

Analysis of effects of stimulation in vitro of ovalbumin primed lymph node cells on adoptive transfer of experimental immune mediated blepharoconjunctivitis in Lewis rats

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Abstract

Aim—To analyse the role of stimulation in vitro of lymphocytes on the augmentation of experimental immune mediated blepharoconjunctivitis (EC, formerly EAC) in Lewis rats induced by adoptive transfer.

Methods—Two weeks after immunisation with ovalbumin (OVA), rat draining lymph nodes were collected and 50×10^6 cells were injected into naive syngeneic recipients either directly or after culture in vitro with OVA, concanavalin A (Con A), or purified protein derivative (PPD) for 3 days. Four days after injection the rats were topically challenged with OVA. 24 hours later, they were sacrificed and eyes and spleens were harvested for histology and proliferation assay. In some experiments, naive recipient rats were irradiated with 7 Gy γ ray before transfer. The expression of adhesion molecules and cytokine profile of OVA primed lymph node cells were also investigated.

Results—Both infiltrated cell number and splenocyte proliferation in the recipients of stimulated cells were higher than those of unstimulated cells. In vitro stimulation with OVA or Con A induced a severe cellular infiltration, while stimulation with PPD did not. Irradiation markedly diminished cellular infiltration. Stimulation in vitro upregulated the CD4/CD8 ratio by four times and augmented expression of CD25, I-A, ICAM-1 molecules on OVA primed lymph node cells by about five times. IFN- γ was detected in OVA primed cells by stimulation in vitro, while IL-4 mRNA was extinguished by stimulation in vitro.

Conclusions—Augmentation of EC by stimulation in vitro of transferred lymphocytes might depend on the upregulation of expression of cell surface molecules and cytokine shift as well as augmented antigen specificity.

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primed lymph node cells augmented the infiltration of inflammatory cells into the conjunctiva. Stimulation in vitro of lymphocytes has been demonstrated to transfer their function efficiently not only in our system but also in many other systems such as experimental autoimmune encephalomyelitis (EAE),^{2,3} experimental autoimmune uveitis (EAU),^{4,5} or experimental lens induced uveitis.⁶ Although several factors such as adhesion molecules were identified to be involved in augmentation of the disease transfer by stimulation in vitro of lymphocytes, the mechanism of this augmentation has not been fully elucidated.

We can easily imagine that stimulation in vitro of lymphocytes with antigens augments the ability of transferred lymphocytes to gather at the inflammatory sites, probably as a result of upregulation of the affinity of transferred lymphocytes with the antigen which exists at the inflammatory sites. Although the number of inflammatory cells in the conjunctiva of rats with EC was increased by stimulation in vitro, infiltrated cells in the conjunctiva were not identified to be from transferred cells, recipient's cells, or a combination of both. The first question is whether the increased number of inflammatory cells at the site is due to only infiltration of transferred cells or partially to cells from recipients together with transferred cells.

In EAU or EAE, the adoptive transfer of cells stimulated with either concanavalin A (Con A) or disease related antigens induced the disease, while in other systems such as experimental lens induced uveitis models, the transfer of Con A stimulated cells were unable to induce the disease⁶ (and unpublished data). This information indicated that both the level of activation and antigen specificity are important for the induction of diseases, and that the degree of involvement of each factor seems to depend on the nature of diseases. Therefore, we compared three stimulants, OVA, Con A, and purified protein derivative (PPD) for their ability to induce EC on stimulation of OVA primed lymph node cells.

Stimulation of lymphocytes in vitro changes various characters of lymphocytes. Variables which we usually regard as the activated state of cells include cell surface molecules,⁷⁻¹⁰ cytokine production, cell proliferation,¹¹⁻¹³ and so on. Cell surface molecules such as CD25 or CD69 are known as activation markers¹⁴ and

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We previously reported¹ that ovalbumin (OVA) primed lymph node cells but not OVA primed sera were able to adoptively transfer experimental immune mediated blepharoconjunctivitis (EC, formerly EAC) in Lewis rats. We also demonstrated that stimulation in vitro of OVA

cell adhesion molecules such as LFA-1¹⁵ are also upregulated during stimulation *in vitro*. These adhesion molecules are regarded as important for homing and invading of cells into target tissues. The next question is what are the changes in the expression of cell surface molecules, probably related to augmentation of inflammation, detected in stimulated cells compared with unstimulated cells?

Other important changes seen during stimulation *in vitro* are cellular proliferation and cytokine production.¹¹⁻¹³ It has been revealed in many experimental systems that IFN- γ ^{16, 17} is a tissue destructive factor compared with IL-4,^{18, 19} which is called an immunoregulatory cytokine. In general, allergic diseases are mediated by IgE and the IgE production is promoted by IL-4 and inhibited by IFN- γ .²⁰ On the other hand, EC in Lewis rats exhibited a dominant involvement of cellular immunity but not humoral immunity in both induction and effector phases.¹ The last question is to determine cytokines related to the development of EC by testing changes of cytokine profiles of OVA primed lymph node cells by stimulation *in vitro*.

Materials and methods

RATS

Six to 8 week old male Lewis rats were purchased from a commercial source (Seac Yoshitomi, Fukuoka, Japan) and maintained in a specific pathogen free animal facility at Kochi Medical School. All animal procedures conformed with institutional guidelines and the ARVO Resolution on Use of Animals in Research.

IMMUNISATION

Rats were injected with 100 μ g of ovalbumin (OVA, grade V, Sigma Immunochemicals, St Louis, MO, USA) emulsified with complete Freund's adjuvant (CFA, Yatoron, Tokyo, Japan) to a final volume of 100 μ l in their left hind footpads. In some experiments, rats were immunised with only CFA without any antigens.

ADOPTIVE TRANSFER OF DISEASE

Methods have been described previously.¹ In brief, cells from immune inguinal lymph nodes were harvested 14 days after immunisation, teased, and washed three times with RPMI-1640 medium (Mediatech, Herndon, VA, USA) supplemented with L glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). After washing, naive recipients received 50 \times 10⁶ fresh unstimulated cells by intravenous injection in the dorsal penile vein. The remainder of the cells was stimulated at the concentration of 10⁷ cells/ml with 50 μ g/ml of OVA in the medium containing 5 \times 10⁻⁵ M 2-mercaptoethanol (2-ME) and 10% fetal calf serum (FCS, ICN Biomedical Japan Co, Tokyo, Japan). In some experiments, cells were stimulated with 1 μ g/ml of concanavalin A (Con A, Sigma Immunochemicals, St Louis, MO, USA) or protein purified derivative (PPD, Japan BCG, Tokyo, Japan) at 10 μ g/ml instead of OVA. Three days later, culture

supernatants were harvested and used for cytokine ELISA. Then 50 \times 10⁶ stimulated cells were injected intravenously into the syngeneic recipients. Four days after transfer, the recipients were challenged with OVA by eye drops. Eyes and spleens were harvested 24 hours after challenge for histology and proliferative responses respectively. The disease was evaluated by the number of infiltrated cells per field detailed in Fukushima *et al.*¹

PROLIFERATION ASSAY

Spleens from recipient rats were combined by group and experiments were repeated as recorded. Lymphocyte proliferation assay was set up in quadruplicate in a 96 well flat bottom plate. Splenocytes (3 \times 10⁵ cells/well) were cultured in 0.2 ml final volume of the RPMI-1640 medium supplemented with 5% FCS, 2-ME (5 \times 10⁻⁵ M), L glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Cells were stimulated with OVA at the final concentration of 0.1, 1, 10, and 100 μ g/ml each. They were also stimulated with Con A at 1 μ g/ml or with PPD at 10 μ g/ml. After incubation for 72 hours at 37°C in a humidified atmosphere with 5% carbon dioxide, cultures were pulsed for 16 hours with 0.5 μ Ci/well of ³H thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). Cultures were then harvested and the radioactivity was measured by the standard technique. Data were expressed as stimulation indices.

IRRADIATION OF RECIPIENT RATS

Recipient rats were irradiated with a dose of 7 Gy using a gamma irradiator 1 day before adoptive transfer.

FLOW CYTOMETRIC ANALYSIS

Either stimulated or unstimulated cells were stained with the following anti-rat antibodies. Anti-CD4 and anti-CD8 (Pharmingen, San Diego, CA, USA) were FITC labelled. Anti-CD3 and anti-CD25 (Pharmingen, San Diego, CA, USA), anti-I-A (OX-3), and anti-CD45RA (OX-33) (Serotec, Oxford), anti-CD11a (LFA-1 α), and anti-CD54 (ICAM-1) (Caltag Laboratories, Burlingame, CA, USA) were biotin labelled. The cells were incubated for 45 minutes on ice with the primary antibodies mentioned above (0.08–2 μ g/2 \times 10⁶ cells) and washed with cold 5% FCS-PBS. Streptavidin-PE (Pharmingen, San Diego, CA, USA) was added to the cells stained with biotin conjugated antibodies for 10 minutes and the cells were washed again. Then the cells were analysed using FACscan (Becton Dickinson, Mountain View, CA).

CYTOKINE ELISA USING CULTURE SUPERNATANT

Lymph nodes from three to eight OVA primed rats were combined for culture. Culture supernatants were harvested at the end of culture before the adoptive transfer mentioned in the section "adoptive transfer of disease". They were assayed by commercial cytokine ELISA (BioSource International, Camarillo, CA, USA) for IFN- γ concentrations strictly following the recommended method.

Table 1 Sequences of primers of rat cytokines and β actin

mRNA	Sense primer		Antisense primer		PCR product size
	5'	3'	5'	3'	
β actin	TTGTAACCAACTGGGACGATATGG		GATCTTGATCTTCATGGTGCTAGG		759
IL-2	CAAAGGAAACACAGCAGCACCTGG		TCCTCAGAAATTCACCCACAGTTG		342
IL-4	TGATGGGTCTCAGCCCCACCTTGC		CTTTCAGTGTGTGAGCCGTGGACTC		378
IL-10	TCAGCACTGCTATGTTGCCTGCTC		GAGTGTACCGTAGGCTTCTATGC		512
IFN- γ	ATCTGGAGGAACTGGCAAAGGACG		CCTTAGGCTAGATTCTGGTGACAGC		288

RT-PCR ANALYSIS OF CYTOKINES

Fresh lymph node cells or stimulated lymph node cells before the adoptive transfer were used. Cellular RNA was isolated by acid/guanidine isothiocyanate/phenol/chloroform extraction.²¹ RNA was transcribed into cDNA in a 20 μ l reaction mixture containing 1 μ g of total RNA. A cDNA equivalent to 1 μ g of total RNA was amplified in a 100 μ l reaction mixture containing 250 μ M of dNTP, 0.2 μ M of the primer pair, 8 μ l of a 10-fold dilution of PCR buffer, and 2.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus, Emeryville, CA, USA). The sense and antisense primer sequences derived from rat IL-2, IL-4, IL-10, IFN- γ , and β actin cDNA are summarised in Table 1. PCR was performed on a DNA thermal cycler (Perkin Elmer, Foster City, CA, USA) for 30 cycles (94°C for 30 seconds, 51°C

for 30 seconds, and 72°C for 1.5 minutes). The PCR products were electrophoresed in 3% agarose gels and visualised with ultraviolet light. The molecular size marker used in this study was ϕ X174/Hae III digest (Wako, Osaka, Japan).

STATISTICAL ANALYSIS

Statistical comparison of the number of infiltrated cells between two groups was performed using Student's *t* test.

Results

STIMULATION IN VITRO AUGMENTS THE NUMBER OF INFILTRATED CELLS IN THE CONJUNCTIVA

Figure 1A shows the number of infiltrated cells in the conjunctiva of the recipients of OVA primed lymph node cells either with or without stimulation *in vitro* as described in the Materials and methods. Stimulation *in vitro* with OVA increased the number of infiltrated cells (stimulated cells: 512.3, unstimulated cells: 45.2) by 12 times ($p < 0.001$). In addition, although splenocytes from the recipients of stimulated cells showed lower responses to OVA compared with the OVA primed lymph node cells, they exhibited higher proliferative responses to OVA than those of the recipients of fresh cells (Fig 1B). These results indicated that stimulation *in vitro* efficiently augmented the adoptive transfer of EC and cellular immunity.

ANTIGEN SPECIFIC PRIMING AND STIMULATION IN VITRO ARE IMPORTANT FOR EFFICIENT ADOPTIVE TRANSFER OF EC

To rule out the possibility that EC induced by the adoptive transfer of cells does not depend on the antigen specific reaction, we set up two experiments. Firstly, we transferred primed lymph node cells from rats injected with only CFA to demonstrate the essential role of antigen specific priming in inducing EC. We also compared the stimulants on OVA primed cells to investigate the role of antigen specific stimulation *in vitro* in the adoptive transfer of EC. The transfer of *in vitro* stimulated lymph node cells from rats injected with only CFA did not

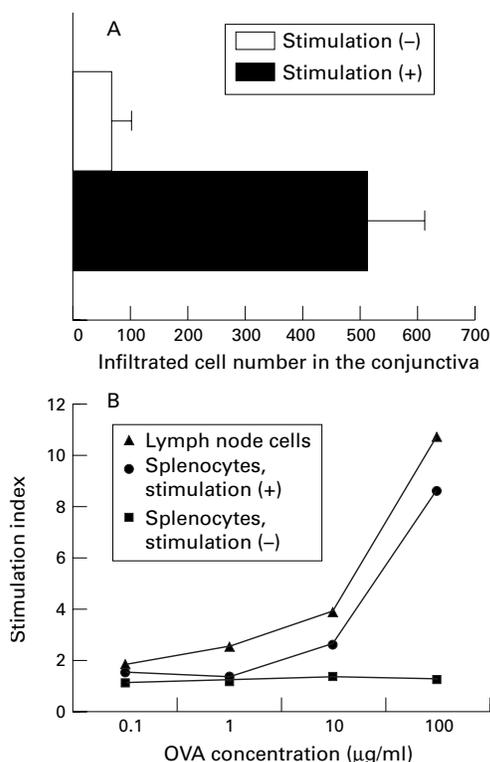


Figure 1 (A) Infiltrated cell number in the conjunctival fornix of recipients of OVA primed cells (50×10^6) either stimulated *in vitro* or not. X axis indicates the mean number of infiltrated cells of three rats. Error bars indicated standard deviations. (B) Proliferative responses of lymph node cells from donor or spleen cells from recipients transferred with OVA primed lymph node cells either stimulated *in vitro* or not. Data are means of SI values of three rats of each group. Similar results were obtained in two more separate experiments. The combined mean cpm values in the unstimulated control cultures of rats were: donor's lymph node cells: 470 (43), recipient's splenocytes with stimulation *in vitro*: 3321 (166), recipient's splenocytes without stimulation *in vitro*: 3301 (104).

Table 2 Comparison of stimulants for adoptive transfer of EC, IFN- γ production, and cellular proliferation

	OVA	Con A	PPD
Infiltrated cell number (SD)*	145.3 (49.1)	118.3 (12.4)	6.5 (1.4)
IFN- γ production†	1884	7831	2012
Cellular proliferation‡	11.2	46.1	9.6

*Infiltrated cells per field were counted in the palpebral conjunctiva.

†The amount of IFN- γ in the culture supernatant (pg/ml).

‡Data were recorded as stimulation index.

Table 3 Flow cytometric analysis of OVA primed lymph node cells

	CD25		I-A		LFA-1		ICAM-1	
	% positive*	MFI†	% positive	MFI	% positive	MFI	% positive	MFI
Before stimulation in vitro	6.7	52.5	26.1	444.6	97.7	50.2	4.4	43.5
After stimulation in vitro	36.3	132.4	24.4	2319.8	95.3	50.9	22.3	46.9

*% positive = percentage of cells expressing the molecule in all cells.

†MFI = mean fluorescence intensity of positive cells.

induce any cellular infiltration into the conjunctiva of the recipient rats challenged with OVA (data not shown). Inflammatory cell infiltration was not detected in the conjunctiva of the recipients of OVA primed lymph node cells stimulated with PPD at 10 µg/ml. The proliferative response of OVA primed lymph node cells for PPD at 10 µg/ml is almost equal to that of OVA at 100 µg/ml (Table 2). There were no remarkable differences in the number of infiltrated cells in the conjunctiva between the recipients of cells stimulated with OVA and those with Con A (average infiltrated cell number; OVA = 145.3, Con A = 118.3) (Table 2).

NOT ONLY STIMULATED DONOR CELLS BUT ALSO CELLS FROM RECIPIENTS GATHER AT THE INFLAMMATORY SITE

We tested whether donor cells merely infiltrated into the inflammatory sites or triggered inflammation and promoted the gathering of recipients' cells at the inflammatory sites. We irradiated the recipients 1 day before the adoptive transfer to deplete haematopoietic cells in the recipients. No apparent histological changes in the conjunctiva of irradiated rats were observed by the routine histological study using haematoxylin and eosin staining. As shown in Figure 2, irradiation drastically reduced the number of infiltrated cells in the conjunctiva (the average number of infiltrated cells of the recipients without irradiation = 306.1; that with irradiation = 56.2) ($p < 0.001$). Taking the data of Figure 1A into consideration, it can be interpreted that stimulation in vitro augmented not only the homing ability of donor cells but also the ability of inducing recipient cells in the conjunctiva.

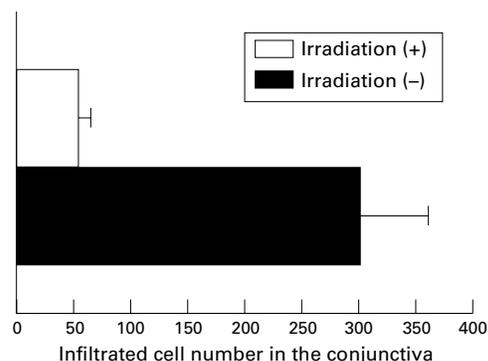


Figure 2 Infiltrated cell number in the conjunctival fornix of recipients either irradiated or not. All recipients received 50×10^6 stimulated OVA primed lymph node cells. X axis indicates the mean number of infiltrated cells per field of three rats in each group. Error bars indicated standard deviations.

PHENOTYPIC CHANGES OF TRANSFERRED CELLS BY IN VITRO STIMULATION

To test the changes of cell surface markers on the cells after stimulation in vitro compared with cells before stimulation, FACScan analysis was performed. The percentage of the total number of cells, which was based on the same form of side scatter-forward scatter profile, and mean fluorescence intensity (MFI) of positive cells was indicated. Although the T/B ratio (before stimulation: 76.7%/19.4%, after stimulation: 79.2%/16.7%) was not changed, the CD4/CD8 (before stimulation: 56.1%/24.9%, after stimulation: 57.0%/6.8%) ratio was increased dramatically by stimulation in vitro with OVA. Percentage positive cells and/or mean fluorescence intensity of positive cells concerning CD25, I-A and ICAM-1 molecules were increased (Table 3).

COMPARISON OF STIMULANTS ON PRODUCTION OF IFN- γ BY OVA PRIMED LYMPH NODE CELLS

To compare three stimulants (OVA, Con A, and PPD) for IFN- γ production of OVA primed lymph node cells, ELISA was performed using culture supernatants. In parallel with the cellular proliferation, Con A stimulated cells produced IFN- γ four times more than OVA stimulated cells, while PPD stimulated cells produced almost as much IFN- γ as that of OVA stimulated cells (Table 2).

IL-4 MRNA WAS EXTINGUISHED BY STIMULATION IN VITRO

By cytokine ELISA, we were able to see only the production of cytokine after stimulation in vitro. To compare the cytokine RNA from cells before stimulation in vitro with that after stimulation, RT-PCR using RNA from cells either before or after stimulation was carried out. As shown in Figure 3, a marginal IL-4 and IL-2 signal was obtained in cells that were not stimulated, whereas stimulation resulted in the absence of the IL-4 signal and a small increase in the IL-2 signal. IL-10 and IFN- γ were detected in both samples.

Discussion

While IgE mediated immune responses are known to be the major effector mechanism for allergic conjunctivitis, cellular immunity is also considered to be involved in the induction of certain types of allergic conjunctivitis such as vernal keratoconjunctivitis.^{22, 23} To clarify the involvement of cellular immunity in the development of allergic conjunctivitis, we established EC in Lewis rats by either active or passive immunisation.¹ The purpose of this study is to further investigate the mechanisms by which EC is induced by passive immunisation.

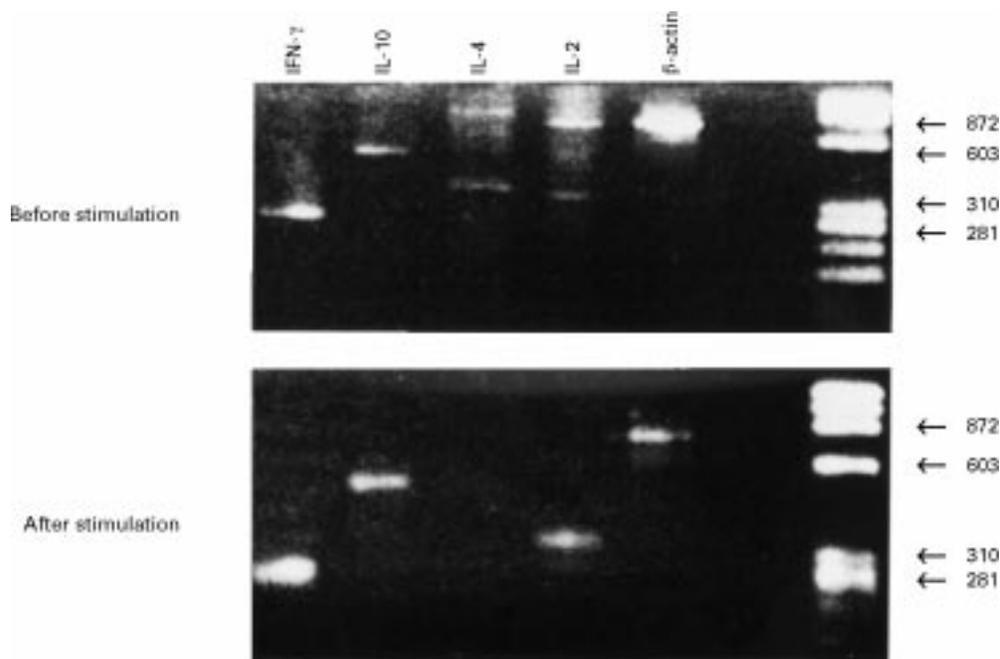


Figure 3 RT-PCR of RNA extracted cells either before or after stimulation *in vitro*. RT-PCR was performed as in Materials and methods. Primers used were rat IL-2, IL-4, IL-10, IFN- γ , and β actin. PCR products were electrophoresed in 3% agarose gels and visualised with ultraviolet light.

Haematoxylin and eosin staining of the recipients' conjunctiva demonstrated that mononuclear cells predominantly infiltrated into the conjunctiva to cause inflammation. Although we did not perform immunohistochemical staining such as CD4 cell surface marker, few plasma cells were detected. In addition, we demonstrated that the transfer of CD4⁺ T cell line specific for OVA peptide 323–339 induced EC (submitted for publication). In keeping with our previous report that EC could not be transferred by OVA primed serum, not humoral immunity but cellular immunity is essential for the induction and development of EC in our system.

The data from Figure 1A and Figure 2 may be interpreted to show that the inflammatory cells were composed of both donor cells and cells from recipients. Concerning the mechanism of infiltration of cells from the recipients, it is possible that stimulated cells release some chemoattractant factors to induce cellular infiltration of cells from the recipients. It is also possible that stimulated cells destroy conjunctival vasculature more effectively than unstimulated ones, thus inducing a massive leakage of recipient cells.

Stimulation of cells *in vitro* with antigen is believed to augment the antigen specificity and function of the cells. To investigate the importance of antigen specificity, antigens were compared for their ability to induce EC. The failure to induce EC by CFA immunisation indicated that antigen specific priming is at least necessary for the induction of EC. Similar to data reported in EAU, mitogenic stimulation as well as antigen specific stimulation induced EC. Although Con A stimulated cells proliferated most vigorously and produced the largest amount of IFN- γ compared with other two stimulants, the intensity of the

disease induced by Con A stimulated cells was slightly milder than that by OVA stimulated cells. These results suggest that antigen specific activation is important for the transfer of EC and an extensive non-specific mitogenic stimulation can compensate the transfer of EC for the antigen specific activation.

The increase of CD4/8 ratio indicated that our culture condition tends to increase CD4⁺ T cells relatively, which probably work as effector cells in EC, thus leading to augmentation of EC. As the T/B ratio and %CD4⁺ cells were not changed by stimulation *in vitro*, the increase of CD4/CD8 ratio appeared to depend on the loss of CD8⁺ cells or the increase of CD4⁺CD8⁺ T cells. Although the exact reason for these phenotypical changes remained unclear, the loss of CD8⁺ T cells, which are known to work as suppressor cells in certain circumstances,^{24, 25} may break suppression of the development of EC. As expected, stimulation upregulated the expression of CD25, I-A, ICAM-1 (Table 3). These molecules proved to be directly involved in the induction of experimental diseases such as EAU, because injection of monoclonal antibodies against these molecules inhibited the development of EAU.^{26–29} Therefore, it is suspected that these molecules are also involved in the development of EC. It is obviously necessary to block these molecules by monoclonal antibodies to prove their roles in the development of EC.

IFN- γ and IL-4 are known as key cytokines to modulate the immune reaction towards either cellular or humoral responses. These two cytokines are usually measured for analysis of involvement of the cellular and humoral immunity mediated by T cells. Because reliable rat IL-4 ELISA has not been available so far, we were unable to test IL-4 production in the

culture supernatant of OVA primed lymph node cells with stimulation in vitro. Therefore, to investigate cytokine production by OVA primed lymph node cells, we tested both ELISA and RT-PCR. Stimulation in vitro induced IFN- γ production, while it downregulated IL-4 gene transcription (Table 2, Fig 3). Although these data should be interpreted with caution since the conditions in which the PCR was performed may not yield quantitative data, it is suspected that our culture system promoted OVA primed lymph node cells toward Th1 type dominant cells. To determine the direct involvement of cytokines on the development of disease, treatment with monoclonal antibodies against cytokines and/or cytokine receptors in vivo, which were reported in other systems,³⁰⁻³² is crucial.

In conclusion, stimulation in vitro of OVA primed lymph node cells upregulated the CD4/CD8 ratio and cell surface expression of CD25, I-A molecules on them. It also induced IFN- γ production and stopped the expression of mRNA of IL-4. These changes as well as upregulation of antigen specificity are possibly involved in the augmentation of EC by the adoptive transfer. These results further support our previous report that cell mediated immunity was dominantly involved in the development of EC.

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