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A method to generate antigen-specific suppressor T cells in vitro from peripheral blood T cells of honey bee venom-sensitive, allergic patients

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Peripheral blood mononuclear cells of patients allergic to honey bee venom were stimulated with denatured bee venom phospholipase A₂, and the antigen-activated T cells were propagated for 4 days by human IL-2 in the presence or absence of recombinant human lipocortin I. Upon antigenic stimulation with the denatured phospholipase A₂ and autologous monocytes or by cross-linking of CD3 by anti-CD3 antibody, the activated T cells, which had propagated by IL-2 alone, formed *N*-glycosylated IgE-binding factors and glycosylation enhancing factor (GEF), while those propagated in the presence of lipocortin formed unglycosylated IgE-binding factors and glycosylation inhibiting factor (GIF). The GEF and GIF formed by the antigen- or anti-CD3-stimulated T cells had affinity for bee venom phospholipase A₂ and could be purified by using anti-lipomodulin Sepharose. In the mouse lymphocyte system, the major cell source of GIF is antigen-specific suppressor T cells, and the antigen-binding GIF from the cells suppressed the *in vivo* antibody response in an antigen (carrier)-specific manner. In view of the findings in the mouse system, the present results may provide an immunological maneuver to generate allergen-specific suppressor T cells, and to obtain allergen-specific suppressor factor from T cell populations in the peripheral blood of allergic patients.

Key words: Suppressor T cell factor; Lipocortin; Bee venom phospholipase A₂; IgE-binding factor

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Abbreviations: GIF, glycosylation inhibiting factor; GEF, glycosylation enhancing factor; DNP-OVA, dinitrophenyl derivatives of ovalbumin; KLH, keyhole limpet hemocyanin; TsF, antigen-specific suppressor T cell factor; D-PLA₂, denatured phospholipase A₂; rIFN- γ , recombinant human interferon- γ ; PBMC, peripheral blood mononuclear cells; MGG, mouse IgG; E'-IgE, IgE-coated fixed, ox erythrocytes; RFC, rosette-forming cells; DPBS, Dulbecco's phosphate-buffered saline; IgE-BF, IgE-binding factor.

Introduction

In our previous studies on the regulation of the IgE antibody response, we described two types of T cell factors which have affinity for IgE and either enhance or suppress the IgE synthesis (Ishizaka, 1984). Subsequent experiments revealed that the major difference between the IgE-potentiating factor and the IgE-suppressive factor is carbohydrate moieties in the molecules (Yodoi et al., 1982), and that the two IgE-binding factors (IgE-BF) probably share a common polypeptide

(Martens et al., 1987). Under physiological conditions, the post-translational glycosylation process of the peptide appears to be controlled by two T cell factors which either enhance or inhibit the *N*-glycosylation of the peptide and thereby determine the biologic activities of IgE-binding factors (Ishizaka, 1984). The major cell source of glycosylation inhibiting factor (GIF) is antigen-specific suppressor T cells (Jardieu et al., 1984), and this factor binds to a monoclonal antibody against rat lipomodulin, a phospholipase inhibitory protein (Uede et al., 1983). Indeed, the lymphokine exerts phospholipase inhibitory activity upon dephosphorylation with alkaline phosphatase. It was also found that repeated injections of affinity-purified GIF into antigen-primed BDF1 mice suppressed the primary IgE and IgG antibody responses. Analysis of the mechanisms of immunosuppression by GIF indicated that GIF treatment switches a subset of T cells in vivo from the formation of IgE-potentiating factor to the formation of IgE-suppressive factor, and suggested that the treatment facilitated the generation of antigen-specific suppressor T cells, which produce their own GIF (Akasaki et al., 1986).

This hypothesis was supported by subsequent experiments using spleen cells of BDF1 mice primed with alum-absorbed ovalbumin (OVA) for persistent IgE antibody formation. Antigenic stimulation of their spleen cells by OVA resulted in the formation of IgE-potentiating factor, i.e., glycosylated IgE-BF, and glycosylation enhancing factor (GEF) (Uede et al., 1984). However, propagation of the antigen-stimulated T cells by IL-2 in the presence of GIF facilitated the generation of antigen-specific T cells that produce their own GIF. Stimulation of the T cells by OVA-pulsed syngeneic macrophages resulted in the formation of IgE-suppressive factor, i.e., unglycosylated IgE-BF, and GIF that had affinity for OVA (Iwata and Ishizaka, 1987). Our separate experiments indicated that the OVA-binding GIF suppressed the anti-hapten antibody response of BDF1 mice to dinitrophenyl derivatives of OVA (DNP-OVA), but not the antibody response to DNP derivatives of keyhole limpet hemocyanin (DNP-KLH). Indeed, the OVA-binding GIF is similar to some of the antigen-specific suppressor factor (TsF) in

terms of affinity for antigen and association of I-J determinant with the molecules (Jardieu et al., 1987). Thus, we constructed T cell hybridomas from OVA-specific T cells that had been propagated in the presence of GIF. Some of the hybridomas formed OVA-specific GIF upon stimulation with OVA-pulsed syngeneic macrophages, and this factor suppressed the antibody response of BDF1 mice to DNP-OVA in a carrier-specific manner (Iwata and Ishizaka, 1988).

The series of experiments described above suggested a maneuver to generate antigen-specific suppressor T cells in vitro from antigen-primed T cell populations. The present experiments were carried out to determine whether the findings obtained with mouse lymphocytes can be reproduced with peripheral blood lymphocytes of allergic patients who are sensitive to honey bee venom. It is known that the major allergen in honey bee venom is phospholipase A₂ (PLA₂) (Sobotka et al., 1976). As the enzyme is cytotoxic, we employed denatured PLA₂ (D-PLA₂) to stimulate antigen-specific T cells. King et al. (1976) reported that treatment of bee venom PLA₂ with 6 M guanidine HCl in the presence of reducing reagent completely denatures the protein and that the protein loses both enzymatic activity and the antigenic determinant involved in binding to anti-PLA₂ antibodies by the treatment. However, we expected that antigen-primed T cells could be stimulated by the denatured antigen in the presence of syngeneic macrophages or monocytes. Since human GIF was not available, we employed recombinant human lipocortin (Wallner et al., 1986), which can switch mouse T cells from the formation of IgE-potentiating factor to the formation of IgE-suppressive factor, and facilitates the generation of GIF-producing T cells from antigen-primed T cell populations (Ohno et al., 1989). The results of the experiments show that stimulation of peripheral blood mononuclear cells of honey bee venom-sensitive patients with D-PLA₂ followed by propagation of antigen-stimulated T cells by IL-2 in the presence of recombinant lipocortin results in the generation of antigen-specific T cells that produce antigen-binding GIF upon stimulation with the antigen-pulsed autologous monocytes.

Materials and methods

Immunoglobulins and antibodies

Human E myeloma protein P.S., monoclonal rat IgE from IR-162 immunocytoma and monoclonal mouse IgE from the hybridoma H-1-DNP- ϵ -26 (Liu et al., 1980) were purified from either the serum of the myeloma patient or rodent ascitic fluid by the method previously described (Ishizaka, 1985). The purity of IgE in the preparations was 97–99.5%. Dimeric human IgE was the same preparation as that previously described (Kisaki et al., 1987). IgE-dimer was labeled with ^{125}I by the chloramine-T method. The mouse monoclonal antibody (H107) specific for human $\text{Fc}\gamma\text{RII}$ (Noro et al., 1986) was kindly supplied by Dr. J. Yodoi, Kyoto University, Japan. The preparation was the gamma globulin fraction of ascitic fluids of BALB/c nude mice injected with B cell hybridoma. The mouse monoclonal antibody against rabbit lipomodulin 141B9 (Iwata et al., 1984) was the same preparation as that previously described (Akasaki et al., 1986). The mouse monoclonal antibodies OKT3 and OKIa1 were supplied by Ortho Diagnostic Systems Inc., Raritan, NJ. The preparation of OKT3 was the IgG fraction of ascitic fluids. Specifically purified goat antibodies against mouse IgG (anti-MGG) were described in a previous article (Suemura et al., 1980). The antibodies contained both anti- γ chain and anti-light chain antibodies. Human IgE and monoclonal antibody 141B9 were coupled to Sepharose CL-4B; approximately 5 mg of protein were coupled to 1 ml of Sepharose.

Antigens and interleukins

Lyophilized phospholipase A_2 (PLA_2) from bee venom was purchased from Sigma Chemical Co., St. Louis, MO. 5 mg of the protein was coupled to 1 ml of Sepharose CL-4B. Denatured PLA_2 was prepared by the method described by King et al. (1976). 5 mg of PLA_2 were dissolved in 0.1 M Tris-HCl buffer, pH 8.6 and denatured in 6 M guanidine HCl in the presence of 5 mg/ml dithiothreitol. After 18 h at 25°C , the reduced protein was alkylated with iodoacetic acid. The denatured protein was dialyzed thoroughly against 0.02 N acetic acid. As the solubility of D- PLA_2

was low at neutral pH, the protein in acetic acid was kept at -40°C .

Chromatographically purified human interleukin-2 was purchased from Electro Nucleonics, Silver Spring, MD. The preparation did not contain a detectable amount of interferon- γ nor of phytohemagglutinin which was used to stimulate human T cells. Recombinant human interferon- γ (rIFN- γ) was a gift of the Schering, Bloomfield, NJ.

Cell preparation and cell cultures

Peripheral blood was obtained from five patients with allergy to honey bee venom and from two non-allergic individuals. The mononuclear cell fraction of the blood was obtained by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). To obtain monocytes, the mononuclear cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and antibiotics ($5 \times 10^6/\text{ml}$) and were placed in tissue culture wells (24 well plate, Costar, Cambridge, MA). After 2 h at 37°C , nonadherent cells were removed, and adherent cells were cultured for 5–7 days in the RPMI culture medium containing 10 ng/ml rIFN- γ . Preliminary experiments showed that more than 60% of monocytes were stained by OKIa1 in immunofluorescence.

To activate antigen-primed T cells, peripheral blood mononuclear cells (PBMC) from each individual were suspended in RPMI 1640 culture medium as described above. 2 ml each of the cell suspension containing 3×10^6 nucleated cells/ml were placed in wells of Linbro tissue culture plates (Flow Laboratories, McLean, VA) and were cultured for 3 days in the presence of 10 $\mu\text{g}/\text{ml}$ D- PLA_2 . Nonadherent cells were recovered, resuspended in fresh culture medium (2×10^5 nucleated cells/ml), and then cultured for 4 days with purified IL-2 (60 U/ml) in the presence or absence of 3 $\mu\text{g}/\text{ml}$ recombinant lipocortin.

T cells propagated by IL-2 were then stimulated by antigen. The lymphoblastoid cells ($1 \sim 3 \times 10^6/\text{ml}$) were co-cultured for 24 h with a 1/10th number of autologous monocytes in the presence of 10 $\mu\text{g}/\text{ml}$ D- PLA_2 . In some experiments, the T lymphoblastoid cells were stimulated by the monoclonal antibody OKT3. Linbro plate wells

were coated with 10 $\mu\text{g}/\text{ml}$ of specifically purified anti-MGG antibody overnight at 4°C. After washing, 10 $\mu\text{g}/\text{ml}$ of OKT3 antibody were placed in the wells. After 4 ~ 6 h at 4°C, the wells were washed, and a 2 ml suspension of lymphoblastoid cells ($1 \sim 3 \times 10^6/\text{ml}$) in RPMI culture medium was placed in each well. The cells were cultured for 24 h to recover culture supernatants.

Cell lines

RPMI 8866 lymphoblastoid cells were cultured in the same RPMI 1640 culture medium as described above. The rat-mouse T cell hybridomas 23A4 cells (Huff et al., 1982) and mouse T cell hybridoma 12H5 cells (Iwata et al., 1988) have been described before. The 23A4 cells formed unglycosylated IgE-BF upon incubation with rat IgE, while the 12H5 cells formed *N*-glycosylated IgE-BF, when they were cultured with mouse IgE. Both hybridomas were maintained in high glucose Dulbecco's modified Eagle's medium, described in a previous publication (Huff et al., 1982).

Purification and detection of IgE-BF

Culture supernatants of antigen-stimulated or OKT3-stimulated T cells were filtered through Diaflo YM 100 membranes (Amicon Corp., Lexington, MA), and the filtrates were concentrated ten-fold by ultrafiltration through YM 5 membranes. The concentrated culture filtrates were fractionated on human IgE-coupled Sepharose by the described procedures (Ishizaka and Sandberg, 1981). The presence of IgE-BF in the acid eluates from IgE-Sepharose was assessed by inhibition of rosette formation of $\text{Fc}_\gamma\text{R}^+$ B lymphoblastoid cell line RPMI 8866 cells with human IgE coated ox erythrocytes (E'-IgE). The procedure for rosette inhibition has been described (Kisaki et al., 1987). E'-IgE were preincubated with an equal volume of test sample, and RPMI 8866 cells ($10^7/\text{ml}$) were added to the suspension for rosette formation. The proportion of rosette-forming cells (RFC) in 300 RPMI 8866 cells was determined in triplicate and was expressed as the average $\pm$ SD. Considering variations in the proportion of rosette-forming cells in replicate slides, less than 10% rosette inhibition was not significant.

Rodent IgE-BF formed by the 23A4 cells and 12H5 cells were detected by the same procedures

as described above, except that rat IgE-coated ox erythrocytes were employed as indicator cells, and mesenteric lymph node cells of Lewis strain rats infected with *Nippostrongylus brasiliensis* were used as a source of $\text{Fc}_\gamma\text{R}^+$ B cells (Yodoi and Ishizaka, 1980).

Human IgE-BF in the eluates from IgE-Sepharose was also detected by radioimmunoassay using monoclonal anti- $\text{Fc}_\gamma\text{RII}$, H107, which cross-reacts with T cell-derived IgE-BF (Kisaki et al., 1987). The method was described in detail in a previous publication (Carini et al., 1988). In principle, the eluate fraction from IgE-Sepharose was applied to the anti- $\text{Fc}_\gamma\text{RII}$ antibody-coated wells, and the binding of IgE-BF was detected using ^{125}I -labeled IgE dimer. Background count with DPBS, instead of a sample, was subtracted. Previous experiments with several negative controls indicated that binding of more than twice the background count was never obtained with any sample lacking IgE-BF.

Detection of GIF and GEF

GIF was detected using T cell hybridoma 12H5 cells (Iwata et al., 1988) and GEF was detected using 23A4 cells (Iwata et al., 1984). A suspension of the hybridoma cells was mixed with an equal volume of test sample, and the cell suspensions were cultured for 24 h either with 10 $\mu\text{g}/\text{ml}$ mouse IgE (12H5 cells) or with the same concentration of rat IgE (23A4 cells). Culture supernatants were filtered through CF 50A membranes, and filtrates containing IgE-BF were fractionated on lentil lectin Sepharose by exactly the procedures previously described (Yodoi et al., 1980). Proteins bound to lentil lectin Sepharose were eluted with 0.2 M α -methyl-mannoside. Both the effluent and eluate fractions from lentil lectin Sepharose were dialyzed against DPBS and assessed for the presence of IgE-BF by the rosette inhibition technique. When the 12H5 cells were cultured with mouse IgE alone, essentially all IgE-BF formed by the cells had affinity for lentil lectin and were recovered by elution with α -methylmannoside. If a sufficient amount of GIF was added to the culture of 12H5 cells, the majority of IgE-BF formed by the cells lacked affinity for lentil lectin (Iwata et al., 1988). Thus, GIF was taken as (+), if the ratio of the percent rosette inhibition be-

tween the effluent/eluate fraction was 2.0 or higher. In contrast, essentially all IgE-BF formed by the 23A4 cells lacked affinity for lentil lectin when the cells were cultured with rat IgE alone. If the same cells were cultured with rat IgE in the presence of a sufficient concentration of GEF, however, the majority of IgE-BF formed by the cells bound to lentil lectin (Iwata et al., 1984), and recovered in the eluate with α -methylmannoside. The presence of GEF in a test sample was taken as (+) when the ratio of the percent rosette inhibition between the effluent and eluate fractions was 0.2 or lower.

Fractionation and treatment of GIF and GEF

GIF and GEF were fractionated on PLA₂-coupled Sepharose. EGTA (2 mM) and galactose (50 mM) were added to the samples, and a 1 ~ 1.5 ml aliquot of a sample was mixed with 0.4 ml of PLA₂-coupled Sepharose for 24 h at 4°C. The mixture was packed into a small column (diameter 0.75 cm) and the beads were washed with 1.5 ml of PBS containing EGTA. The PLA₂-Sepharose was then eluted with 1.5 ml of glycine-HCl buffer, pH 3.0 and the eluate was adjusted to pH 7.0 with Tris-HCl buffer. The effluent and eluate fractions were concentrated to 0.5 ml and dialyzed against RPMI 1640 medium for the assay of either GEF or GIF activity.

GIF was partially purified by affinity chromatography on anti-lipomodulin (141B9)-coupled Sepharose by the method previously described (Akasaki et al., 1987). Culture supernatants were mixed with the immunosorbent and after washing the immunosorbent with DPBS, proteins bound to the immunosorbent were eluted with glycine-HCl buffer, pH 3.0.

Results

Effect of lipocortin during propagation of antigen-activated T cells on the nature of IgE-BF formed

The experimental design to test the effect of lipocortin is illustrated in Fig. 1. Peripheral blood mononuclear cells from three patients and one normal individual were cultured with 10 μ g/ml D-PLA₂ for 3 days. Nonadherent cells were recovered, and resuspended in fresh culture medium.

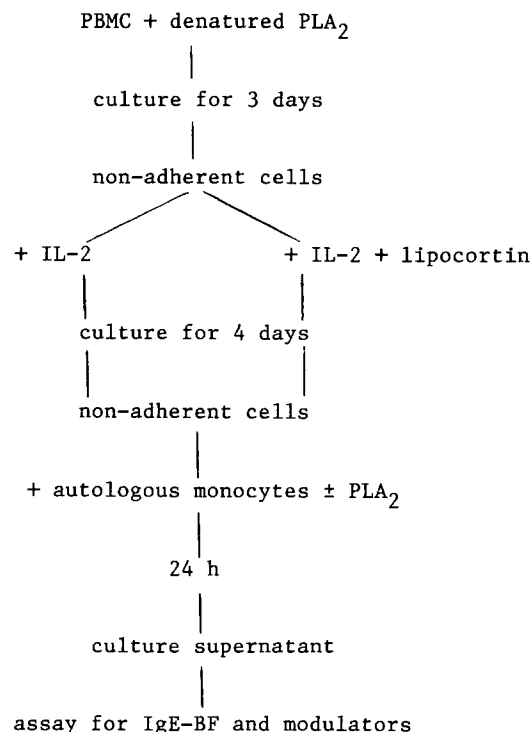


Fig. 1. A protocol for the generation of GIF-producing T cells from peripheral blood mononuclear cells of honey bee venom sensitive patients.

One half of the cell suspension from each individual was cultured with IL-2 in the presence of 3 μ g/ml recombinant lipocortin, and the remainder was cultured with IL-2 alone. After 4 days of culture, T cells propagated by IL-2 were recovered, resuspended in fresh culture medium (2×10^6 nucleated cells/ml) and incubated for 24 h with autologous monocytes in the presence of 10 μ g/ml D-PLA₂. Aliquots of the activated T cells from one patient (DF), which were propagated in the presence of lipocortin, were incubated with autologous monocytes in the presence or absence of the antigen (c.f. Table I). Culture filtrates were fractionated on IgE-sepharose and the acid eluate fractions from the beads were assessed for the presence of IgE-BF (Fig. 2). As shown in Table I, activated T cells from bee venom-sensitive patients formed IgE-BF upon incubation with D-PLA₂ and autologous monocytes, while those from a normal individual failed to form a detectable amount of IgE-BF. It was also found that T cells from one of the patients (DF) failed to form a

TABLE I

EFFECT OF LIPOCORTIN DURING PROPAGATION OF ACTIVATED T CELLS BY IL-2 ON THE NATURE OF IgE-BF FORMED BY THE T CELLS UPON ANTIGENIC STIMULATION ^a

Source of PBL	Allergy to bee sting	Lipocortin in culture	Antigenic stimulation ^b	IgE-BF		Presence of modulator ^c	
				Rosette inhibition ^c (%)	RIA ^d (cpm)	GIF	GEF
GB	+	+	+	24 (22/2)	724	29/3 (+)	ND
		—	+	22 (3/20)	449	2/30 (—)	8/34 (+)
JW	+	+	+	32 (29/5)	745	32/2 (+)	ND
		—	+	21 (2/22)	422	0/33 (—)	4/36 (+)
DF	+	+	+	21	438	25/6 (+)	ND
		+	—	3	44	21/8 (+)	ND
KI	—	+	+	6	31	35/3 (+)	ND
		—	+	3	0	2/38 (—)	31/3 (—)
Control ^f						0/34	40/2

^a Experimental design is shown in Fig. 1.

^b T cells propagated in the presence or absence of lipocortin were incubated with autologous monocytes in the presence or absence of D-PLA₂.

^c IgE-BF in culture supernatants were assessed following the design shown in Fig. 2. The numbers in the column represent the percentage of rosette inhibition. Numbers in parentheses represent the percentage of rosette inhibition by the effluent/eluate fractions from lentil lectin Sepharose. The proportion of IgE-RFC in the absence of IgE-BF was 35.0 ± 1.5 (SD) %.

^d Background count in the assay was 118 cpm, which was subtracted from cpm with test samples. >120 cpm in the column were taken as significant.

^e Presence of GIF or GEF was assessed by using the 12H5 cells or 23A4 cells (c.f. materials and methods section). (+) sign represents the presence of GIF or GEF. The numbers represent the percentage of rosette inhibition by the effluent/eluate fractions from lentil lectin Sepharose. The proportion of IgE-RFC in the absence of IgE-BF in the GIF and GEF assays were 21.7 ± 1.5 (SD) % and 20.5 ± 1.2 (SD) %, respectively.

^f The 12H5 cells (GIF assay) and 23A4 cells (GEF assay) were cultured with IgE, and IgE-BF in culture filtrates were fractionated on lentil lectin Sepharose. All IgE-BF formed by the 12H5 cells bound to lentil lectin, while essentially all IgE-BF formed by the 23A4 cells failed to bind to the lectin.

detectable amount of the factors when they were incubated with autologous monocytes alone.

In order to determine the effect of lipocortin during the propagation of antigen-specific T cells, affinity-purified IgE-BF from two patients (JW and GB) were fractionated on lentil lectin Sepharose. The results showed that essentially all IgE-BF formed by the T cells that had been propagated by IL-2 alone bound to lentil lectin Sepharose and were recovered by elution with α -methylmannoside, whereas the factors formed by the T cells propagated in the presence of lipocortin failed to bind to lentil lectin Sepharose (Table I). The results indicated that addition of lipocortin during propagation of T cells changed the nature of the IgE-BF formed by the T cells.

We determined the GIF/GEF activity in culture filtrates of T cells which had been propagated in the presence or absence of lipocortin. In order to detect GIF, the effluent fraction from IgE-Sepharose, described above, was added to hybridoma 12H5 cells and the cell suspension was cultured for 24 h in the presence of 10 μ g/ml mouse IgE (c.f. Fig. 2). As shown in Table I, culture supernatants of antigen-stimulated T cells that had been propagated in the presence of lipocortin switched the 12H5 cells to form unglycosylated IgE-BF, indicating that the fraction contained GIF. If the same T cells were propagated in the absence of lipocortin, however, culture filtrate of the T cells failed to change the nature of IgE-BF formed by the 12H5 cells. Thus, we de-

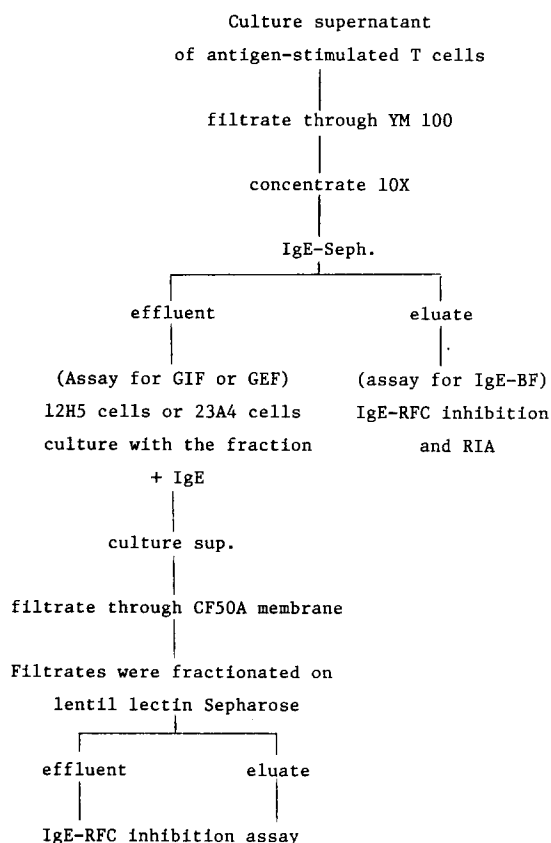


Fig. 2. Protocol for the analysis of IgE-binding factors and for the detection of either glycosylation inhibiting factor or glycosylation enhancing factor in culture supernatant of antigen-stimulated T cells.

terminated whether the latter preparation contained GEF (c.f. materials and methods section). As shown in Table I, the culture filtrate of T cells from two patients (GB and JW) switched the 23A4 cells to form IgE-BF that had affinity for lentil lectin, while those from the normal individual (KI) failed to do so. The results collectively indicate that T cells of the two patients (GB and JW) that had been propagated by IL-2 alone formed GEF, whereas the same cells propagated by IL-2 in the presence of lipocortin released GIF.

Since the cross-linking of CD3 results in the activation of T cells (Landergren et al., 1984), we determined whether the T cells of bee venom-sensitive patients might respond to monoclonal anti-CD3 for the production of IgE-BF. Thus peripheral blood mononuclear cells of two patients (DF and JW) and those of a normal individual (VC)

were cultured with D-PLA₂, and the activated T cells were propagated by IL-2 in the presence or absence of lipocortin. Nonadherent cells recovered from the cultures were resuspended in fresh medium and the suspensions were placed in OKT3-coated wells. After 24 h incubation, culture filtrates were fractionated on IgE-Sepharose and the presence of IgE-BF in the acid eluate fractions was assessed. As shown in Table II, activated T cells from allergic patients formed IgE-BF upon incubation in OKT3-coated wells, but the same cells cultured in anti-MGG-coated wells failed to form a detectable amount of IgE-BF. It was also found that T cells of normal individuals cultured with IL-2 failed to form a detectable amount of IgE-BF. We have also determined the presence of GEF or GIF in the effluent fraction from IgE-Sepharose. The results confirmed that antigen-stimulated T cells propagated by IL-2 alone released GEF, while the same cells propagated in the presence of lipocortin formed GIF (Table II).

Presence of antigen-binding GIF or GEF in culture supernatants of antigen-stimulated T cells

Previous studies in rodent lymphocyte systems indicated that antigen-specific suppressor T cell hybridomas released antigen-binding GIF upon incubation with antigen-pulsed syngeneic macrophages, while the same cells constitutively released nonspecific GIF (Jardieu et al., 1987). Experiments were carried out to determine whether GIF released from antigen-stimulated T cells of bee venom-sensitive patients has affinity for PLA₂. Peripheral blood mononuclear cells of four patients and one normal individual were stimulated with D-PLA₂ and the activated T cells were propagated by IL-2 in the presence of lipocortin. As shown in Table III, T cell blasts from three patients (JW, GB, RH) were incubated with autologous monocytes in the presence of D-PLA₂, and those of two patients (JW, DF) were placed in OKT3-coated wells. As a control, the same T cells from one patient (JW) were cultured for 24 h without stimulation. Culture filtrates were absorbed with IgE-Sepharose, and the effluent fractions, which did not contain a detectable amount of IgE-BF, were fractionated on PLA₂-Sepharose. GIF activity in the effluent and eluate fractions

TABLE II

FORMATION OF IgE-BF BY ANTIGEN-SPECIFIC T CELLS UPON STIMULATION WITH THE MONOCLONAL ANTIBODY OKT3

Source of PBL	Allergy to bee sting	Lipocortin in culture	Stimulation with OKT3 ^a	IgE-BF ^b		Presence of modulator ^c	
				Rosette inhibition (%)	RIA (cpm)	GIF	GEF
DF	+	+	+	20	542	38/2 (+)	ND
		—	+	17	462	6/38 (—)	4/38 (+)
JW	+	+	+	37	598	31/4 (+)	
		+	—	8	110	23/9 (+)	
		—	+	21	564		0/34 (+)
VC	—	+	+	5	0	ND	ND
		—	+	4	0	ND	ND
Control ^d						0/41	42/1

^a T cells propagated by IL-2 in the presence or absence of lipocortin were cultured in OKT3 anti-MGG-coated wells or anti-MGG-coated wells.

^b IgE-BF in culture filtrates were assessed by rosette inhibition and RIA. The numbers represent the percentage of rosette inhibition. The proportion of IgE-RFC in the absence of IgE-BF was 37.0 ± 2.0 (SD) %. Background count in RIA was 138 cpm, which was subtracted from all values. >140 cpm in the column were taken as significant.

^c GIF or GEF in the effluent fraction from IgE-Sepharose (c.f. Fig. 2) was assessed by using the 12H5 cells or 23A4 cells. The numbers represent the percentage of rosette inhibition by the effluent/eluate fraction from lentil lectin Sepharose. The proportion of IgE-RFC in the absence of IgE-BF was $24.5 \pm 1.3\%$ in GIF assay and $26.3 \pm 1.3\%$ in GEF assay.

^d See footnote of Table I.

TABLE III

GIF OBTAINED UPON ANTIGENIC STIMULATION HAS AFFINITY FOR PLA₂

Cell source of PBL	Allergy to bee stings	Stimulation ^a with	GIF in PLA ₂ -Seph. ^b		
			Effluent	Eluate	Control ^c
JW	+	D-PLA ₂ + MO	12/23 (—)	32/2 (+)	0/34 ^d
		OKT3	10/18 (—)	31/4 (+)	0/30 ^e
		No stimulation	25/8 (+)	0/32 (—)	0/30 ^e
GB	+	D-PLA ₂ + MO	10/19 (—)	32/0 (+)	0/29 ^f
RH	+	D-PLA ₂ + MO	24/20 (±)	34/9 (+)	0/41 ^g
DF	+	OKT3	0/24 (—)	32/0 (+)	0/29 ^f
KI	—	D-PLA ₂ + MO	39/6 (+)	3/42 (—)	0/41 ^g

^a Activated T cells propagated in the presence of lipocortin were cultured with autologous monocytes + D-PLA₂ or in OKT3-coated wells. A portion of the cells from J.W. were cultured in anti-MGG-coated wells (no stimulation).

^b Culture supernatants were absorbed with IgE-Sepharose and then fractionated on PLA₂-Sepharose. GIF activity in the effluent and eluate fraction from PLA₂-Sepharose was assessed by using 12H5 cells. The numbers represent the percentage of rosette inhibition by the effluent/eluate fractions from lentil lectin Sepharose. (+, —) indicate the presence or absence of GIF.

^c The 12H5 cells were cultured with IgE alone and IgE-BF in culture filtrates were fractionated on lentil lectin Sepharose. All IgE-BF were recovered in the eluate.

^{d-g} The proportion of IgE-RFC in the absence of IgE-BF in the GIF assay (d-g) were 22.3 ± 0.7 (SD) %; 24.5 ± 1.3 (SD) %; 22.3 ± 0.7 (SD) %; and 23.8 ± 1.2 (SD) %, respectively.

TABLE IV

AFFINITY OF HUMAN GIF FOR MONOCLONAL ANTI-LIPOMODULIN ANTIBODY 141B9

Preparation	Fraction from 141B9-Sepharose	GIF activity ^c effluent/eluante
Culture filtrate ^a	Unbound	7/19 (-)
	Bound ^b	28/3 (+)
Human lipocortin	Unbound	38/0 (+)
	Bound ^b	0/39 (-)
Control		0/39

^a Culture filtrates of antigen-activated T cells were concentrated ten-fold, absorbed with IgE-Sepharose and then fractionated on anti-lipomodulin Sepharose.

^b Proteins bound to 141B9-Sepharose were eluted with glycine-HCl buffer, pH 3.0.

^c GIF activity was determined by the same procedures as shown in Fig. 2 using 12H5 cells. Numbers in the column represent the percentage of rosette inhibition by the effluent/eluante fraction from lentil lectin Sepharose. Control indicate the distribution of IgE-BF formed by the 12H5 cells incubated with IgE alone. The proportion of IgE-RFC in the absence of IgE-BF in the assay was 20.5 ± 1.5 (SD) %.

from PLA₂-Sepharose was assessed using 12H5 cells and the results are summarized in Table III. When T cells from the patients were stimulated with antigen-pulsed monocytes, the majority of GIF activity in culture supernatant bound to PLA₂-Sepharose and was recovered by elution at acid pH. In one case (RH), weak GIF activity may

have been present in the effluent fraction, but the eluate fraction had a higher GIF activity. It was also found that GIF released from unstimulated T cells of one patient (JW), as well as that released from normal T cells, failed to bind to PLA₂-Sepharose. An interesting finding was that T cells from two patients (DF, JW) released antigen-binding GIF when they were cultured on OKT3-coated dishes. The results suggested that antigen-specific T cells released GIF that had affinity for the nominal antigen when they were stimulated through their T cell receptors (TcR).

We determined whether human GIF and/or human recombinant lipocortin binds to monoclonal anti-lipomodulin (141B9). Antigen-stimulated T cells of an allergic patient (DF) were propagated by IL-2 in the presence of lipocortin and the cells were incubated with autologous monocytes in the presence of D-PLA₂. Culture filtrates were absorbed with IgE-Sepharose and unbound protein was concentrated ten-fold. 1 ml of this preparation or 6 µg of human recombinant lipocortin in 1.0 ml DPBS was mixed with 0.5 ml of 141B9-Sepharose overnight. The effluent and washing with 3 ml DPBS were combined and the 141B9 Sepharose was eluted with 4 ml of glycine HCl buffer. After dialysis, both the effluent and eluate fractions were assessed for GIF activity. The results shown in Table IV indicate that GIF from T cells bound to 141B9 Sepharose and was

TABLE V

GEF RELEASED FROM PATIENT'S T CELLS UPON ANTIGENIC STIMULATION HAS AFFINITY FOR PLA₂ ^a

Cell source of PBL	Allergy to bee sting	Stimulation with	GEF in PLA ₂ -Sepharose ^b		
			Effluent	Eluate	Control ^c
JW	+	OKT3	18/11 (-)	0/34 (+)	30/3 ^d
		No stimulation	0/31 (+)	25/4 (-)	30/3 ^d
RH	+	D-PLA ₂ + MO	23/20 (±)	5/33 (+)	28/1 ^e
KI	-	D-PLA ₂ + MO	31/5 (-)	28/3 (-)	28/1 ^e

^a PBMC were stimulated by D-PLA₂, and activated T cells were propagated in the absence of lipocortin. Nonadherent cells were cultured with autologous monocytes + D-PLA₂ or in OKT3-coated wells. A portion of the cell suspension from J.W. was cultured without stimulation.

^b Culture supernatants were absorbed with IgE-Sepharose and then fractionated on PLA₂-Sepharose. GEF activity in the effluent and eluate fractions from PLA₂-Sepharose was assessed by using 23A4 cells. The numbers represent the percentage of rosette inhibition by the effluent/eluante fraction from lentil lectin Sepharose. (+, -) indicate the presence or absence of GEF.

^c The 23A4 cells were cultured with IgE alone, and IgE-BF in culture filtrates were fractionated on lentil lectin Sepharose. The numbers represent the percentage of rosette inhibition by the effluent/eluante fractions from lentil lectin Sepharose.

^{d,e} The proportion of IgE-RFC in the absence of IgE-BF in the rosette inhibition assay (d, e) were 24.6 ± 1.3 (SD) % and 23.9 ± 0.8 (SD) %, respectively.

recovered by elution at acid pH, whereas recombinant human lipocortin failed to bind to the immunosorbent.

The results of the experiments in Tables I and II indicated that antigen-specific T cells propagated in the absence of lipocortin formed GEF. Thus we determined whether the GEF formed by antigen-stimulated T cells has affinity for PLA₂. Antigen-activated T cells from two patients (JW, RH) were propagated by IL-2 alone, and these T cells were cultured with antigen plus autologous monocytes, or in OKT3-coated dishes. An aliquot of the T cells from patient JW was cultured without stimulus. Culture filtrates were absorbed with IgE-Sepharose, and the effluent from the beads was further fractionated on PLA₂-Sepharose. The distribution of GEF activity between the effluent and eluate fractions from PLA₂-Sepharose is shown in Table V. The results indicated that GEF formed by antigen-activated T cells or OKT3-stimulated cells bound to PLA₂-Sepharose and was recovered by elution at acid pH, whereas GEF formed by unstimulated T cells failed to bind to PLA₂-Sepharose. The results also confirmed that normal T cells cultured with IL-2 alone did not produce a detectable amount of GEF when they were cultured with antigen-pulsed syngeneic monocytes.

Discussion

The data described in this paper indicate that antigenic stimulation of allergen-primed human T cells, followed by propagation of the activated T cells by IL-2 in the presence of lipocortin, facilitated the generation of 'antigen-specific' T cell populations that produce GIF. If the same antigen-stimulated cells were propagated by IL-2 alone, GEF rather than GIF was detected in the culture supernatants of the T cells (Table I). Since lipocortin can inhibit the *N*-glycosylation of IgE-BF (Ohno et al., 1989), the possibility may be considered that the 'GIF' detected in the culture supernatants might be due to a carry-over of lipocortin. However, this possibility was excluded by the fact that GIF in the culture supernatant bound to the monoclonal anti-lipomodulin antibody 141B9, whereas recombinant human lipo-

cortin failed to do so (Table IV). The results reproduced previous findings obtained with OVA-primed mouse spleen cells. Stimulation of the cells with OVA-pulsed macrophages followed by propagation of the activated T cells with IL-2 in the presence of rodent GIF (Iwata and Ishizaka, 1988) or human lipocortin (Ohno et al., 1989) facilitated the generation of antigen-specific suppressor T cells that produce their own GIF.

In both human and mouse systems, antigen-specific T cells propagated in the presence of lipocortin or GIF produced unglycosylated IgE-BF and GIF upon antigenic stimulation, whereas those propagated in the absence of lipocortin/GIF formed glycosylated IgE-BF and GEF (Table I; Iwata and Ishizaka, 1988). The mechanisms for the generation of GIF-producing cells are unknown. In the mouse lymphocyte system, not only GIF and lipocortin but also a synthetic PLA₂ inhibitor facilitated the generation of GIF-producing cells (Ohno et al., 1989). The results indicate that PLA₂ inhibitory activity of GIF/lipocortin is responsible for the generation of GIF-producing cells. We speculated that the presence of lipocortin or GIF during the propagation of T cells changed the proportions of GEF-producing T cells and GIF-producing T cells (Iwata and Ishizaka, 1988). However, it is also possible that lipocortin/GIF enhanced the production of GIF and inhibited the formation of GEF by T cells. Indeed, recent experiments in our laboratory (Ohno et al., unpublished data) demonstrated that culture of the GEF-producing hybridoma, 12H5 cells, with GIF stopped the formation of GEF.

When T cells in the D-PLA₂-activated mononuclear cells were propagated by IL-2 in the presence or absence of lipocortin, and then stimulated with D-PLA₂ plus autologous monocytes, the cells released IgE-BF and GIF/GEF, the latter of which had affinity for PLA₂. Incubation of the T cells with either D-PLA₂ alone or autologous monocytes alone failed to induce the formation of these factors. IgE-BF and antigen-binding GIF/GEF were also obtained when the same cells were cultured in OKT3-coated wells. These findings indicate that the formation of IgE-BF and antigen-binding GIF/GEF is the result of the activation of antigen-specific T cells through T cell receptors. Indeed, cross-linking of TcR_{αβ} or CD3 on OVA-

specific GIF/GEF-producing mouse T cell hybridomas, i.e., 231F1 and 12H5 cells, resulted in the formation of IgE-BF and OVA-binding GIF/GEF (Iwata et al., 1989). It appears that both IgE-BF and antigen-binding GIF/GEF are derived from antigen-specific T cells.

The question was raised why T cell-derived factors could bind to nominal antigen such as bee venom PLA₂. We wondered if the affinity of GIF for PLA₂ is related to the property of this lymphokine to inhibit PLA₂. To minimize this possibility, we fractionated GIF preparations on PLA₂-Sephacrose in the presence of EGTA. It is known that PLA₂ is a Ca²⁺-dependent enzyme and that lipomodulin binds to PLA₂ in the presence of Ca²⁺ (Hirata et al., 1984). In the presence of EGTA, GIF from unstimulated T cells failed to bind to PLA₂-sephacrose, while those obtained from antigen-stimulated T cells bound to the beads (Table III). It was also found that PLA₂-binding GIF failed to bind to OVA-coupled Sepharose (results not shown). We have also found that mouse GIF from antigen-stimulated T cell hybridomas, such as 231F1 and 71B4, bound to OVA-Sepharose but not to PLA₂-Sepharose (results not shown). The same principle applies to GEF. As shown in Table V, GEF from unstimulated T cells failed to bind to PLA₂-Sepharose, whereas that from antigen-activated cells bound to the antigen. These findings collectively suggest that GIF and GEF from antigen-stimulated cells bound to PLA₂-Sepharose in an antigen-specific manner. Recent experiments in our laboratory on OVA-binding mouse GIF from the T cell hybridoma 231F1 cells have shown that the OVA-binding factors bind to the same epitope as that recognized by TcR_{αβ} on the cells in the context of class II molecules (Ishizaka et al., 1989). The experiments also showed that the OVA-binding factors bind to monoclonal anti-TcR_{αβ}. These findings support the idea that the antigen-binding GIF/GEF are derived from antigen-specific T cells, and have a close relationship to TcR_{αβ}.

Our previous studies in the mouse system indicated that antigen-binding GIF shares common properties with the NP-specific TsF3 (Furusawa et al., 1984), GAT-specific TsF2 (Turck et al., 1986) and KLH-specific TsF (Saito and Taniguchi, 1984). All of the TsFs as well as antigen-binding

GIF bind to nominal antigen (hapten), possess I-J determinant(s) and selectively suppress the antibody response in an antigen (carrier)-specific manner. Furthermore, evidence was obtained that KLH-specific TsF, NP-specific TsF3 and OVA-specific GIF (Jardieu and Ishizaka, 1987) are composed of two polypeptide chains, one of which binds to homologous antigen, and another peptide has an I-J determinant. The possible relationship between TsF and GIF is supported by the fact that not only GIF but also both NP-specific TsF₁ and TsF₃ bound to anti-lipomodulin (141-B9)-Sepharose and were recovered by elution at acid pH (Steele et al., 1989). We speculate that PLA₂-binding human GIF obtained in the present experiments is an antigen-specific TsF. We have previously constructed the mouse T cell hybridoma 71B4 from the OVA-specific mouse T cells that had been propagated by IL-2 in the presence of GIF (Iwata and Ishizaka, 1988). The T cell hybridoma produced OVA-binding GIF upon antigenic stimulation, and this factor suppressed the anti-hapten antibody responses of BDF1 mice to DNP-OVA in a carrier-specific manner. One may expect that a sufficient amount of PLA₂-binding GIF (TsF) could be obtained by constructing T cell hybridomas from PLA₂-specific human T cells that had been propagated by IL-2 in the presence of recombinant lipocortin, and by stimulating the hybridoma with anti-CD3 antibody. In more recent experiments, which will be published separately, we have constructed human T cell hybridomas from such T cells and obtained GIF-producing hybridomas. Considering the antigen (carrier)-specific immunosuppressive effects of antigen-binding GIF in the mouse system, one may expect that PLA₂-specific GIF (TsF) from such a human hybridoma is useful to suppress the *in vivo* IgE antibody response of the donors of the original T cells to the allergen.

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