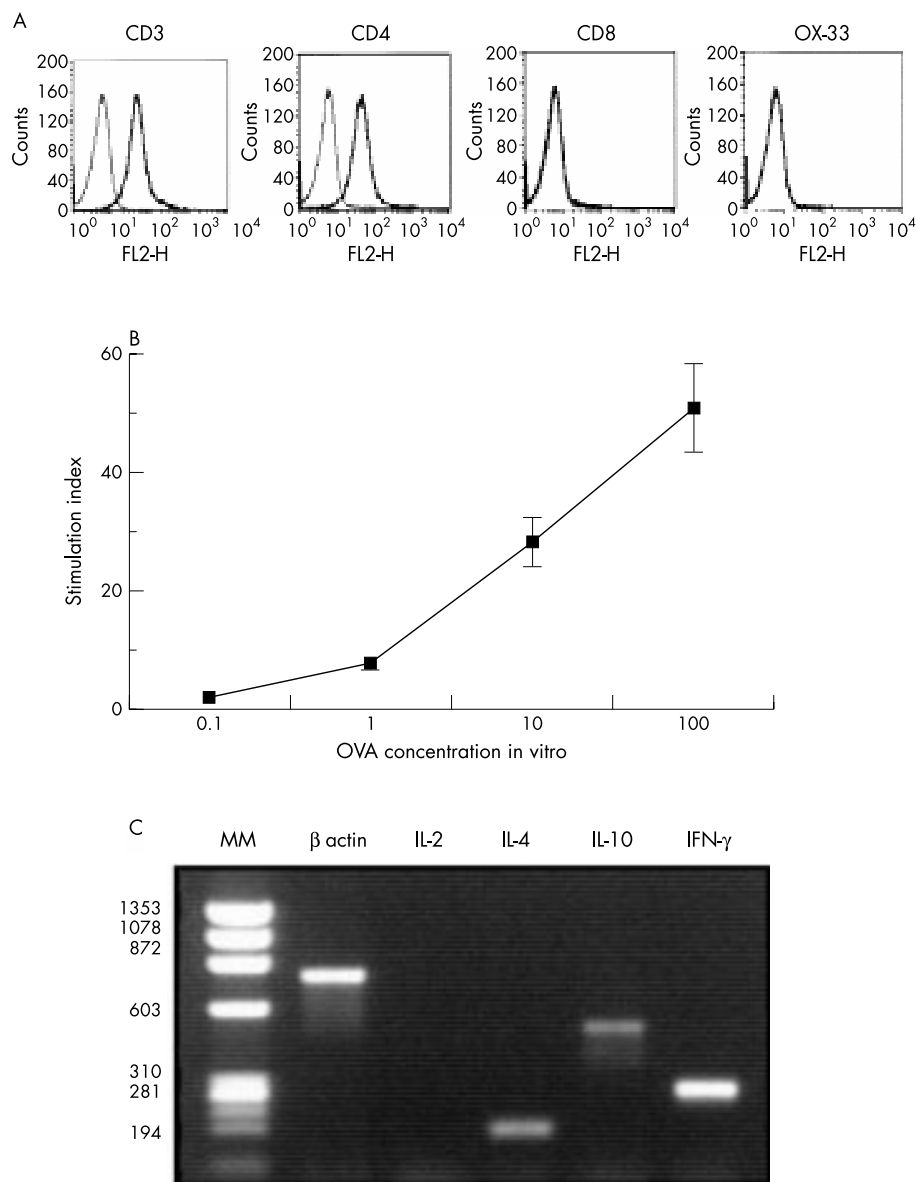


**Figure 1** Effects of IFN- $\gamma$  on EC induced by active immunisation. BN rats were actively immunised with OVA and 13 days later they were challenged with OVA. IFN- $\gamma$  (10  $\mu$ g per injection) or PBS was injected intraperitoneally every other day from the day of immunisation until challenge. Twenty four hours after challenge, the eyes were harvested for histology. Mononuclear cells were counted per field, whereas eosinophils were counted throughout the section. Eosinophilic infiltration was attenuated by IFN- $\gamma$  treatment, while infiltration of mononuclear cells was not affected. (A, B) PBS treated group. (C, D) IFN- $\gamma$  treated group. (A, C)  $\times 80$ , (B, D)  $\times 320$ . One representative of each group is shown. (E) Number of infiltrating mononuclear cells per field and number of infiltrating eosinophils throughout the section. An average of the data from six rats is shown for each group. \* $p < 0.05$ . Error bar indicates standard deviation.

doses of IFN- $\gamma$  (0.1  $\mu$ g and 1  $\mu$ g per injection) did not affect EC (data not shown). Thus, excess amounts of IFN- $\gamma$  inhibited the development of EC during the induction phase. These data agree with the previous result that cellular infiltration into the conjunctiva was much more extensive in IFN- $\gamma$  knockout mice<sup>11</sup> than in wild type mice.

To study the role of IFN- $\gamma$  on the development of EC during the effector phase, we established OVA specific T cell lines. These cell lines were CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup>, and CD45RA<sup>-</sup> (Fig 2A). These cells vigorously proliferated against OVA (Fig 2B). They expressed IL-4, IL-10, and IFN- $\gamma$  but not IL-2, indicating that they were mixture of Th1 and Th2 cells (Fig 2C). We induced EC in BN rats by transfer of these T cells and then injected IFN- $\gamma$  intraperitoneally. Because lower doses of IFN- $\gamma$  (0.1 and 1  $\mu$ g per injection) were ineffective for the inhibition of EC induced by active immunisation, effects of IFN- $\gamma$  were tested only at 10  $\mu$ g per injection. No difference was observed between the IFN- $\gamma$  injected group and the control group (Fig

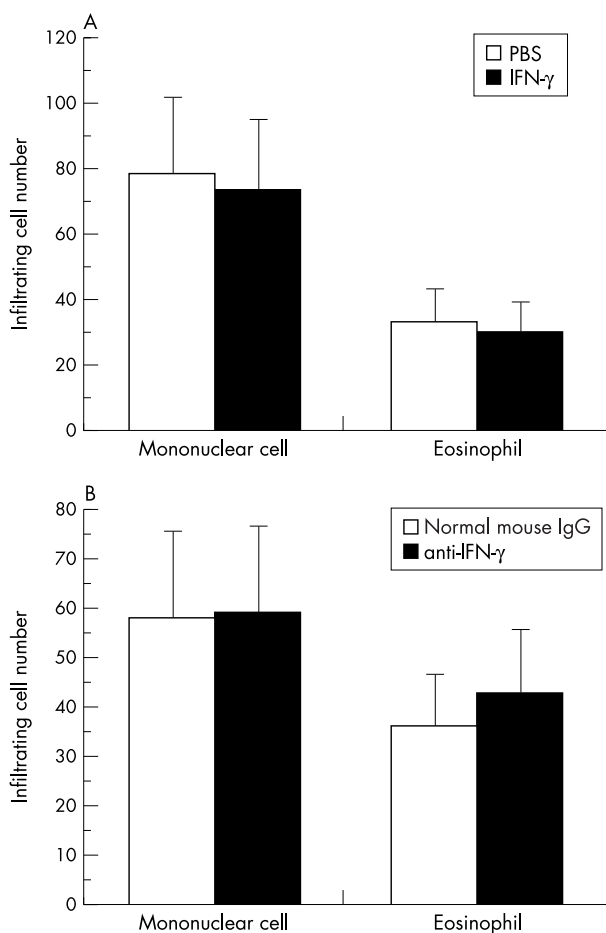
3A) at the same dose of IFN- $\gamma$  that suppressed eosinophilic infiltration in actively immunised BN rats (Fig 1). Next, to investigate the involvement of endogenous IFN- $\gamma$  in the effector phase of EC, we injected an anti-IFN- $\gamma$  antibody into rats developing EC. No differences in cellular infiltration into the conjunctiva were noted between the anti-IFN- $\gamma$  treated group and the normal mouse IgG treated group (Fig 3B). The ineffectiveness of IFN- $\gamma$  during the effector phase was further demonstrated by the finding that topical administration of IFN- $\gamma$  just before antigen challenge did not affect EC (data not shown). These data suggest that IFN- $\gamma$  is not likely to be involved in the effector phase of EC in BN rats. Together with the data that high doses of IFN- $\gamma$  inhibit eosinophilic infiltration in BN rats induced by active immunisation (Fig 1), we can conclude that IFN- $\gamma$  is an inhibitory cytokine for the development of EC and that it exerts suppressive effects only during the induction phase.



**Figure 2** Characterisation of OVA specific T cell lines. OVA specific T cell lines were established by repeated stimulation with OVA in the presence of irradiated thymocytes as APC. (A) Expression of cell surface molecules. After a third stimulation with OVA, cells were analysed by FACscan. This cell line was identified to be CD4 positive T cells. One of three representative experiments was shown. (B) Antigen specific proliferation. After a second stimulation with OVA, cells were rested with rIL-2 for 4 days. Then, proliferative capability of these cells against OVA was examined in the presence of APC. These T cells proliferated against OVA vigorously. Results were the average of three independent cell lines. Background cpm was 1767 (SD 449). (C) Expression of cytokine mRNA. After a third stimulation with OVA, mRNA was extracted from these T cells for RT-PCR. IL-4, IL-10, and IFN- $\gamma$  were detected, whereas IL-2 was not detectable. MM = molecular marker.

To ascertain the cellular effects of IFN- $\gamma$  treatment, we examined phenotypic compartments in draining lymph node cells especially focusing on expression of MHC class II, because IFN- $\gamma$  promotes MHC class II expression. Compared with the control group, no difference was noted on T cell compartment including CD25 expression (Fig 4A). On the contrary, expression of MHC class II, particularly RT1D, was augmented in the IFN- $\gamma$  treated group (Fig 4A). In parallel, CD45RA, which is a B cell marker, expressing cells were increased. Thus, it could be considered that treatment protocol here activated B cell compartment, leading to an increased number of MHC class II positive cells. Next, we tested antigen specific cellular and humoral immunity in rats that received active immunisation. IFN- $\gamma$  treatment did not appear to affect antigen specific proliferation of lymph node cells (Fig 4B) or the total IgE level in serum (Fig 4C). Additionally, antigen specific IgE production in serum was similar between the two

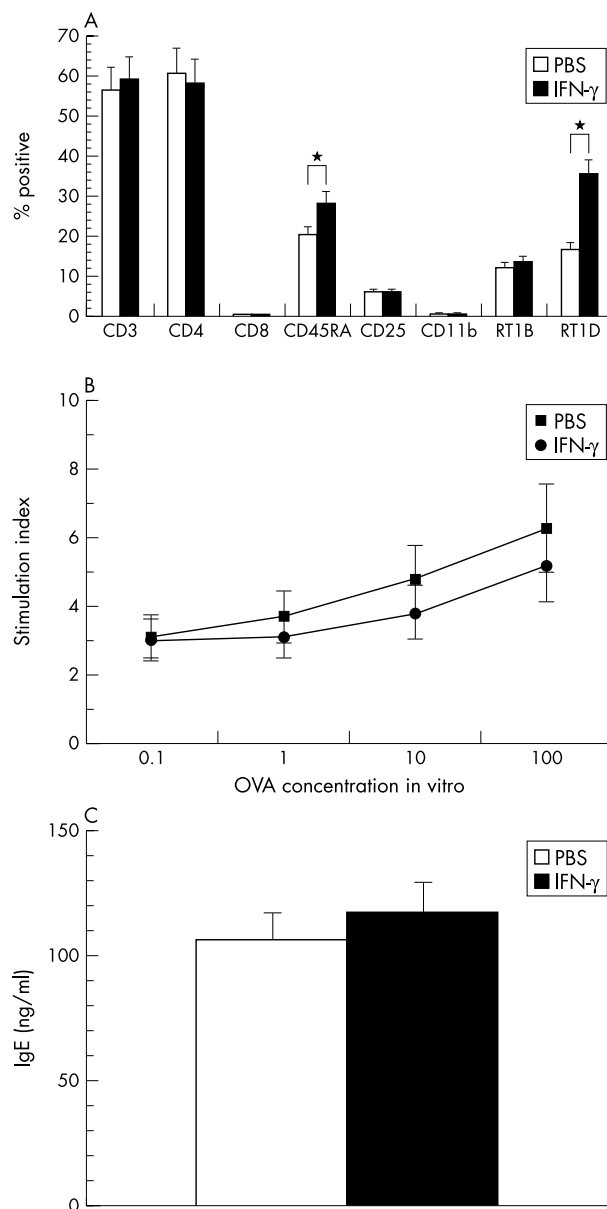
groups (data not shown). Because IgE was not suppressed by IFN- $\gamma$  treatment, IFN- $\gamma$  treatment did not profoundly affect Th2 immunity. Recently, it was demonstrated that sensitivity to IFN- $\gamma$  is different even among Th2 subpopulations,<sup>15</sup> in that IFN- $\gamma$  affects IL-5 expression rather than IL-4 expression. These data are in good agreement with our findings that IL-4 dependent IgE production was not affected but IL-5 dependent eosinophilic infiltration was attenuated by IFN- $\gamma$  treatment. It may also be that endogenous IFN- $\gamma$  is relatively abundant and inhibits Th2 immune responses, so that the effects of additional IFN- $\gamma$  are not remarkable. Although it is well known that fully differentiated Th2 cells are more sensitive to IFN- $\gamma$  than Th1 cells,<sup>16</sup> treatment with IFN- $\gamma$  during the induction phase might have affected the development of Th1 and Th2 cells equally, thus not affecting the Th1/Th2 balance.



**Figure 3** Effects of IFN- $\gamma$  and an anti-IFN- $\gamma$  monoclonal antibody on EC induced by transfer of OVA specific T cells. OVA specific T cells were transferred into syngeneic BN rats. Four days later, the rats were challenged with OVA. IFN- $\gamma$  (10  $\mu$ g per injection) or PBS was intraperitoneally injected daily from the day of immunisation to the day of challenge (A). An anti-IFN- $\gamma$  monoclonal antibody or normal mouse IgG (3 mg per injection) was injected on day 3 and day 4 (B). Twenty four hours after challenge, the eyes were harvested for histology. The number of infiltrating mononuclear cells and eosinophils in rats treated with either IFN- $\gamma$  (A) or anti-IFN- $\gamma$  (B) are shown. Neither IFN- $\gamma$  nor anti-IFN- $\gamma$  treatment affected the progression of EC induced by transfer of OVA specific T cells. Averages of data from six rats (A) and four rats (B) are shown for each group. Error bar indicates standard deviation.

Considering the effects of IFN- $\gamma$  on immune responses, it is necessary to pay attention to the involvement of innate immunity<sup>17</sup> as well as acquired immunity discussed as above. Involvement of innate immunity could be less in EC by passive immunisation than that by active immunisation, because passive EC is putatively mediated by antigen specific T cells. This differential involvement of innate immunity on the development of EC between by active and passive immunisation might be related to the fact that suppressive effects of IFN- $\gamma$  were noted in active EC but not passive EC. Although we have not investigated the interaction of IFN- $\gamma$  linking the innate immune system and acquired immune system,<sup>18</sup> this interaction, at least in part, should take a role in the suppressive effects of IFN- $\gamma$  on EC. One important cell compartment for this linkage is dendritic cells (DC).<sup>19</sup> Because IFN- $\gamma$  was shown to activate DC function to promote IL-12 production,<sup>20</sup> increased Th1 level might have suppressed Th2 level, thus leading to less eosinophilic infiltration.

In conclusion, IFN- $\gamma$  exerts its suppressive effects on EC during the induction phase, but not the effector phase.



**Figure 4** Effects of IFN- $\gamma$  on immune responses. Lymph node cells and sera were collected from actively immunised rats treated with IFN- $\gamma$  or PBS, as detailed in the Materials and methods. (A) Expression of cell surface molecules in lymph node cells evaluated by flow cytometric analysis. Data were presented as an average of percentages of positive cells expressing a particular molecule. IFN- $\gamma$  treatment increased B cell compartment and RT1D positive cells, compared to PBS treatment. Averages of six individual rats per groups are shown. \* $p < 0.05$ . Error bar indicates standard deviation. (B) Antigen specific proliferation of lymph node cells against OVA. No apparent differences were noted between the two groups. Data from six individual rats were combined in each group. (C) Total IgE in serum. A similar amount of IgE was detected in both groups. Data from six individual rats were combined in each group.

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