Full title; The extract of syngeneic keratinocytes enhances IgE production from BALB/c mouse splenic lymphocytes *in vitro*.

Kenichi Morimoto,[*] Yukiko Nabeshima,[*] Mitsuru Mochizuki,[*] Souichi Oomizu,[†] Shoso Yamamoto,[*] and Michihiro Hide[*]

[*]Department of Dermatology, Division of Molecular Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

[†]Hiroshima Prefectural Institute of Industrial Science and Technology, Higashi-Hiroshima, Japan

Corresponding author; Michihiro Hide M.D., Ph.D.

1-2-3, Kasumi, Minami-ku, Hiroshima, 734-8551, Japan

tel. +81-82-257-5238, fax. +81-82-257-5239

e-mail: mhide@hiroshima-u.ac.jp

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and by the Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER) of Hiroshima Prefectual Institute of Industrial Science and Technology.

Abstract

Background: The increase of serum IgE levels is closely associated with atopic dermatitis. We have previously revealed that cellular extract of PAM212 cells (PAM-extract), BALB/c mouse keratinocyte cell line, induced a remarkable increase of serum IgE levels, *in vivo*, when subcutaneously injected into BALB/c mice. However, precise mechanism of IgE-increasing activity was unclear.

Objective: To elucidate the mechanism of IgE-increase in sera of BALB/c mice induced by PAM-extract, we explored the direct influence of PAM-extract on immunoglobulin production and class-switching in the culture of splenic lymphocytes and purified B-cells, *in vitro*.

Methods: Splenic lymphocytes or purified B-cells obtained from BALB/c mice were cultured with various combinations of IL-4, anti-CD40 antibody, and PAM-extract for seven days. IgE and IgG concentrations of culture supernatants were measured by ELISA. Epsilon germ-line transcriptions were assessed by RT-PCR from the cultured cells.

Results: IgE and IgG concentrations in culture supernatant of splenic lymphocytes were increased by an addition of PAM-extract in the presence of both IL-4 and anti-CD40 antibody. Epsilon germ-line transcript was also induced in parallel to the increase of IgE

production. Similar results were obtained when purified B-cells were employed in stead of whole splenic lymphocytes.

Conclusion: The cellular extract of keratinocyte promotes immunoglobulin class-switching to IgE and IgE production from mouse splenic B-cells in an IL-4- and CD40-stimuli-dependent manner. Such enhancement may account for the increase of serum IgE in patients with dermatitis in association with a Th2 microenvironment.

Key words; immunoglobulin E, keratinocyte, B-lymphocyte, immunoglobulin class switching, atopic dermatitis

Introduction

Atopic diseases, such as asthma, atopic dermatitis and allergic rhinitis, are closely associated with the increase of serum IgE levels [1, 4, 7, 10] and a genetic background predisposed to Th2 type reactions [7]. Clinical observations have suggested a correlation between serum IgE levels and the extent and/or severity of the disease, especially of the cutaneous lesions [4, 10, 17]. Based on these findings, we hypothesized that skin damage, especially destruction of the epidermis, raises serum IgE. We previously revealed that serum IgE level of BALB/c mouse increased when injected with syngeneic keratinocyte extract, and that this phenomenon was not clearly observed in CBA/j mouse [19], suggesting the close association of genetic background with the effect of keratinocyte extract. It is well known that B-cells produce IgE protein via immunoglobulin class-switching triggered by epsilon germ-line transcription under the influence of both interleukin (IL)-4 and CD40L-CD40 interaction [9]. However, continual injections of the extract of keratinocytes also induced the increase of serum IgG_{2b} [19], which is rather decreased by IL-4 [15], *in vivo*. Thus, the precise mechanism of the serum IgE increase by keratinocyte extract remained unclear. To address this question, we studied the effect of keratinocyte extract on *in vitro* production of IgE and IgG subclasses by whole splenic

lymphocytes of BALB/c mice in the presence or absence of IL-4 and anti-CD40 antibody. We also studied direct effect of keratinocyte extract on B-cells that produced IgE, using negative magnetic cell sorting from splenic lymphocytes. The effect on class switching for IgE was investigated by assessing the change of epsilon germ-line transcription with both whole splenic lymphocytes and isolated B-cells from the spleen.

Materials and Methods

Preparation of cellular extract of PAM212 cells

PAM 212, a murine keratinocyte cell line, derived from BALB/c mouse skin [13], was cultured in α-minimal essential medium (GIBCO-BRL, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (GIBCO-BRL), penicillin G (100 IU /ml) and streptomycin (100 μg/ml) (Meiji, Tokyo, Japan). PAM 212 cells were cultured in 225 cm^2 tissue culture flasks (Corning, Corning, NY), harvested at sub-confluency by using a cell scraper (Sumitomo Bakelite, Tokyo, Japan) and were collected by centrifugation at 300 *g* for 10 min. The cells were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS(-)) and resuspended in five volumes of ice-cooled PBS(-) relative to the weight of the pellet. The cells were sonicated for 30 seconds with 30 seconds intervals for ten times in total. The homogenates were centrifuged at 105,000 *g* for 60 min. The supernatant was collected and filtered by 0.2 μm membrane filter (Acrodisc, Pall Corporation, Ann Arbor, MI) as a cellular extract (PAM-extract) and stored at –80°C until use.

The protein concentration of PAM-extract was approximately 3.6 mg/ml, determined by Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA).

Gel-filtration of PAM-extract

A Superose-6 HR 10/30 column (Amersham Bioscience, Tokyo, JAPAN) connected to the ÄKTA explorer HPLC system (Amersham Bioscience) was equilibrated in PBS(-) at room temperature. A 1.0 ml volume of PAM-extract was loaded onto the column, eluted by PBS(-) at a flow rate of 0.4 ml/min, and each 1.0 ml fraction was collected. Molecular size markers (Bio-Rad, Tokyo, JAPAN) were eluted by the same method as PAM-extract and the chromatogram pattern was recorded simultaneously.

Heat-treatment of PAM-extract

PAM-extract was heated at 85 °C for 15 minutes and filtered to remove insoluble products.

Isolation of mouse splenic lymphocytes

Splenic lymphocytes were isolated from 8-week-old BALB/c mice as previously described [19]. Briefly, spleens were isolated from 8-week-old BALB/c mice and minced in Iscove's Modified Dulbecco's Minimum Essential Medium (GIBCO-BRL) with 10% heat-inactivated fetal bovine serum, penicillin G (100 IU /ml) and streptomycin (100

μg/ml). The lymphocyte suspension was obtained with a nylon mesh filter (Cell Strainer 352350, Beckton Dickinson Labware, Franklin Lakes, NJ) and a density separation medium (Lympholyte-M, Cedarlane Laboratories, Hornby, Canada). This study was carried out in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University Faculty of Medicine, as well as the "Principles of Laboratory Animal Care".

B-cell isolation from splenic lymphocytes

Splenic B-cells were isolated using Magnetic-Activated Cell Sorting (MACS^{TM}) according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the splenic lymphocytes were incubated with anti-CD43 coated metal microbeads, applied to Auto-MACS^{TM} (Miltenyi Biotec), and the unbound cells were collected as splenic B-cells. The purity of the B-cells was assessed by flow cytometry. Briefly, Aliquots of lymphocytes before and after B-cell isolation were stained respectively by FITC-labeled anti-CD4 and anti-CD8 antibody, PE-labeled anti-CD19 antibody, and biotin-labeled anti-Gr1 antibody and streptoavidin-APC conjugate (BD Pharmingen, Tokyo, Japan), and analyzed by fluorescence-activated cell sorting

(FACSCalibur^{TM}, Becton Dickinson, San Jose, CA). Dead cells were gated out by 7AAD staining [14]. The resulting cell population contained >97% $CD19^+$, <1% $CD4^+$ or $CD8^+$, and <0.1% $Gr1^+$ cells.

Cell culture

Splenic lymphocytes and B-cells prepared as described above were washed and re-suspended in Iscove's Modified Dulbecco's Minimum Essential Medium (GIBCO-BRL) with 10% heat-inactivated fetal bovine serum, penicillin G (100 IU /ml) and streptomycin (100 μg/ml), respectively. Each group of cell preparations were distributed into 96-well cluster plates (Corning) ($2x10^6$ cells/200 μl/well) and maintained at 37°C in a 5% CO_2 atmosphere with cellular extracts or other stimulants in the presence or absence of 100 ng/ml (1000 U/ml) IL-4 (R&D systems, Inc. Minneapolis, MN) and 10 μg/ml anti-CD40 antibody (Serotec, Oxford, UK) and prostaglandin-E_2 (PGE_2) (SIGMA, Tokyo, Japan) at various concentrations in the medium described above. Seven days afterwards, the supernatants of the cell cultures were collected by centrifugation and stored at –30°C until use.

Measurement of IgE and IgG subtypes

The IgE concentrations in cell culture media were determined by use of an enzyme-linked immunosorbent assay (ELISA) kit (Yamasa, Chiba, Japan) according to the manufacturer's instruction. The IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 concentrations were assayed by use of ELISA kits (Bethyl, Montgomery, TX) according to the manufacturer's instruction.

Semi-quantitative reverse transcription-PCR (RT-PCR) of the epsilon germ-line transcription

Total cellular RNA was prepared from spleen cell cultures with RNEasy mini kit (QIAGEN, Tokyo, Japan) according to manufacturer's instruction, and re-suspended in 20 μl of ultra pure water prepared in the kit. First strand cDNA was synthesized using the SuperScript™ II reverse-transcription kit (Life Technologies, Gaithersburg, MD) according to manufacturer's recommended protocol. Briefly, reverse transcription of mRNA was performed in a final volume of 20 μl containing 1 μl $Oligo(dT)_{12\text{-}18}$ primer, 4 μl of 5X First Strand Buffer (250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$), 2 μl of 0.1 M DTT, 1 μl of 0.1 M dNTP Mix, and 1 μl of SuperScript™ II reverse-transcriptase. PCR amplifications were performed using AmpliTaq Gold™ PCR kit (Applied Biosystems Japan, Tokyo, Japan). Briefly, each 1.0

μl of cDNA sample and 24 μl of master mix, containing 2.5 μl of 10X PCR buffer (with 15mM $MgCl_2$), 2.0 μl of 2.5 mM dNTP, 17.4 μl of distilled water, 0.1 μl of AmpliTaq Gold™ DNA polymerase, and 1.0 μl of each PCR primer, were mixed. Epsilon germ-line primers were selected from the upstream Iε exon and downstream $Cε_1$ exon to amplify a 365-bp DNA product as reported previously: upstream primer, 5'-GCACAGGGGGCAGAAGAT-3' (nucleotides 799-816); downstream primer, 5'-CGTTGAATGATGGAGGAT-3' (nucleotides 377-394) [6]. cDNA was also amplified with primers specific for HGPRT, a housekeeping gene, to control for sample to sample variation in RNA isolation and integrity, RNA input, and reverse transcription: upstream primer, 5'-GTTGGATACAGGCCAGACTTTGTTG-3' (nucleotides 514-538); downstream primer, 5'-GATTCAACTTGCGCTCATCTTAGGC-3' (nucleotides 652-678) [6]. Both PCR amplification cycle number and RNA input for each pair of primers were decided empirically to establish conditions that resulted in a linear correlation between RNA input and PCR product and that would therefore allow reliable comparison of the relative levels of epsilon germ-line transcripts and HGPRT mRNA in different samples. The following reaction conditions were chosen as optimal: Iε-$Cε_1$ and HGPRT PCRs were incubated at 95 °C for 10 minutes for 1 cycle to activate the AmpliTaq Gold™ DNA polymerase, then at 95 °C for 30 seconds, 50 °C for 30 seconds,

and 72 °C for 1 minute for 28 cycles. All PCR amplifications were performed at least twice with multiple sets of experimental RNAs. PCR products were analyzed on 2.5% agarose gels. Gels were stained by Ethidium Bromide and exposed to ultraviolet light. Gel-image was obtained and analyzed with LS-9000 (Fuji Film, Tokyo, Japan) to assess the amount of epsilon germ-line transcript and HGPRT.

One-step real-time RT-PCR of the epsilon-germline transcription

Total cellular RNA was prepared from spleen cell cultures with RNEasy 96 kit (QIAGEN) according to manufacturer's instruction, and re-suspended in 120 μl of ultra pure water prepared in the kit. Reverse transcription and real-time quantitative PCR were performed in an optical 96-well plate with an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), using QuantiTect™ SYBR® Green RT-PCR kit (QIAGEN). Each 50 μl reactions contained 25 μl 2x QuantiTect™ SYBR® Green RT-PCR Master Mix containing inactive form of HotStarTaq™ DNA polymerase and 5 mM $MgCl_2$, 0.5 μl QuantiTect™ RT mix, 1.0 μl of each forward and reverse primer, 5 μl RNA template, and 17.5 μl RNase free water provided in the kit. Reactions were first incubated at 50 °C for 30 min for reverse transcription, and 95 °C for 15 min for inactivate reverse transcriptase and activate HotStarTaq™ DNA polymerase.

Amplification was performed for 60 cycles of a sequential incubation at 95 °C for 15 sec and 60 °C for 1 min. The RT-PCR products were checked for their specificity by agarose gel electrophoresis. The expression of epsilon germ-line transcription and HGPRT were quantified by comparative threshold cycle method for real-time PCR as described in the manufacturer's instructions.

Results

IgE production from mouse splenic lymphocytes:

PAM-extract enhanced IgE production from splenic lymphocytes in a dose-dependent manner in the presence of 100 ng/ml of IL-4 and 10 μg/ml of anti-CD40 antibody. The maximum concentration of IgE was 168.6 +/- 20.2 ng/ml, 3.4 fold higher than that of the control, and was produced in the presence of 10% PAM-extract in the cell culture. No IgE was detected in the absence of either IL-4 or anti-CD40 antibody even in the presence of the maximum concentration of PAM-extract (fig.1).

Epsilon germ-line transcription:

To elucidate the mechanism of PAM-extract, we studied the effect of PAM-extract on epsilon germ-line transcription which is essential to class-switch recombination to IgE. The amount of the transcription increased up to 3.5-fold higher by PAM-extract in a dose-dependent manner over 0.1% to 10%, when observed either by Semi-quantitative RT-PCR (fig. 2a) or by one-step real-time RT-PCR (fig. 2b). PAM-extract induced no or only a subtle increase of the amount of HGPRT, indicating PAM-extract induced epsilon

germ-line transcription rather than cell proliferation (Fig. 2a, 3). This effect was not observed in the absence of IL-4 or anti-CD40 antibody (fig. 3).

The effect of PAM-extract on B-cells:

To study the direct effect of PAM-extract on B-cells, splenic B lymphocytes were purified and cultured in the presence or absence of PAM-extract. PAM-extract also enhanced IgE production in a dose-dependent manner upto 5.6-fold higher than control (fig. 4a).

PAM-extract also induced epsilon germ-line transcription in the absence of non-B-cell lymphocytes. The amount of the transcription assessed by one-step real-time RT-PCR in the presence of 10% PAM-extract was 32-fold higher than that in the absence of PAM-extract (fig. 4b).

Biochemical properties of IgE-inducing factor in PAM-extract:

To characterize the biochemical properties of IgE-inducing factor in PAM-extract, PAM-extract was heat-treated at 85 °C for 15 min, or gel-fractionated before the culture with splenic lymphocytes. The heat treatment did not affect the activity of PAM-extract for enhancing IgE production (Fig. 5). Fractions of gel-chromatography

eluted between molecular marker for 1.3 kDa and that for 17 kDa enhanced IgE production in the culture (fig. 6).

The effect of PAM-extract on the production of IgG subtypes:

To study the effect of PAM-extract on IgG subclasses, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 concentrations in culture supernatants of splenic lymphocytes were assessed by ELISA. PAM-extract enhanced the production of IgG_1, IgG_{2b}, and IgG_3, as well as that of IgE from splenic lymphocytes in a dose-dependent manner in the presence of 100 ng/ml of IL-4 and 10 μg/ml of anti-CD40 antibody (Fig. 7).

The effect of PGE_2 on IgE production from splenic lymphocytes:

PGE_2 was added at 0.01, 0.1, or 1.0 μM to the culture of splenic lymphocytes with 100 ng/ml of IL-4, 10 μg/ml of anti-CD40 antibody, and various concentrations of PAM-extracts. No effect of PGE_2 on IgE production was observed under these conditions (Fig. 8).

Discussion

In this study, we demonstrated that the keratinocyte extract directly enhanced IgE production and IgE class-switching in splenic lymphocyte of BALB/c mouse in the presence of IL-4 and anti-CD40 antibody. These phenomena were abolished in the absence of either IL-4 or anti-CD40 antibody, indicating that the effect of PAM-extract is dependent on both IL-4 and CD40 activation. The requirement of IL-4 and CD40 activation for PAM-extract may account for the increase of serum IgE concentration observed selectively in Th2-predisposed mouse strains injected with syngeneic keratinocyte extract [19], and the presence of patients with atopic dermatitis without elevation of serum IgE. This may also be the reason why extensive destruction or proliferation of keratinocytes observed in many non-atopic skin disorders does not accompany the increase of serum IgE.

The signaling effect of damaged-cell components released by necrosis on the immune system was first postulated in 1994 by Matzinger [8]. She proposed that “danger signal” or destruction of cell constructions is critical rather than whether the component is derived from self or non-self tissues. Danger signals can be divided into two large subclasses according to their sources; endogenous ones and exogenous ones. Many substances, such as ATP, UTP, heat-shock proteins, reactive oxygen intermediates, and

the degradation products of heparan sulfate or hyaluronan, are reported to be endogenous danger signals which can activate dendritic cells (DCs) to produce TNF-α, IL-12 or interferon-γ, to drive the immune system to Th1-type reactions [3].

DCs are considered to play a key role in initiating immune responses against pathogens. Immature DCs constitutively intake various substances around themselves such as autoantigens by endocytosis, and then process and present them on MHC class II molecules to T-cells without co-stimulation. The interaction between DCs and T-cells without co-stimulation does not activate T-cells but converts T-cells to non-responders. When a tissue is damaged by pathogen or other offending stimuli, immature DCs intake various substances around themselves together with "danger signals", produced endogenously or exogenously, and present them to T-cells with co-stimulating molecules such as CD80 or CD86. Thus, the "danger signal" is received by DCs to initiate immune reactions [11]. Our data suggests that the extract of syngeneic keratinocytes may contain a molecule, which acts like a "danger signal". However, the role of this molecule may be different from that of previously reported "danger signals", in that it activates Th2-type rather than Th1-type immune reactions. Moreover, the effect is not inherent, but is an enhancing one for immune reactions induced by IL-4 and anti-CD40 antibody. We have demonstrated that PAM-extract directly acts on B-cells to enhance IgE production in the

presence of IL-4 and CD40 stimulation. However, this result cannot exclude the possibility that the extract may also act on DCs or monocytes as well as B-cells, which produce IgE. Moreover, B-cells may also act as APCs, susceptible to “danger signals” as well as other APCs. Further investigation to define the target molecule for keratinocyte extract is necessary to understand the precise mechanism of the enhancement of IgE production.

The biochemical properties of the factor contained in PAM-extract, heat-stable and the molecular weight between 1.3 and 17 kDa, suggest that it may be a peptide, a small protein such as cytokine, or a lipid mediator.

Keratinocytes produce and/or contain various immunologically active substances, such as IL-18 [16, 17], and PGE_2 [2, 12], which may enhance Th2 type reactions and/or IgE production by B cells in certain conditions. However, IL-18 has not been reported to have a direct effect on B-cells to induce IgE production, and the concentration of IL-18 protein in PAM-extract was below the detection limit of the ELISA kit (< 23 pg/ml) employed in this study (data not shown).

We did detect the presence 0.83 μM PGE_2 in PAM-extract, and studied its effect on IgE production in our culture system. However, no effect of PGE_2 in the concentration up to 1.0 μM was observed on IgE production, regardless of the presence of PAM-extract

(Fig. 8). We then prepared PGE_2-reduced PAM-extract by culturing PAM-212 with 10 μg/ml indomethacin for 10 days and studied its effect as ordinary PAM-extract. The PGE_2-reduced PAM-extract showed similar enhancing activity on IgE production to that of ordinary PAM-extract (data not shown). Finally, gel-chromatography has indicated that the molecular weight of IgE-enhancing factor contained in PAM-extract was larger than 1.3 kDa, namely, much larger than that of PGE_2 (M.W. 319.5). Thus we conclude that PGE_2 is not crucial in PAM-extract for the enhancement of IgE production *in vitro*.

Likewise, the presence of IL-4, IL-12p40, IL-13 and GM-CSF were not detected in PAM-extract and/or proved to have no effect on IgE production in our system (data not shown).

Some individuals with atopic dermatitis show IgE auto-reactivity toward cells of their own tissues including keratinocytes [5] such as IgE autoantibody against cultured keratinocyte in the patients with atopic dermatitis and anti-BP180 IgE autoantibodies in bullous pemphigoid. Therefore, it is feasible that the extract from syngeneic keratinocytes may induce the production of auto-antibodies against its constituents. However, the effect of PAM-extract *in vitro* shown in this study suggests that it enhances rather than initiate the production of IgE. It is unlikely that purified B-cells obtained from non-treated mice produce antigen-specific IgE during the seven days culture without dendritic cells and/or

T-cells. Moreover, antigen-specific IgE against keratinocyte extract was not observed *in vivo* as demonstrated in previous report [19] and *in vitro* studied by dot-blot under the condition that the PAM-extract was dotted on a methyl-cellulose membrane at the concentration of xx mg/ml and the diluted culture supernatant containing about xx ng/ml IgE used as the detection antibody (data not shown). Thus we conclude that the PAM-extract provide the suitable environment for the production of broad, non-specific IgE.

IgE-targetting therapy using anti-IgE antibody is not effective for the patients with atopic dermatitis due to insufficient dosing of anti-IgE antibody because of the very high levels of IgE in these patients. But the therapy is drastically effective for the patients with asthma and allergic rhinitis, suggesting that the controlling of IgE is still one of the key strategies for management of atopic dermatitis. The identification of this factor(s) and elucidation of the precise mechanism of IgE-produce-enhancement by endogenous skin destruction may contribute to the development of a new therapy for atopic dermatitis, or Th2 type immune diseases.

Acknowledgements

We are grateful to Ms. K. Fujiwara, K. Ishii, and K. Uchida for laboratory works, Prof. Nakanishi (Hyogo Medical University) for the measurement of IL-18, Dr. Yuspa (NCI, NIH, Bethesda MD) for kind gift of PAM212 cells, and Prof. M. Kanno (Hiroshima University) and Prof. K. Inaba (Kyoto University) for helpful discussions.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and by the Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER) of Hiroshima Prefectual Institute of Industrial Science and Technology.

References

1. Clendenning WE, Clack WE, Ogawa M, Ishizaka K: Serum IgE studies in atopic dermatitis. J Invest Dermatol 1973; 61: 233-6.

2. Fedyk ER, Phips RP: Prostaglandin E2 receptors of the EP2 and EP4 subtypes regulate activation and differenciation of mouse B lymphocytes to IgE-secreting cells. Proc Natl Acad Sci 1996; 93: 10978-83.

3. Gallucci S, Matzinger P: Danger signals: SOS to the immune system. Curr Opin Immunol 2001; 13: 114-9. DOI 10.1016/S0952-7915(00)00191-6

4. Holt PG, Rose AH, Batty JE, Turner KJ: Induction of adjuvant-independent IgE responses in inbred mice: primary, secondary, and persistent IgE responses to ovalbumin and ovomucoid. Int. Arch. Allergy. Appl Immunol 1981; 65: 42-50.

5. Kortekangas-Savolainen O, Peltonen S, Pummi K, Kalimo K, Savolainen J: IgE-binding components of cultured human keratinocytes in atopic eczema/dermatitis syndrome and their crossreactivity with Malassezia furfur. Allergy 2004; 59: 168-73. DOI 10.1046/j.1398-9995.2003.00330.x

6. Linehan LA, Warren WD, Thompson PA, Grusby MJ, Berton MT: STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. J

Immunol 1998; 161: 302-10.

7. Matsuda H, Watanabe N, Geba GP, *et al*: Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 1997; 9: 461-6.

8. Matzinger P: Tolerance, danger, and the extended family. Annu Rev Immunol 1994; 12: 991-1045. DOI 10.1146/annurev.iy.12.040194.005015

9. Monticelli S, Vercelli D: Molecular regulation of class switch recombination to IgE through ε germline transcription. Allergy 2001; 56: 270-8. DOI 10.1034/j.1398-9995.2001.00129.x

10. Morita E, Kaneko S, Hiragun T, *et al*: Fur mites induce dermatitis associated with IgE hyperproduction in an inbred strain of mice, NC/Kuj. J Dermatol Sci 1999; 19: 37-43. DOI 10.1016/S0923-1811(98)00047-4

11. Pennisi E: Teetering on the brink of danger. Science 1996; 271: 1665-7.

12. Roper RL, Brown DM, Phipps RP: Prostaglandin E2 promotes B lymphocyte Ig isotype switching to IgE. J Immunol 1995; 154: 162-70.

13. Sawamura D, Sato T, Kon A, *et al*: Mouse 230-kDa bullous pemphigoid antigen gene: structural and functional characterization of the 5'-flanking region and interspecies conservation of the deduced amino-terminal peptide sequence of the protein. J Invest Dermatol 1994; 103: 651-5.

14. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV: Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry 1992; 13:204-8.

15. Snapper CM, Finkelman FD, Paul WE: Regulation of IgG1 and IgE production by interleukin 4. Immunol Rev 1988; 102: 51-75.

16. Stoll S, Muller G, Kurimoto M,*et al*: Production of IL-18 (IFN-gamma-inducing factor) messenger RNA and functional protein by murine keratinocytes. J Immunol 1997; 159: 298-302.

17. Takai S, Tateno M, Hirano T, Kondo N, Hirose S, Yoshiki T: Increased IgE level as marker of host-versus-graft disease: inhibition of this HVGD with a monoclonal antibody to IL-4. Cell Immunol 1993; 149: 1-10. DOI 10.1006/cimm.1993.1131

18. Tani M, Komura A, Horikawa T: 1-alfa, 25-dihydroxyvitamin D3 modulates Ia antigen expression induced by interferon-gamma and prostaglandin E2 production in PAM 212 cells. Br J Dermatol 1992; 126: 266-74.

19. Yamamoto T, Kaneko S, Hide M, Mochizuki M, Morimoto K, Yoshimura M, Yamamoto S: Increase in serum IgE levels following injection of syngeneic keratinocyte extracts in BALB/c mice. Arch Dermatol Res 2002; 294: 117-23. DOI 10.1007/s00403-002-0292-4

Figure Legends

Figure 1

Keratinocyte extract (PAM-extract) enhances IgE production from splenic lymphocytes cultured with both IL-4 and anti-CD40 antibody. Splenic lymphocytes were stimulated with different combinations of IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), and PAM-extract (final concentration; 0 to 10%). Columns represent the IgE concentration in culture supernatants after 7-days stimulation. Results are expressed in ng/ml (as mean +/- S.D. of quadricate values) and are representative of one of 8 separate experiments.

Figure 2

Keratinocyte extract (PAM-extract) induces epsilon germ-line transcription of splenic lymphocytes. Splenic lymphocytes were stimulated with IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), and various concentrations of PAM-extract (final concentration; 0 to 10%). (a) Semi-quantitative RT-PCR analysis. Columns represent the ratios of epsilon germ-line transcription (epsilon GT) over house-keeping gene, HGPRT after 5-day stimulation. (b) One-step real-time RT-PCR analysis. Columns are expressed

as mean +/- S.D. of quadricate values, and are representative for one of four separate experiments.

Figure 3

Keratinocyte extract (PAM-extract) induces epsilon germ-line transcription in the presence of both IL-4 (100 ng/ml) and anti-CD40 antibody (10 μg/ml). Splenic lymphocytes were stimulated with IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), and non-treated or heat-treated PAM-extract (final concentration; 0 to 10%). Columns represent the ratio of epsilon germ-line transcription (epsilon GT) over house-keeping gene, HGPRT after 5-days stimulation.

Figure 4

Keratinocyte extract (PAM-extract) enhances IgE production and epsilon germ-line transcript from splenic B-cells cultured with both IL-4 and anti-CD40 antibody. B-cells were purified from splenic lymphocytes by Magnetic-Activated Cell Sorting and stimulated with different combinations of IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), and PAM-extract (final concentration; 0 to 10%). (a) Columns represent the IgE concentration in culture supernatants after 7-day stimulation. Results are expressed in

ng/ml (as mean +/- S.D. of quadricate values). (b) Columns represent the ratios of epsilon germ-line transcription (epsilon GT) over house-keeping gene, HGPRT after 5-day stimulation, measured by one-step real-time RT-PCR. Results are representative of one of 3 separate experiments.

Figure 5

Heat-treated PAM-extract enhances IgE production from splenic lymphocytes cultured with both IL-4 and anti-CD40 antibody as well as non-treated PAM-extract. Splenic lymphocytes were stimulated with IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), and PAM-extract (final concentration, 10%). Columns represent the IgE concentration in culture supernatants after 7-days stimulation. Results are expressed in ng/ml (as mean +/- S.D. of quadricate values).

Figure 6

Fractions of PAM-extract eluted between 1.3 and 17 kDa enhances IgE production from splenic lymphocytes. PAM-extract was fractioned by gel-chromatography. Splenic lymphocytes were stimulated with IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), and

each fraction of PAM-extract (final concentration; 10%). Columns represent the IgE concentration in culture supernatants after 7-day stimulation, and are expressed in ng/ml (as mean +/- S.D. of quadricate values). The bold line shows the chromatographic pattern (OD 280nm). The arrows are size markers.

Figure 7

Keratinocyte extract (PAM-extract) enhances IgG_1, IgG_{2b}, and IgG_3 production as well as IgE production from splenic lymphocytes. Splenic lymphocytes were stimulated with IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), and various concentrations of PAM-extract (final concentration; 0 to 10%). Columns represent IgE concentration in culture supernatants after 7-day stimulation. Results are expressed in ng/ml (as mean +/- S.D. of quadricate values) and are representative of one of 4 separate experiments.

Figure 8

PGE_2 has no significant effect on IgE production from splenic lymphocytes in the presence of IL-4, anti-CD40 antibody, and the extract of keratinocytes. Splenic lymphocytes were stimulated with IL-4 (100 ng/ml), anti-CD40 antibody (10 μg/ml), PAM-extract (final concentration; 0 to 10%), and PGE_2 (final concentration; 0 to 1.0 μM).

Columns represent the IgE concentration in culture supernatants after 7-days stimulation. Results are expressed in ng/ml (as mean +/- S.D. of quadricate values) and are representative of one of 2 separate experiments.

Abbreviations;

IL-4, Interleukin 4; PAM-extract, cellular extract of PAM212 cells; HGPRT, Hypoxanthine-Guanine Phosphoribosyltransferase; DC, dendritic cell; APC, antigen-presenting cell

Fig. 1

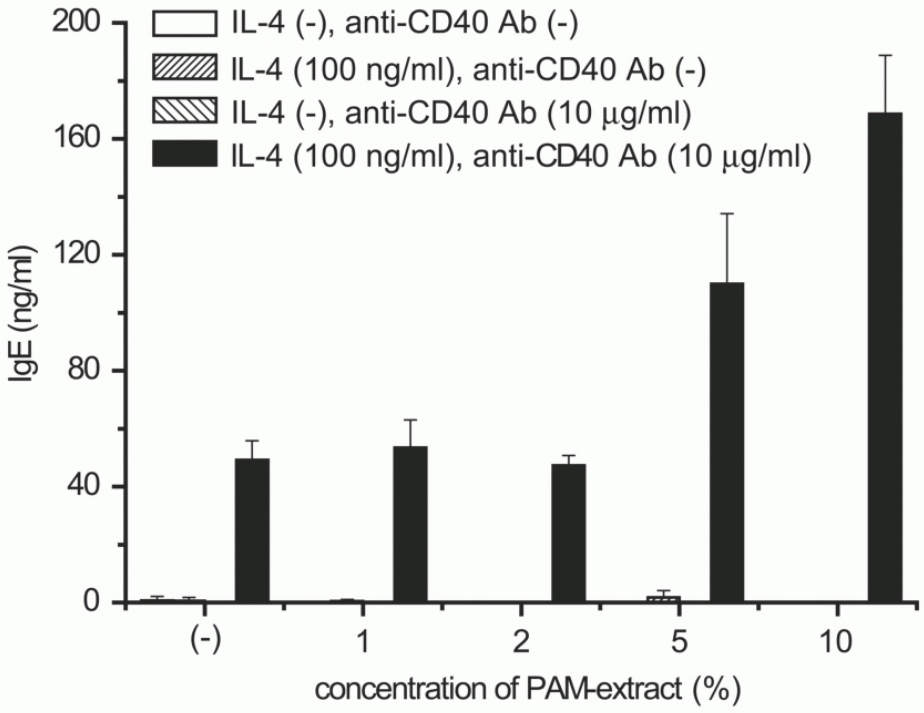
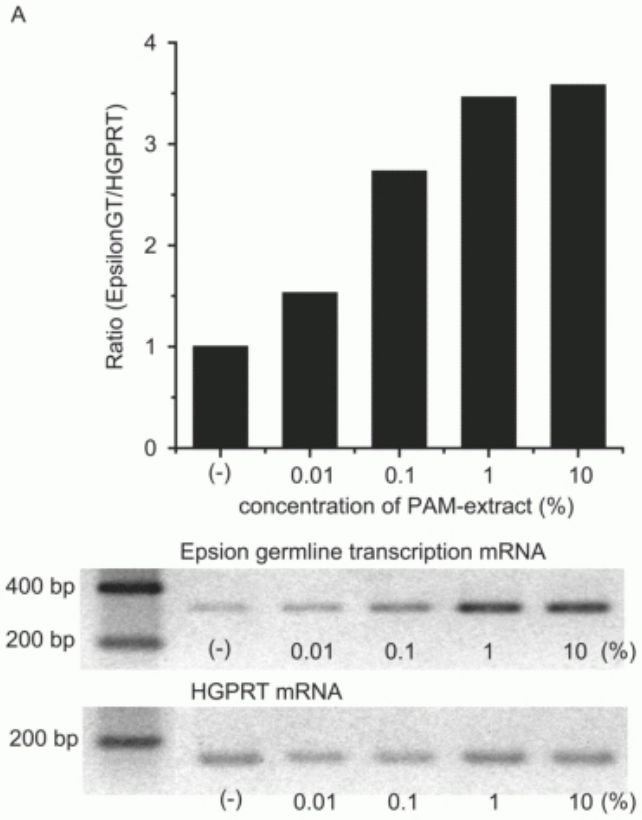

IL-4 (-), anti-CD40 Ab (-)
IL-4 (100 ng/ml), anti-CD40 Ab (-)
IL-4 (-), anti-CD40 Ab (10 μg/ml)
IL-4 (100 ng/ml), anti-CD40 Ab (10 μg/ml)
IgE (ng/ml)
200
160
120
80
40
0
(-)
1
2
5
10
concentration of PAM-extract (%)

Fig. 2


A
Ratio (EpsilonGT/HGPRT)
0
1
2
3
4
(-)
0.01
0.1
1
10
concentration of PAM-extract (%)
Epsion germline transcription mRNA
400 bp
200 bp
(-)
0.01
0.1
1
10 (%)
HGPRT mRNA
200 bp
(-)
0.01
0.1
1
10 (%)

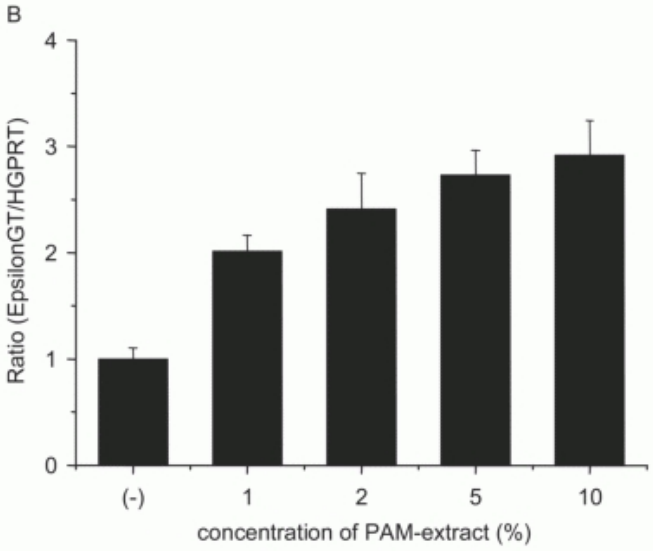

B
Ratio (EpsilonGT/HGPRT)
0
1
2
3
4
(-)
1
2
5
10
concentration of PAM-extract (%)

Fig. 3

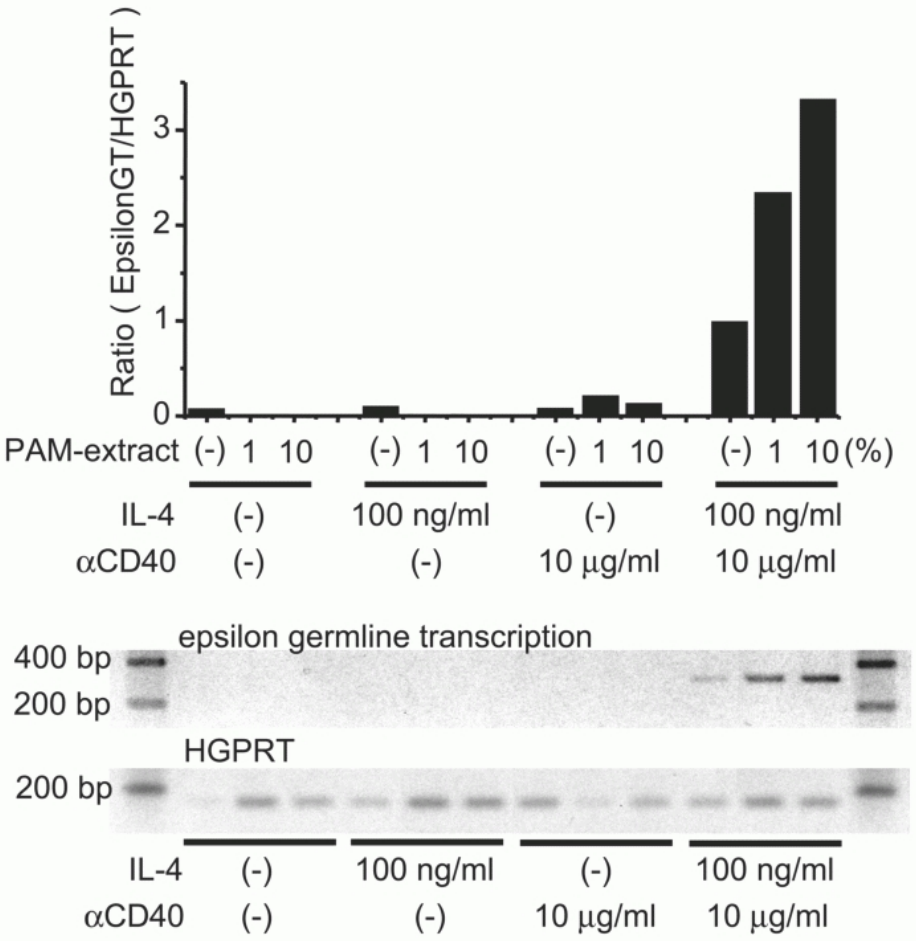

Ratio (EpsilonGT/HGPRT)
0
1
2
3
PAM-extract (-) 1 10 (-) 1 10 (-) 1 10 (-) 1 10 (%)
IL-4 (-) 100 ng/ml (-) 100 ng/ml
αCD40 (-) (-) 10 μg/ml 10 μg/ml
epsilon germline transcription
400 bp
200 bp
HGPRT
200 bp
IL-4 (-) 100 ng/ml (-) 100 ng/ml
αCD40 (-) (-) 10 μg/ml 10 μg/ml

Fig. 4

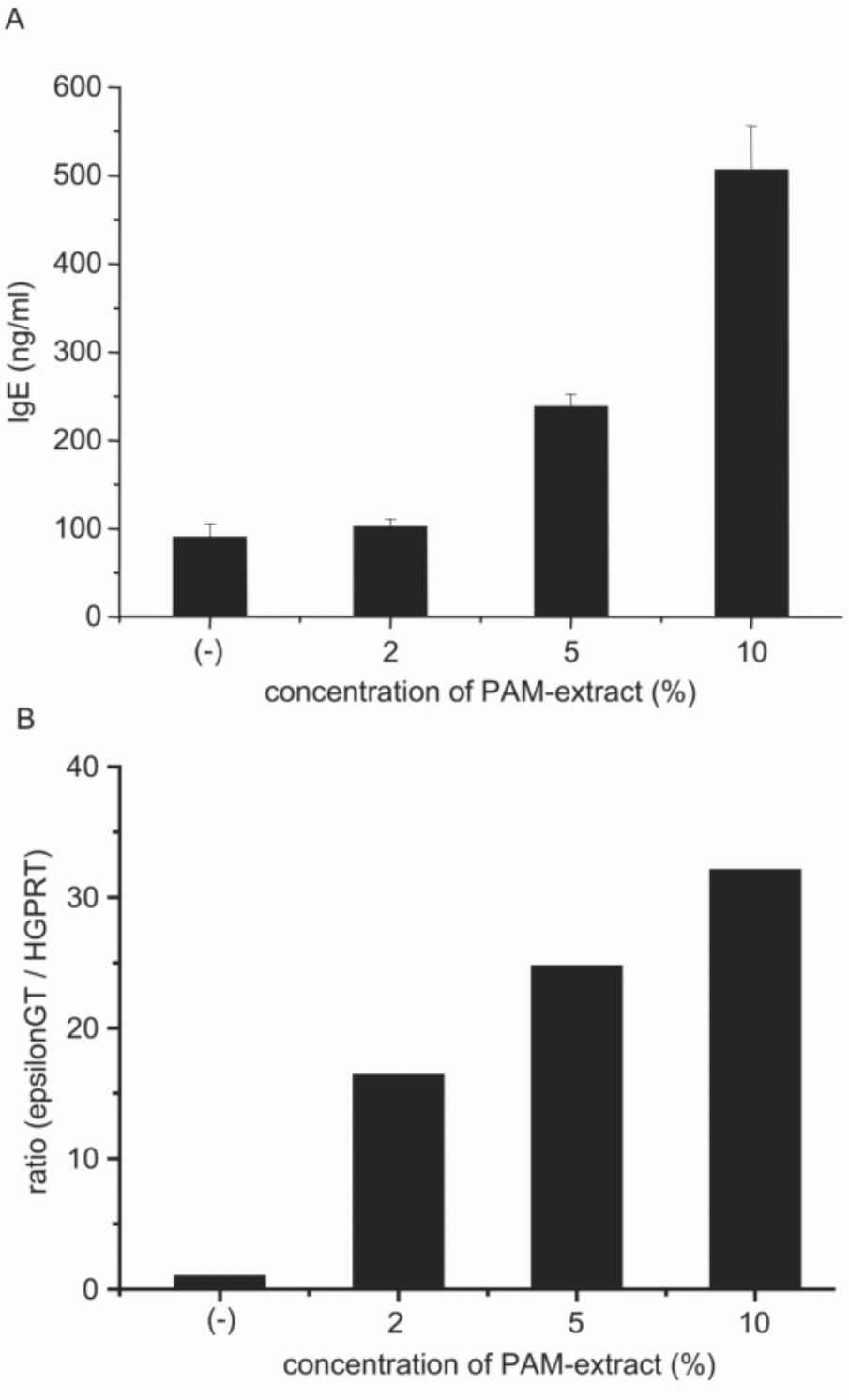

A
600
500
400
300
200
100
0
IgE (ng/ml)
(-)
2
5
10
concentration of PAM-extract (%)
B
40
30
20
10
0
ratio (epsilonGT / HGPRT)
(-)
2
5
10
concentration of PAM-extract (%)

Fig. 5

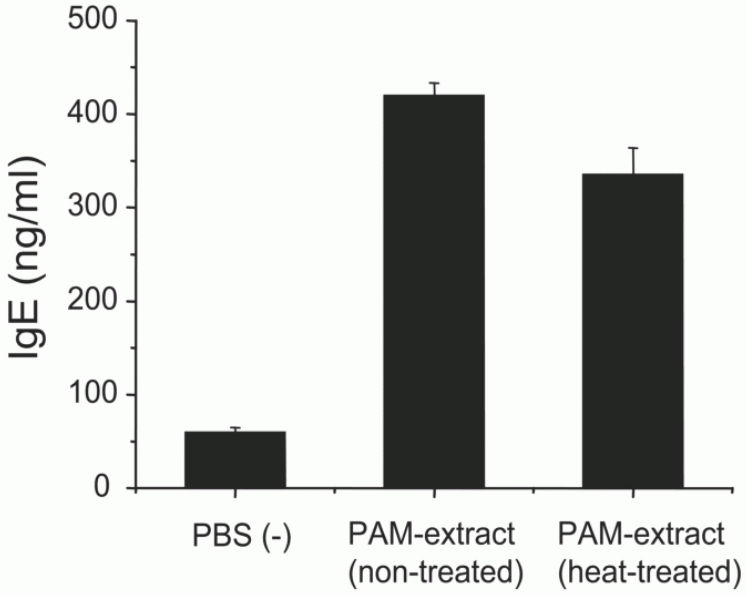

500
400
300
200
100
0
IgE (ng/ml)
PBS (-)
PAM-extract
(non-treated)
PAM-extract
(heat-treated)

Fig. 6

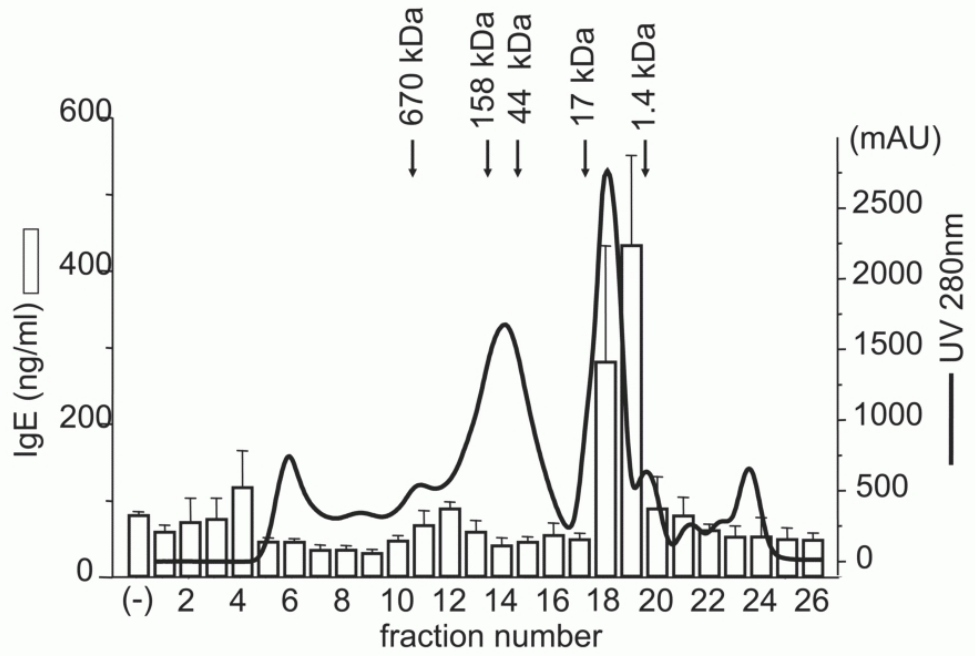

670 kDa
158 kDa
44 kDa
17 kDa
1.4 kDa
600
400
200
0
IgE (ng/ml)
(mAU)
2500
2000
1500
1000
500
0
UV 280nm
(-) 2 4 6 8 10 12 14 16 18 20 22 24 26
fraction number

Fig. 7

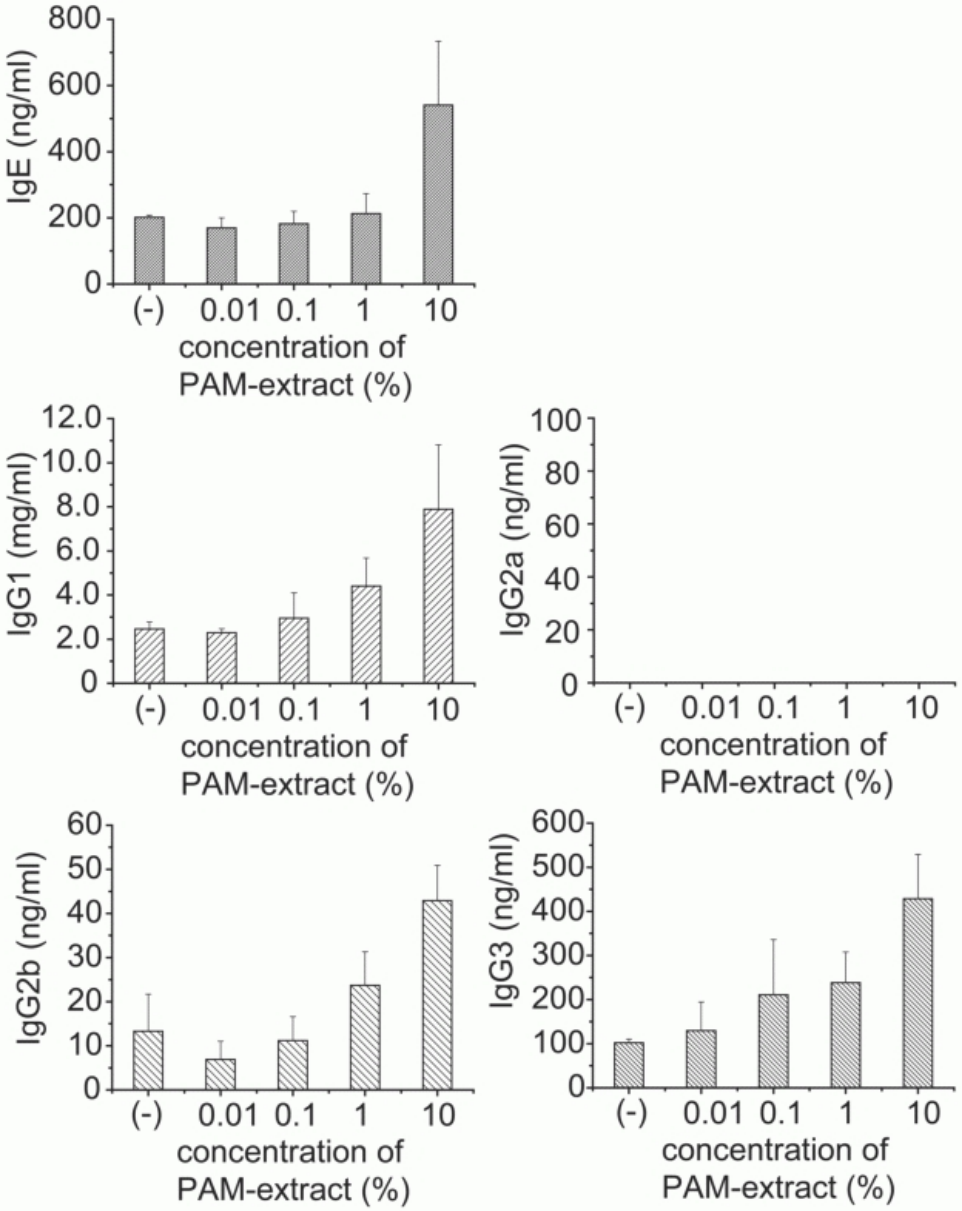
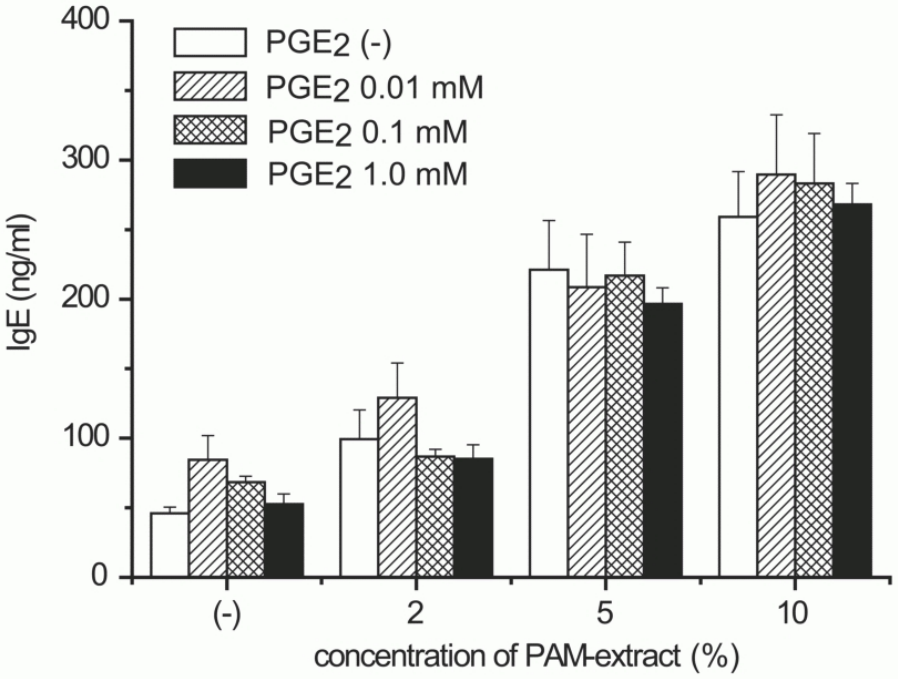

IgE (ng/ml)
800
600
400
200
0
(-) 0.01 0.1 1 10
concentration of PAM-extract (%)
IgG1 (mg/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0
(-) 0.01 0.1 1 10
concentration of PAM-extract (%)
IgG2a (ng/ml)
100
80
60
40
20
0
(-) 0.01 0.1 1 10
concentration of PAM-extract (%)
IgG2b (ng/ml)
60
50
40
30
20
10
0
(-) 0.01 0.1 1 10
concentration of PAM-extract (%)
IgG3 (ng/ml)
600
500
400
300
200
100
0
(-) 0.01 0.1 1 10
concentration of PAM-extract (%)

Fig. 8


400
300
200
100
0
IgE (ng/ml)
PGE2 (-)
PGE2 0.01 mM
PGE2 0.1 mM
PGE2 1.0 mM
(-)
2
5
10
concentration of PAM-extract (%)