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Epitope specificity of bee venom phospholipase A₂-specific suppressor T cells which produce antigen-binding glycosylation inhibiting factor

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Abstract

From the spleen cells of BALB/c mice primed with bee venom phospholipase A₂ (PLA₂), we established seven T cell hybridomas which constitutively secreted glycosylation inhibiting factor (GIF), expressed both CD3 and TCR $\alpha\beta$, and responded to antigen-pulsed antigen presenting cells (APC) for the formation of IgE-binding factor. Upon stimulation with antigen-pulsed APC, four of the seven hybridomas produced GIF having affinity for native PLA₂. The antigen-binding GIF could suppress the anti-hapten antibody response of BALB/c mice to dinitrophenyl (DNP) – PLA₂ conjugates in a carrier-specific manner and bound to immunosorbents coupled with either the mAb 14-12 or anti-TCR α chain, H28-710. Analysis of the epitope specificity of the TCR on the GIF-producing T hybridomas indicated that all of the hybridomas which could produce antigen-binding GIF upon antigenic stimulation recognized the synthetic peptide representing amino acid residues 19–34 in PLA₂ molecules in the context of the product of the I-A^d subregion and the antigen-binding GIF formed by the cells had affinity for the peptide. The 3-D structure of bee venom PLA₂ indicates that the sequence of amino acid 14–24 forms a loop in the PLA₂ molecule and represents an external structure of the antigen, while peptide 25–37 forms an α helix. Evidence was obtained which suggests that the sequence of 25–34 contains amino acid residues interacting with Ia molecules, while peptide 19–24 contains residues involved in the interaction of p19–34 – Ia complexes with TCR on the hybridomas. It was also found that not only the synthetic peptide 19–34, but also the peptides 13–28 and 19–30 inhibited the binding of antigen-binding GIF to PLA₂-coupled Sepharose, while peptide 25–40 failed to do so. The results collectively indicate that the antigen-binding GIF and TCR on the cell source of the factor interact with a common epitope which is exposed on the surface of a nominal antigen.

Introduction

In the course of previous studies on the regulation of IgE antibody response, we have obtained evidence that antigen-specific suppressor T cells constitutively secrete glycosylation inhibiting factor (GIF), that could switch a subset of T cells and T cell hybridomas from the formation of glycosylated IgE-binding factors (IgE-BF) to the formation of unglycosylated IgE-BF (1). The resulting unglycosylated IgE-BF suppressed IgE synthesis in an isotype-specific manner. Subsequent experiments indicated that GIF is probably a subunit of antigen-specific suppressor T cell

factors (TsF). An ovalbumin (OVA)-specific suppressor T hybridoma, 231 F1 cells, constitutively secretes the 15 kDa GIF, having no affinity for OVA but no IgE-BF. Upon stimulation with OVA-pulsed syngeneic macrophages, however, the same cells produced IgE-BF and the 80 kDa GIF, the latter of which had affinity for OVA (2). The OVA-binding GIF appeared to consist of an antigen-binding polypeptide chain and a non-specific GIF chain (3), and suppressed both IgE and IgG1 antibody responses of syngeneic mice to DNP – OVA in a carrier-specific manner (2).

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Furthermore, the OVA-binding GIF bound to anti-TCR α chain antibody and mAb 14-12, the latter of which was believed to be specific for the antigen-binding chain of effector type TsF (4). Thus, the OVA-binding GIF shares antigenic determinants, physicochemical properties, and immunosuppressive activities with the effector-type TsF, such as keyhole limpet hemocyanin-specific TsF (5), GAT-specific TsF₂ (6), and NP-specific TsF₃ (7).

Several investigators indicated a close relationship between antigen-specific TsF and TCR. TsF derived from T cell hybridomas and suppressor T clones are bound by the monoclonal anti-TCR α chain antibody (4,8–10). Evidence has been presented that the TCR α chain is a critical component of TsF (11). Variants of suppressor T cell hybridomas that failed to express the TCR α chain cDNA did not produce TsF and transfection of the TCR α chain cDNA into these cells restored TsF bioactivity (12,13). These findings clearly show that TsF is actually a product of T cells and utilizes gene(s) encoding the TCR α chain. However, the most difficult question to be answered is why TsF have affinity for the nominal antigen and could be enriched by binding to antigen-coupled immunosorbent. It is well established that the TCR on helper and cytotoxic T cells recognizes processed antigen in the context of a MHC product and nominal antigen itself cannot stimulate these cells (14). Thus, we anticipated that the cell source of antigen-binding GIF (TsF) may be specific for a unique epitope and studied the epitope specificity of the TCR on the cell source of OVA-binding GIF (15). The results showed that TCR on all of seven T cell hybridomas, which produce OVA-binding GIF (TsF), recognized a synthetic peptide corresponding to residues 307–317 in the OVA molecules in the context of class II molecules on antigen presenting cells (APC). In contrast, the other three GIF-producing hybridomas, which recognized the major immunogenic epitope represented by residues 323–339 in the OVA molecules, failed to form OVA-binding GIF upon antigenic stimulation. Furthermore, OVA-binding GIF produced by the former group of hybridomas had affinity for the peptide 307–317 for which the TCRs on the cell source of the factors are specific (4). These findings supported the concept that TsF (or antigen-binding GIF) is related to the TCR.

Since the OVA-binding GIF had affinity for p307–317, we speculated that the epitope represents an external structure in OVA molecules (4). In this series of experiments, however, we could not provide evidence that the epitope is actually exposed to the surface of the antigen molecules. In the present experiments, attempts were made to construct GIF-producing T cell hybridomas from spleen cells of mice that had been immunized with bee venom phospholipase A₂ (PLA₂), whose crystal structure is known (16). The present experiments provide the evidence that the TCR on the cell source of antigen-binding GIF is actually specific for an external structure in the antigen and that the antigen-binding factor shares epitope specificity with the TCR.

Methods

Antigen and antibodies

Lyophilized bee venom PLA₂ was purchased from Sigma (St Louis, MO). Denatured PLA₂ and CNBr-treated PLA₂ were prepared by the methods described by King *et al.* (17). Detailed procedures were previously described (18). In order to obtain

CNBr-cleaved fragments, 5 mg of CNBr-treated PLA₂ was dissolved in 0.1 M Tris–HCl, pH 8.6, and 5 mg dithiothreitol was added to the solution. After 18 h at room temperature, peptides were incubated at pH 8.0 for 60 min with excess iodoacetamide. After carboxymethylation, the pH was adjusted to 3.0–3.2 and the fragments were applied to a Bio-Gel P-10 column (2.5 × 30 cm), equilibrated with 0.05 M acetic acid. Peptides eluted from the column were lyophilized, and further fractionated by reverse-phase chromatography using an RPC (C₁₈) column (Beckman, Fullerton, CA). The mobile phase was 0.1% trifluoroacetic acid (Sigma) and peptides were eluted with a linear gradient of 2-propanol (Aldrich, Milwaukee, WI) from 0 to 60% in 30 min at a flow rate of 0.5 ml/min. The peptide under each peak was lyophilized separately. The amino acid composition of the peptides was analyzed by Dr T. Hugli at Scripps Clinic and Research Foundation. Synthetic peptides corresponding to residues 1–16, 7–22, 13–28, 19–34, and 25–40 in the PLA₂ molecule were synthesized by Cytel Corp. (La Jolla, CA). The peptides corresponding to residues 19–30 and 19–35 were synthesized by Kirin Pharmaceutical (Maebashi, Japan). All of the synthetic peptides were purified by HPLC and their amino acid sequences were confirmed.

Dinitrophenyl derivatives of PLA₂ were prepared using 2,4-dinitrobenzene sulfonic acid (Eastman-Kodak, Rochester, NY). The average number of DNP groups in the DNP–PLA₂ conjugates was 1.7 per PLA₂ molecule. The preparation of DNP–OVA was previously described (19). The conjugates contained 4.3 groups of DNP per molecule of OVA.

Monoclonal mouse IgE (H-1, DNP- ϵ -26) (20), rat IgE (IR 162), and anti-lipomodulin (141-B9) (21) were the same preparations as those described in a previous article (19). Monoclonal anti-mouse CD3 (145-2C11) (22) was supplied by Dr M. Soloski, Johns Hopkins University. Anti-mouse TCR α chain (H-28-710) (23) and anti-mouse TCR β chain mAb (H-57-597) (24) were supplied by Dr R. T. Kubo, National Jewish Center for Immunology and Respiratory Medicine (Denver, CO). Ascitic fluid containing rat mAb 14-12 (25) or 14-30 (26) and the globulin fraction of the ascitic fluid were previously described (4). Anti-I-A^d mAb MKD6 (27) and anti-I-E mAb 14.4.4 (29) employed in the present experiments were the same preparations as those previously described (15).

Immunosorbents

PLA₂ or peptide 19–35 was coupled to CNBr-treated CL-Sepharose 4B at 1 mg protein (peptide) per gel. The globulin fraction of ascitic fluid containing mAb 14-12 or partially purified mAb 141-B9 was coupled to CL-Sepharose 4B at 4–5 mg per gel. Monoclonal anti-TCR α chain was coupled to Tresyl activated Sepharose at 1 mg/ml gel.

Construction of hybridomas

GIF-producing T cell hybridomas were constructed by the procedures described in previous articles (19,29) from spleen cells of BALB/c mice which had been immunized with 10 μ g PLA₂ adsorbed to 4 mg alum. Two weeks after the immunization, their spleen cells suspended in RPMI 1640 medium containing 10% FCS were incubated with 10 μ g/ml CNBr-treated PLA₂ for 3 days. Non-adherent cells were resuspended in fresh culture medium and T cells were propagated for 4 days

by incubation with 50 units/ml recombinant mouse IL-2 in the presence of 2 μ M synthetic PLA₂ inhibitor, ONO-RS-082 [2-(*p*-amylcinnamoyl)-amino-4-chlorobenzoic acid, ONO-Pharmaceutical Co., Osaka, Japan] (29). T cells were then fused with thymoma clone BW5147 cells by the procedures previously described (19). Hybridomas were maintained in complete Dulbecco's modified Eagle's medium (DMEM) that consisted of high glucose DMEM supplemented with antibiotics, 10% FCS, 10% NCTC 135 medium (Gibco, Grand Island, NY), 10 mM HEPES buffer, 0.2 U/ml bovine insulin (Sigma), 50 μ g/ml sodium pyruvate, and 150 μ g/ml oxalacetic acid.

Antigen presentation to T cell hybridomas

T cell hybridomas (1 $\times$ 10⁶ cells/ml) in DMEM were incubated for 24 h with BALB/c-derived B cell line A20.3 or A20-2J cells (2 $\times$ 10⁵ cells) in the presence of 10 μ g/ml CNBr-treated PLA₂ or 5 μ g/ml of an appropriate peptide. In some experiments, A20.3 cells were incubated overnight with 30 μ g/ml PLA₂ and the antigen-pulsed APC were incubated with an appropriate hybridoma cell. The A20.3 cells were fixed with glutaraldehyde by the method described by Shimonkevitz *et al.* (30). In order to determine any possible role of Ia molecules, the fixed cells were treated for 30 min at 37°C with 10 μ g/ml anti-I-A^d or anti-I-E antibody. After washing, the cells were incubated with T cell hybridomas together with synthetic peptide for antigen presentation.

Detection and fractionation of IgE-BF

The factor was detected by inhibition of rosette formation of rat Fc ϵ R⁺ B cells with rat IgE-coated, fixed ox erythrocytes (31). Fixed ox erythrocytes coated with human serum albumin were employed as controls. Mesenteric lymph node cells of Lewis strain rats infected with *Nippostrongylus brasiliensis* were used as a source of Fc ϵ R⁺ cells. Procedures for the detection of IgE-BF were exactly as previously described (31). The proportion of rosette forming cells (RFC) in 300 lymphocytes was determined in triplicate and expressed by the average $\pm$ SD. The percentage of rosette inhibition of IgE-BF was calculated from the decrease in the proportion of RFC in the presence of IgE-BF. The SD in the percentage of rosette inhibition among triplicate assays was usually 3–6%.

In some experiments, culture supernatants were fractionated on rat IgE-coupled Sepharose (32) and the acid eluate fraction from the immunosorbent was tested for the presence of IgE-BF by the procedures described above. In order to determine the nature of IgE-BF, IgE-BF were fractionated on lentil lectin Sepharose (Pharmacia, Piscataway, NJ) by the procedures previously described (32). Proteins bound to the beads were recovered by elution with 0.2 M α -methylmannoside.

Detection and fractionation of GIF

GIF was detected by the ability of this lymphokine to switch the T cell hybridoma 12H5 cells from the formation of glycosylated IgE-BF to the formation of unglycosylated IgE-BF (19). Briefly, 12H5 cells (1 $\times$ 10⁶ cells/ml) were incubated for 24 h with 10 μ g/ml mouse IgE in the presence or absence of a test sample. Culture supernatants were filtered through CF50A membranes to remove IgE and filtrates were fractionated on lentil lectin Sepharose by the method described above. The presence of IgE-BF in the effluent fraction and eluate fraction was then

determined by rosette inhibition. When the 12H5 cells were incubated with mouse IgE alone, essentially all IgE-BF formed by the cells bound to lentil lectin and was recovered in the eluate with α -methylmannoside. If a sufficient amount of GIF was added to the culture of 12H5 cells, all IgE-BF formed by the cells failed to be retained in the lentil lectin Sepharose and was recovered in the effluent fraction. GIF in the test sample was taken as (+) if the ratio of the percent rosette inhibition between the effluent and eluate fraction was 2.0 or higher.

GIF was also detected by its ability to inhibit the development of RFCs with IgE-coated erythrocytes (19). Briefly, mesenteric lymph node cells of rats infected with *N. brasiliensis* were mixed with an equal volume of a sample to be tested and the cells were incubated for 24 h with rat IgE. The proportion of Fc ϵ R⁺ cells in the suspension was determined by rosetting with IgE-coated erythrocytes.

To determine the affinity of GIF for PLA₂ or its peptide, the culture supernatant of hybridomas was first absorbed with IgE–Sepharose. Aliquots of 1 ml of the effluent from IgE–Sepharose were fractionated on 0.25 ml PLA₂–Sepharose by the procedures previously described (18). Binding of GIF to anti-TCR α chain-coupled Sepharose or mAb 14-12-coupled Sepharose was determined in a similar manner (4). Proteins bound to the immunosorbent were eluted with 0.1 M glycine–HCl buffer, pH 3.0, and tested for the presence of GIF by the procedures described above.

Flow cytometry

Hybridoma cells were incubated with a saturating concentration of anti-CD3 or anti-TCR mAb at 0°C, followed by FITC-labeled rabbit anti-hamster IgG (Organon Teknika, Westchester, PA) (15,33). Cell-associated fluorescence was analyzed by using a FACScan (Becton-Dickinson, Mountain View, CA).

Determination of the immunosuppressive effects of GIF

Groups of four BALB/c mice were immunized by an intraperitoneal injection of 2 μ g DNP–PLA₂ included in 2 mg alum or 10 μ g

Table 1. Formation of IgE-BF and antigen-binding GIF by antigenic stimulation of PLA₂-specific hybridomas

Hybridoma	IgE-BF ^a	GIF activity in PLA ₂ –Sepharose ^b	
		flow-through	eluate
6C1	27	0/30 (–)	25/7 (+)
3B3	21	1/28 (–)	33/0 (+)
8C4	22	0/42 (–)	40/5 (+)
2A2	24	5/36 (–)	34/8 (+)
3E6	30	27/4 (+)	1/30 (–)
9A4	19	28/2 (+)	9/22 (–)
7B5	21	32/7 (+)	6/27 (–)
Medium control	–	0/32	–

^aCulture supernatants were absorbed with IgE–Sepharose and IgE-BF in acid eluate fractions was determined by rosette inhibition. Numbers represent percent rosette inhibition.

^bFlow-through fraction from IgE–Sepharose was fractionated on PLA₂–Sepharose and the GIF activity in each fraction was determined by using the 12H5 cells (cf. Methods). Numbers represent percent rosette inhibition by the effluent/eluate fraction from lentil lectin Sepharose. The proportion of RFCs in the absence of IgE-BF was 28.9 $\pm$ 0.6%. The (+) and (–) indicate the presence or absence of GIF.

DNP-OVA absorbed to 2 mg alum. Animals received i.v. injections of 0.2 ml GIF sample on day 0, 2, 4, and 6, and anti-hapten antibodies were measured on day 7, 14, 21, and 28. Anti-DNP IgG1 antibodies were measured by ELISA using monoclonal anti-DNP IgG1 antibody as a standard. Detailed procedures were described in a previous publication (33). IgE antibodies were measured by ELISA with an amplification system (34).

Results

Construction of GIF-producing hybridomas specific for bee venom PLA_2

The hybridomas were constructed from PLA_2 -primed BALB/c spleen cells by the procedure described in Methods. Culture supernatants of 87 hybridomas were assessed for the presence of GIF activity and 39 GIF-producing hybridomas were selected. These hybridomas were incubated with A20.2J cells in the presence of CNBr-treated PLA_2 for antigen stimulation, and the hybridoma clones that produced IgE-BF were then stained with anti-CD3 and anti-TCR β chain by immunofluorescence. Thus, we established seven hybridomas which (i) were well-stained by both anti-CD3 and anti-TCR β chain, (ii) constitutively secreted GIF, and (iii) produced IgE-BF upon antigenic stimulation. None of the unstimulated hybridoma clones constitutively secreted a detectable amount of IgE-BF. These hybridomas were subcloned and the CD3 $^+$ subclone of each hybridoma was incubated with antigen-pulsed A20.2J cells. Culture supernatants were absorbed with IgE-Sepharose and acid eluates of the column were assessed for the presence of IgE-BF. As expected, all of the hybridomas formed IgE-BF upon antigenic stimulation (Table 1). The flow-through fraction from the IgE-Sepharose was further fractionated on a PLA_2 -Sepharose column to determine the distribution of GIF activity. As shown in Table 1, the 6C1, 3B3, 8C4, and 2A2 cells produced GIF having affinity for PLA_2 -Sepharose, whereas GIF from the 3E6, 9A4, and 7B5 cells failed to be retained in PLA_2 -Sepharose.

Table 2. Binding of GIF from antigen-stimulated hybridomas to 14-12-coupled Sepharose

Hybridomas	Cultures of GIF production ^a	GIF in 14-12-coupled Sepharose ^b	
		flow-through	eluate
3B3	unstimulated	22/3 (+)	5/23 (-)
	antigen-stimulated	5/32 (-)	26/8 (+)
9A4	unstimulated	32/0 (+)	0/32 (-)
	antigen-stimulated	8/25 (-)	29/0 (+)
7B5	unstimulated	20/3 (+)	3/30 (-)
	antigen-stimulated	5/25 (-)	31/5 (+)
Medium control		3/26	

^aHybridoma cells (10^6 cells/ml) were cultured with A20.3 cells (2×10^5 cells/ml) in the presence or absence of 3 μ g/ml CNBr-treated PLA_2 for 24 h.

^bPartially purified GIF in culture supernatants was fractionated on 14-12-coupled Sepharose. The GIF activity in the flow-through fraction and acid eluate fraction from 14-12-coupled Sepharose was evaluated by using 12H5 cells. The proportion of IgE-rosetting cells in the absence of IgE-BF was $27.4 \pm 0.8\%$. The (+) and (-) indicate the presence or absence of GIF.

We determined whether the mAb 14-12 can bind GIF from antigen-stimulated PLA_2 -specific hybridomas. The three representative hybridomas, i.e. 3B3, 9A4, and 7B5, were stimulated with antigen-pulsed APC or co-cultured with APC alone. GIF in culture supernatants was partially purified using 141B9-Sepharose, and the affinity-purified GIF was further fractionated on 14-12-Sepharose. It is clear in Table 2 that GIF from unstimulated hybridomas failed to be retained in the 14-12-Sepharose column. In contrast, GIF from antigen-stimulated hybridomas bound to the immunosorbent and was recovered by acid elution. It should be noted that GIF from antigen-stimulated 7B5 and 9A4 cells failed to be retained on PLA_2 -coupled Sepharose (cf. Table 1), but bound to 14-12-Sepharose. In order to confirm the specificity of the immunosorbent, aliquots of the GIF preparations from antigen-stimulated 3B3 cells and 9A4 cells were fractionated on Sepharose coupled with mAb 14-30, as isotype controls. As expected from previous findings (4,33), GIF activity failed to be retained in the 14-30-Sepharose (results not shown).

Previous experiments indicated that the OVA-binding GIF from T cell hybridomas shared an antigenic determinant with TCR α chain (4). Thus, we determined as to whether the PLA_2 -binding GIF may bind to Sepharose coupled with anti-TCR α chain. The PLA_2 -binding GIF from the 3B3 cells was affinity-purified by using PLA_2 -coupled Sepharose and the preparation was fractionated on anti-TCR α chain-coupled Sepharose. Determination of the GIF activity in the flow-through fraction, washing, and acid eluate fractions clearly showed that the PLA_2 -binding GIF bound to the immunosorbent and was recovered by elution at acid pH. As expected from a previous publication on GIF from OVA-specific hybridomas (33), the presence of the antigenic determinant recognized by anti-TCR α chain was not restricted to PLA_2 -binding GIF. When the 9A4 cells were stimulated with antigen-pulsed A20.2J cells, GIF in the culture supernatant bound to anti-TCR α -coupled Sepharose and was recovered by acid elution. In contrast, GIF from

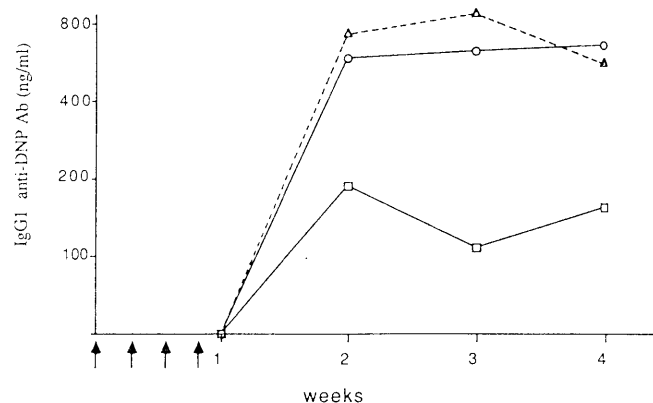


Fig. 1. Suppression of anti-hapten antibody response to DNP- PLA_2 by PLA_2 -binding GIF. BALB/c mice were immunized with alum-adsorbed DNP- PLA_2 and the IgG1 anti-hapten antibody concentration in their serum was determined. Three groups of mice received an i.v. injection of PLA_2 -binding GIF (□), non-specific GIF (Δ) or saline (○) on the days indicated by arrows.

unstimulated 3B3 cells and 9A4 cells failed to bind to the immunosorbent.

We determined whether the PLA₂-binding GIF from 3B3 cells could suppress the antibody response of BALB/c to DNP-PLA₂ conjugates. The PLA₂-binding GIF was purified from the culture supernatant of antigen-stimulated cells using 14-12-Sephrose, while non-specific GIF was purified from culture supernatants of unstimulated cells using 141B9-Sephrose. Both the PLA₂-binding GIF and non-specific GIF preparations could switch the 12H5 cells at a dilution of 1:100. Three groups of BALB/c mice were immunized with alum-absorbed DNP-PLA₂. One group was treated by i.v. injections of PLA₂-binding GIF, while another group of mice were treated with non-specific GIF. The third group received i.v. injections of DPBS. The IgG1 anti-DNP antibodies in their sera were measured by an ELISA assay and the results are illustrated in Fig. 1. It is evident that the PLA₂-binding GIF markedly suppressed anti-DNP antibody response, while non-specific GIF failed to do so. Further, the PLA₂-binding GIF failed to suppress the anti-DNP IgG1 or IgE antibody response of BALB/c mice to alum-absorbed DNP-OVA (results not shown). The results collectively indicated that PLA₂-binding GIF is an antigen-specific TsF.

Epitopes recognized by the TCR on GIF-producing hybridomas

As preliminary experiments, we confirmed that not only CNBr-treated PLA₂ but also denatured PLA₂ could stimulate the PLA₂-specific hybridomas for the formation of IgE-BF when they were cultured with APC and the antigen. Since the denatured antigen was obtained by treatment of PLA₂ with 6 M guanidine-HCl in the presence of 5 mg/ml dithiothreitol, followed by alkylation (17), it appears that neither disulfide bonds nor the conformational structure in the original PLA₂ was required for stimulation of the T cell hybridomas. Attempts were therefore made to stimulate the hybridomas by fragments of PLA₂. The amino acid sequence of bee venom PLA₂ (35) indicates that CNBr treatment followed by cleavage of the disulfide bonds should produce three fragments representing amino acid residues 1-36 (p1-36), 42-86 (p42-86), and 87-134 (p87-134), and a small fragment representing amino acid

residues 37-41. Thus, CNBr-treated PLA₂ were reduced, alkylated, and cleavage products were fractionated by gel filtration through a Biogel P-10 column. The results of chromatography are shown in Fig. 2. The first small peak (Fr I) corresponded to the elution volume of the native PLA₂, while the second and the third peaks represented CNBr cleavage products. No protein or peptide was detected in Fr IV. Further fractionation of Fr II and Fr III by reverse-phase column chromatography on a C₁₈ column showed that Fr II was composed of two peptides (II₁ and II₂), while Fr III represented a single peptide. Incubation of the 3B3 cells with each of the three fragments in the presence of A20.2J cells showed that the Fr II₁ peptide stimulated the 3B3 cells for the formation of IgE-BF and antigen-binding GIF. Neither Fr II₂ nor Fr III induced IgE-BF formation, and GIF present in culture supernatants of the Fr II₂ or Fr III-stimulated cells failed to bind to PLA₂-coupled Sepharose. Similar experiments with 9A4 cells gave the same results. Fr II₁ peptide, but neither Fr II₂ nor Fr III, stimulated the 9A4 cells for the formation of IgE-BF. Since the GIF from antigen-stimulated 9A4 cells failed to bind to PLA₂-Sepharose but bound to the 14-12-Sephrose (cf. Tables 1 and 2), the flow-through fractions from IgE-Sephrose were fractionated on 14-12-Sephrose. It was found that GIF in Fr II₁-stimulated 9A4 cells bound to the 14-12-Sephrose, while the GIF formed by both the Fr II₂-stimulated and Fr III-stimulated cells failed to be retained in the immunosorbent. The amino acid composition of Fr II₁ and Fr III clearly showed that Fr II₁ corresponds to the N-terminal peptide representing amino acid residues 1-36, while Fr III is the C-terminal peptide representing amino acid residues 87-134. Thus it appears that both the 3B3 and 9A4 hybridomas responded to the N-terminal fragment 1-36 in the context of MHC product.

We tested the ability of five synthetic peptides representing residues 1-16, 7-22, 13-28, 19-34, and 25-40 to stimulate the hybridoma cells in the presence of APC. Since unstimulated hybridomas did not secrete IgE-BF, the ability of the peptides was evaluated by detecting IgE-BF in their supernatant of antigen-stimulated cells. The results, summarized in Table 3, show that the 3B3 cells responded to p19-34, while the 3E6 cells responded to p25-40. Thus, we tested the epitope specificity

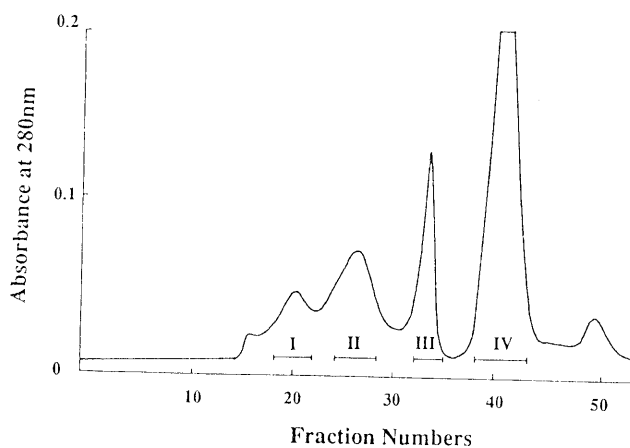


Fig. 2. Gel filtration of CNBr fragments of bee venom PLA₂ on a Biogel P-10 column. Fractions indicated were pooled separately and lyophilized.

Table 3. Formation of IgE-BF by stimulation with synthetic peptides^a

Peptide	Hybridoma						
	3B3	6C1	8C4	2A2	3E6	9A4	7B5
p1-16	2	ND	ND	ND	2	0	24
p7-22	0	0	ND	ND	3	ND	3
p13-28	4	4	0	0	0	2	0
p19-34	23	19	18	20	0	0	5
p25-40	4	5	0	3	19	20	0
CNBr-PLA ₂	21	18	18	16	20	16	22

^aHybridoma cells were cultured with A20-2J cells in the presence of 5 µg/ml synthetic peptide. Culture supernatants were fractionated in IgE-Sephrose and acid eluate fractions were assessed for the presence of IgE-BF by rosette inhibition. The numbers in the table represent percent rosette inhibition. The proportion of IgE RFCs in the absence of IgE-BF was 29.5 ± 0.7%. Less than 5% of rosette inhibition was statistically not significant in this experiment.

Table 4. Stimulation of T hybridomas with synthetic peptide in the context of the I-A^d product

Hybridoma	Fixed APC treated with ^a	Antigen added ^b	IgE-BF ^c (%)
3B3	none	p19–34	28
	anti-I-A ^d	p19–34	0
	anti-I-E	p19–34	30
	none	CNBr–PLA ₂	0
9A4	none	p25–40	20
	anti-I-A ^d	p25–40	0
	anti-I-E	p25–40	18
	none	CNBr–PLA ₂	0
	none	CNBr–PLA ₂	0

^aGlutaraldehyde-fixed cells were treated with either anti-I-A^d or anti-I-E antibody (10 µg/ml) for 30 min at 37°C.

^b3B3 cells and 9A4 cells (10⁶ cells/ml) were incubated with glutaraldehyde-fixed A20.3 cells (5 × 10⁵ cells/ml) for 24 h in the presence of 10 µg/ml p19–34 or p25–40, or CNBr-treated PLA₂.

^cNumbers in this column represent percent inhibition of IgE rosettes. In the absence of IgE-BF, the proportion of RFCs was 29.5 ± 0.6%.

of the other GIF-producing hybridomas. As shown in Table 3, all of the four hybridomas which produce antigen-binding GIF upon antigenic stimulation, i.e. 3B3, 6C1, 8C4, and 2A2 (cf. Table 1), responded to p19–34 for the formation of IgE-BF, while the other three hybridomas, i.e. 7B5, 3E6, and 9A4, which produced GIF having low affinity, if any, for the native antigen, responded to either p1–16 or p25–40, but not to p19–34.

Since our experiments shown in Table 2 indicated that GIF from the antigen-stimulated cells possesses antigenic determinant recognized by mAb 14-12, we determined whether the hybridoma cells stimulated by each of the five synthetic peptides in the presence of APC produced GIF having this determinant. Culture supernatants of the antigen-stimulated hybridoma cells shown in Table 3 were absorbed with 14-12–Sepharose and proteins bound to the immunosorbent were eluted at acid pH. The acid eluate fractions were then assessed for GIF activity. Results of the experiments clearly showed that the 3B3 and 6C1 cells responded to p19–34 for the formation of GIF having the 14-12 determinant, i.e. 14-12⁺ GIF, while the other peptides failed to induce the same cells to form 14-12⁺ GIF. In contrast, the 9A4 cells formed the 14-12⁺ GIF only when they were cultured with p25–40 in the presence of APC. Comparisons of the results with those presented in Table 3 show that the same synthetic peptide induces the formation of IgE-BF and 14-12⁺ GIF in the presence of APC.

We confirmed that processing of the synthetic peptides by APC is not required for stimulation of 3B3 cells and 9A4 cells, and that class II molecules are involved in the presentation of the peptide. The A20.3 cells were fixed with glutaraldehyde and the cell suspensions were treated with either anti-I-A^d or anti-I-E antibody. Aliquots of the 3B3 cell suspension were cultured either with the antibody-treated or with untreated A20.3 cells in the presence of p19–34, and IgE-BF in culture filtrates was determined. The results of the experiment, shown in Table 4, indicate that the fixed APC could present the peptide to the 3B3 cells for the formation of IgE-BF and that the product of the I-A^d, but not I-E subregion, was involved in the presentation of the peptide to 3B3 cells. As expected, the fixed APC and CNBr-treated PLA₂ failed to stimulate the hybridoma cells for the

Table 5. Affinity of peptide 19–34 to PLA₂-binding GIF^a

Experiment	Sepharose coupled with	Peptide present during absorption	GIF activity in ^b	
			flow-through	eluate
I	p19–35 PLA ₂	none	7/26 (–)	20/7 (+)
		none	0/29 (–)	25/0 (+)
II	PLA ₂	p7–22	1/24 (–)	25/2 (+)
		p13–28	25/10 (+)	5/17 (–)
		p19–34	22/3 (+)	0/26 (–)
		p19–30	30/6 (+)	3/26 (–)
		p25–40	0/20 (–)	22/6 (+)
		none	2/21 (–)	28/6 (+)
		medium control	0/28	

^aPLA₂-binding GIF was affinity purified from culture supernatant of antigen-stimulated 3B3 cells using PLA₂–Sepharose. The preparation could switch the 12H5 cells from the formation of glycosylated IgE-BF to the formation of unglycosylated IgE-BF at a 1:10 dilution. The preparation was mixed overnight with PLA₂–Sepharose or p19–35-coupled Sepharose in the presence or absence of an appropriate peptide, and unbound protein Fr (flow through Fr) and acid eluate fraction were recovered.

^bGIF activity was determined by using the 12H5 cells. The proportion of IgE RFCs in the absence of IgE-BF was 28.5 ± 0.3%. The (+) and (–) represent the presence or absence of GIF activity.

formation of IgE-BF. Experiments of the same design with the 9A4 cells and p25–40 showed that the product of the I-A^d subregion is also involved in the presentation of peptide to 9A4 cells (Table 4).

We determined whether the PLA₂-binding factors from the 3B3 cells bind the synthetic peptide 19–35. The results shown in Table 5 indicate that the antigen-binding factors bound to Sepharose coupled with the peptide and was recovered by elution at acid pH. We also determined whether the peptide may inhibit the binding of the antigen-binding GIF to PLA₂–Sepharose. The factor was incubated with PLA₂–Sepharose in the presence of one of the synthetic peptides (0.2 mg/ml). The results of the experiments shown in Table 5 indicate that not only p19–34 but also p13–28 and p19–30 inhibited the binding of the factor to PLA₂–Sepharose, whereas p7–22 and p25–40 failed to do so. The results suggest that amino acid residues 19–28 are sufficient for the binding of p19–34 to antigen-binding factor. However, among the three peptides which blocked the binding of the factor to PLA₂–Sepharose, only p19–34 could stimulate the 3B3 cells for the formation of IgE-BF. Neither p19–30 nor p13–28 induced the 3B3 cells for the formation of IgE-BF in the presence of A20.3 cells (results not shown).

Discussion

In the present experiments, we constructed seven GIF-producing T cell hybridomas specific for the venom PLA₂. All of the hybridomas constitutively secrete GIF, expressed CD3 and TCRαβ, and responded to APC pulsed with CNBr-treated PLA₂ for the formation of IgE-BF. As expected, neither the antigen alone nor the antigen plus glutaraldehyde-fixed APC induced the formation of IgE-BF (cf. Table 4). When the A20.3 cells were

pulsed with 30 µg/ml CNBr-treated PLA₂ for 24 h and then fixed with glutaraldehyde, however, the APC stimulated both 3B3 and 9A4 cells for the formation of IgE-BF. Furthermore, treatment of the antigen-pulsed and glutaraldehyde-fixed A20.3 cells with anti-I-A^d, but not with anti-I-E antibody, prevented the antigen presentation to the hybridoma cells for the formation of IgE-BF (results not shown). These findings collectively indicate that processing of CNBr-treated PLA₂ is required for antigen presentation and that class II MHC molecules were involved in the presentation of processed antigen to the T cell hybridomas.

In our previous experiments on GIF from OVA-specific murine hybridomas, we showed that GIF-producing T cells, which are specific for the epitope representing amino acid residues 307–317, produced OVA-binding GIF upon antigenic stimulation, while those specific for the major immunogenic determinant, i.e. peptide 323–339, formed GIF having no affinity for native OVA (15). Nevertheless, GIF produced by both groups of hybridomas upon antigenic stimulation shared antigenic determinants recognized by mAb 14-12, anti-TCRα chain, and anti-TCRβ chain (33), whereas those constitutively produced by unstimulated cells lacked all of the antigenic determinants. The present experiments reproduced the findings in PLA₂-specific hybridomas. Upon antigenic stimulation, T cell hybridomas specific for p19–34 produced antigen-binding GIF, whereas those specific for p25–40 or p1–16 produced GIF having low or no affinity for the antigen. It was also found that GIF produced by all of the antigen-stimulated hybridomas possessed the 14-12 determinant and TCRα chain determinant, while GIF constitutively produced by the same cells lacked the determinants. Attempts were also made to determine whether the PLA₂-binding GIF bound to an immunosorbent coupled with anti-TCRβ chain (H57-597); however, no convincing result was obtained (results not shown).

An important finding from the biologic viewpoint is that the affinity of GIF for immunogen is essential for its immunosuppressive effects (33). In the OVA-specific system, OVA-binding GIF from the hybridomas specific for the peptide 307–317 suppressed the *in vivo* anti-hapten antibody responses of syngeneic mice to DNP–OVA in a carrier-specific manner, while the 14-12⁺ GIF produced by antigen-stimulated, p323–339-specific hybridomas failed to suppress the antibody response. Furthermore, the OVA-binding GIF failed to suppress the antibody response to DNP conjugates of urea-denatured OVA, which did not bind OVA-binding GIF (33). The present experiments on PLA₂-specific hybridomas confirmed the principle through the demonstration that the PLA₂-binding GIF from the 3B3 cells suppressed the anti-hapten antibody response of BALB/c mice to DNP–PLA₂ conjugates but failed to suppress the antibody response to DNP–OVA. In contrast, non-antigen-binding (but 14-12⁺ and H28-710⁺) GIF from antigen-stimulated 7B5 cells failed to suppress the anti-hapten antibody response of BALB/c mice to DNP–PLA₂ (results not shown). It appears that PLA₂-binding GIF from the 3B3 cells represents an effector-type, antigen-specific TsF.

It should be noted that the hybridomas specific for p19–34 of PLA₂, e.g. 3B3 cells, responded to the peptide in the presence of glutaraldehyde-fixed APC; however, pretreatment of the fixed APC with anti-I-A^d antibody prevented the presentation of the peptide to the hybridomas. This finding indicates that further processing of p19–34 by APC is not

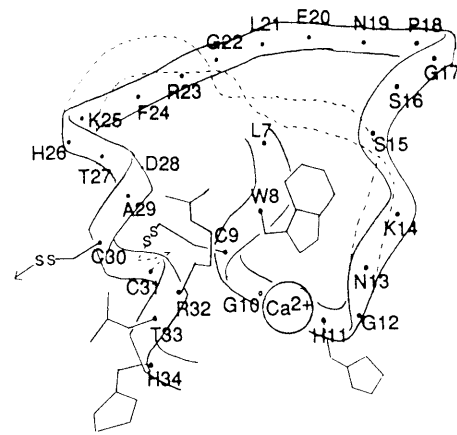


Fig. 3. Molecular model of the amino-terminal one-third of bee venom PLA₂ as compared with bovine pancreatic PLA₂ (dotted line). Amino acid sequence of the peptide is as follows:

10 20 30 40
IIPGTLWCGHGNKSSGPNELGRFKHTDACCRTHDMCPDV

required for antigen presentation and that the TCR on the T cell hybridoma recognizes the peptide in the context of the I-A^d product (Table 4). The same principle applies to the 9A4 cells which are specific for p25–40. The present experiments clearly showed that p19–34, but neither p13–28 nor p25–40, stimulated the 3B3, 6C1, 8C4, and 2A2 cells for the formation of IgE-BF, while the 9A4 cells and 7B5 cells responded to p25–40 and p1–16, respectively, but not to p19–34 (Table 3). The results indicate that all of the four hybridomas which produce the antigen-binding GIF upon antigenic stimulation are specific for a common epitope, p19–34, whereas those producing non-antigen-binding, 14-12⁺ GIF recognize different epitopes.

The present findings showed that the antigen-binding GIF from the 3B3 cells had affinity for p19–34 to which the TCR on the cells responded in the context of class II MHC molecules. The antigen-binding GIF bound to Sepharose coupled with the peptide and was recovered by acid elution. Furthermore, the binding of the factor to PLA₂–Sepharose was prevented by the peptide. These findings are in agreement with previous observations on OVA-specific hybridomas which produce OVA-binding GIF. The OVA-binding factors had affinity for the synthetic peptide, which represents the epitope recognized by the TCR on the cell sources of the factors (4). However, important findings obtained in the PLA₂-binding GIF were that the binding of the antigen-binding factor to PLA₂–Sepharose was inhibited not only by p19–34 but also by p19–30 and p13–28, but not by p25–40 (Table 5). It should be noted that none of the three peptides stimulated the 3B3 cells in the presence of APC. We speculate that both p19–30 and p13–28 lack the epitope for binding to Ia molecules. The results suggest that the sequence of residues 19–25 contains structures involved in the binding of p19–34 to antigen-binding GIF.

The X-ray structure of bee venom PLA₂ shows that 11 amino acids (amino acids 14–24) form a loop that connects the Ca²⁺ binding site (amino acids 8–12) and α helix (amino acids 26–37) which includes one of the catalytic residues, His34 (16). Both the Ca²⁺ binding sites and the catalytic helices in bee venom PLA₂ and in bovine pancreatic PLA₂, whose crystal structure is

also known (36), are sequentially and structurally homologous, while the loop in bee venom PLA₂ is twice as long in length as the loop in bovine PLA₂ (amino acids 34–38) (Fig. 3). Mobile regions in a protein are generally the potential sites responsible for molecular recognition in protein–protein interaction (37). Considering that the loop locates on the surface of the bee venom PLA₂ molecule and that the loop is a long one, it is quite likely that the loop exposed on the surface of the antigen molecule is conformationally flexible and that it can access the ensemble of conformations of the synthetic peptide 19–34. It is also conceivable that His26, Thr27, Asp28, Arg32, and Thr33 in the helix are exposed on the surface of PLA₂ molecules. The molecular model shown in Fig. 3 explains why GIF having affinity for p19–34 (and p19–30 and p13–28) also binds to native PLA₂.

Since p19–34 and p25–40 stimulate 3B3 cells and 9A4 cells, respectively, in the context of the I-A^d product, whereas p19–30 fails to do so, one may speculate that amino acid residues 25–34, which represent a portion of the α helix, contain residues which interact with class II molecules. If this is the case, failure of p25–40 to stimulate the 3B3 cells strongly suggests that the sequence of amino acid 19–24, which represents a portion of the loop, may contain amino acid residues involved in the interaction of the p19–34–Ia complexes with the TCR on the 3B3 cells. As discussed above, the same sequence appears to contain the residues involved in the binding of p19–34 to antigen-binding GIF. It is not known why participation in class II molecules is required for recognition of the epitope by the TCR on 3B3 cells. Neither CNBr-treated PLA₂ nor PLA₂-coupled Sepharose induced 3B3 cells for the formation of IgE-BF.

It was suggested that an immunodominant T cell epitope is an amphipathic α helix or has the ability to fold as an amphipathic structure (38). Indeed, evidence has been presented that the immunodominant epitopes in sperm whale myoglobin, i.e. residues 102–118 and 132–146 (39,40), and residues 52–61 in egg white lysozyme (41), represent α helices in the native antigen molecules. In these immunodominant epitopes, Ia-contact residues are intermingled with residues that presumably are involved in the engagement of the TCR. However, an amphipathic α helix does not appear to be an absolute requirement for immunogenicity. In the case of OVA peptide 323–339, the peptide assumes a β sheet or extended conformation (42). Residues 19–34 in bee venom PLA₂ may represent another type of epitope recognized by T cells. In the native PLA₂, this segment appears to consist of a portion of a loop that is exposed on the surface of the molecule and a portion of the α helix (Fig. 3). As discussed above, the peptide appears to bind to Ia through the portion of the α helix, while the loop contains the residues involved in interaction with the TCR and antigen-binding GIF. Such a T cell epitope may not be unique for bee venom PLA₂. Recently, the crystal structure of OVA was reported by Stein *et al.* (43). According to their analysis, amino acid residues 307–317, which have affinity for OVA-binding GIF (4), represent a portion of a long loop which exists between an α helix and a β sheet. The crystal structure of OVA molecules, together with the fact that seven out of 11 amino acids in p307–317 are hydrophilic amino acids, strongly suggests that the loop is exposed on the surface of the OVA molecule.

If an external structure of PLA₂ is involved in interaction with

the TCR on 3B3 cells, one might expect that native PLA₂ can bind to the TCR. Our preliminary experiments to demonstrate the binding of biotin-coupled PLA₂ to 3B3 cells failed to provide convincing results. However, Silliciano *et al.* (44) reported that multivalent hapten conjugated with synthetic polymers bound to TCR $\alpha\beta$ on MHC-restricted T cell clones specific for FITC and that a high concentration of the monovalent hapten significantly inhibited the binding of the multivalent conjugates to the T cell clone. Kuchroo *et al.* (45) also demonstrated that CD3⁺ NP-specific suppressor T_s hybridoma formed rosettes with NP-coupled sheep erythrocytes and rosette formation was inhibited by NP–BSA conjugates. Experiments are being designed to determine whether p19–30 and p19–34 may have some affinity for the TCR on the 3B3 cells.

It should be noted that neither p19–34 in PLA₂ nor p307–317 in OVA represent an immunodominant epitope. Lymph node cells of BALB/c mice immunized with PLA₂ included in complete Freund's adjuvant failed to respond to p19–34 for proliferation (results not shown). Similarly, lymph node cells of BDF₁ mice primed with OVA and complete Freund's adjuvant failed to respond to p307–317 (15). Further studies are required for an explanation of why T cell clones specific for such epitopes are not present in T helper cell populations. Nevertheless, the present findings are in agreement with the hypothesis that suppressor T cells and T helper cells have distinct epitope specificities (46), and suggest that the TCR on suppressor T cells are specific for an external structure in the antigen molecule.

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Abbreviations

APC	antigen presenting cells
DMEM	Dulbecco's modified Eagle's medium
DNP	dinitrophenyl
GIF	glycosylation inhibiting factor
IgE-BF	IgE-binding factor
MLN	mesenteric lymph node
ONO-RS	ONO-RS-082, 2-(<i>p</i> -amylcinnamoyl)-amino-4-chlorobenzoic acid
PLA ₂	phospholipase A ₂
RFC	rosette forming cells
TsF	antigen-specific suppressor T cell factor
OVA	ovalbumin

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