Some studies concerning the mechanisms of PUFA action have been carried out using cultured cells; however, few in vitro studies on the direct effects of PUFAs on mast cells or basophils have been performed, and the molecular mechanisms by which PUFAs regulate mast cell functions are largely unknown.

Rat basophilic leukemia (RBL-2H3) cells have been used as in vitro systems for the analysis of cellular and molecular mechanisms involved in the secretion of histamine and serotonin by exocytosis [5,6]. RBL-2H3 cells have the same functions as primary mast cells and normal basophils which mediate allergic type I reaction [5,6]. Therefore, in the present study, we studied the effects of several PUFAs on RBL-2H3 cell signal transduction to determine the biological effects of these PUFAs on allergic reactions.

2. Materials and methods

2.1. Reagents

The PUFAs (arachidonic acid [AA], γ-linolenic acid [γ-LN], linoleic acid [LA], α-linolenic acids [α-LN], eicosapentaenoic acid [EPA] and oleic acid [OLE]) and saturated fatty acids (stearic acid [STA] and arachidic acid [AD]) were obtained from the following companies; AA: Funakoshi (Tokyo, Japan), γ-LN and LA: Sigma (St. Louise, MO, USA), OL, SA and AD: Wako Pure Chemical Industries (Osaka, Japan), and α-LN and EPA: Idemitsu Petrochemicals (Tokyo, Japan). A 20-mM ethanol stock solution of each PUFA was made, and stored at −70 °C.

Fura-2-AM was obtained from Dojindo (Kumamto, Japan). An IP_3 assay kit was obtained from Amersham Radiochemical (Amersham, UK).

2.2. Cells

All experiments were performed using a secreting subline of rat basophilic leukemia cells, RBL-2H3 [5]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, as described previously [6].

2.3. Pipes buffer

The Pipes buffer that was used had the following composition: 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM glucose and 10 mM piperazine-*N*-*N*′-bis (2-ethanesulfonic acid) (Pipes; pH 7.4). For the experiments performed in the absence of external calcium, Pipes–EGTA buffer (14 mM NaCl, 5 mM KCl, 1.0 mM EGTA, 5.5 mM glucose and 10 mM Pipes; pH 7.4) was used.

2.4. Measurement of cytosolic free-calcium concentration ($[Ca^{2+}]i$)

RBL-2H3 cells (6×10^5 cells/ml) were loaded with fura-2-AM (6 μM), as described previously. After centrifugation to remove free dye, the cells were resuspended in 1.5 ml of buffer. Fura-2-loading under the above conditions was adequate to monitor the changes in $[Ca^{2+}]i$. The resulting fluorescence was measured in a 1 cm quartz cuvette using a Shimazu RF-5000 spectrophotometer (excitation, 335 or 362 nm; emission, 500 nm) with stirring at 37 °C. Estimation of $[Ca^{2+}]i$ from fura-2 fluorescence data was based on the method described in a previous paper [7].

2.5. Measurement of histamine release from RBL-2H3 cells

The degranulation process was monitored by measuring histamine release, as described previously. Cells were pre-incubated for 16 h at 37 °C on a 24-well microplate (Falcon, No. 3047) in 1 ml/well of DMEM containing 10% fetal calf serum. The cells were washed three times with Pipes buffer and then challenged with 500 μl of each concentration of PUFA for 35 min. The amount of histamine release was determined by HPLC according to the post-column derivatization method using ortho-phthalaldehyde as described previously [6]. The percentage of histamine release (% release) was calculated using the following formula: $100A/(A+B)$, where A is the amount of histamine released from the cells after stimulation,

Table 1
Effects of various polyunsaturated fatty acids on histamine release from RBL-2H3 cells

Drug		Histamine release (%)	Drug		Histamine release (%)	Drug		Histamine release (%)
AA	20 μM	12.31±1.12*	α-LN	20 μM	8.85±0.24	STA	20 μM	10.01±0.47
	40 μM	36.56±1.16**		40 μM	9.37±0.13		40 μM	9.38±1.09
LA	20 μM	11.91±0.39*	EPA	20 μM	9.42±0.23	AD	20 μM	9.36±0.06
	40 μM	23.28±0.22**		40 μM	8.51±0.25		40 μM	10.34±0.18
γ-LN	20 μM	12.36±0.41*	OLE	20 μM	10.37±0.58			
	40 μM	28.35±0.38**		40 μM	11.29±0.11*			

Values are the mean±S.D. of 3 experiments.
The histamine release (HR) % from RBL-2H3 cells treated with a control solvent (0.4% EtOH) was 9.46±0.50%.
Levels of significance from control were determined by the Student's *t*-test. *$p<0.05$, **$p<0.01$.

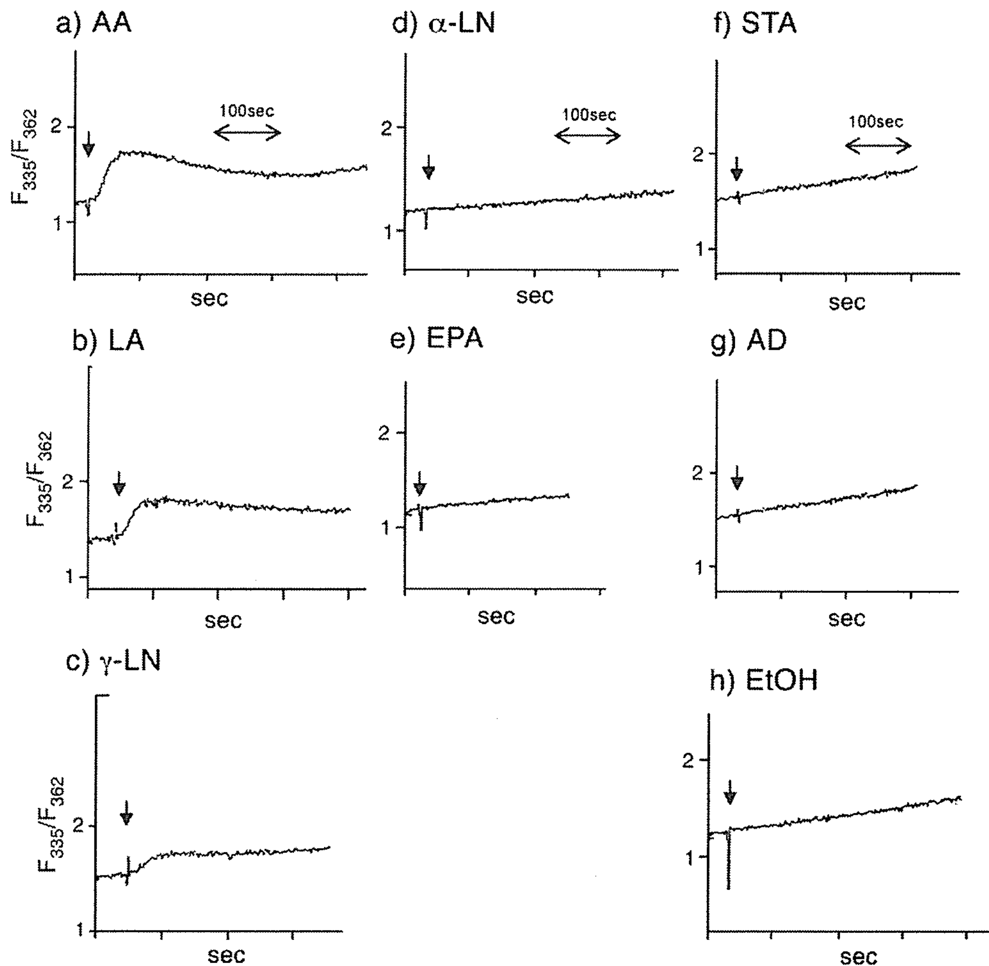

Fig. 1. Effects of PUFAs on [Ca^{2+}]i in RBL-2H3 cells. Changes in [Ca^{2+}]i in RBL-2H3 cells were monitored using the fluorescence Ca^{2+} indicator dye, fura-2. Where indicated, 40 μM of fatty acids (a, AA; b, LA; c, γ-LN; d, α-LN; e, EPA; f, STA; g, AD) or a solvent control (0.4% EtOH) was added to the cuvette, and [Ca^{2+}]i was monitored by measuring the ratio of the fluorescence intensity (F_{335}/F_{362}) of fura-2. The traces are representatives of typical experiments that were repeated at least three times.

and B is the amount of residual histamine remaining in the cells after stimulation.

2.6. Measurement of IP3 formation

IP_3 formation in the RBL-2H3 cells after PUFA stimulation was measured as described previously [6,7]. Briefly, RBL-2H3 cells (8×10^6 cells/ml) were stimulated with PUFA for 20 min. The IP_3 was then extracted, and the concentration in the cells (4×10^5 cells equivalent) was measured using an IP_3 assay kit (Amersham, UK), as described previously [6].

3. Results

3.1. Effects of different PUFAs on histamine release from RBL-2H3 cells

As shown in Table 1, 20–40 μM of AA (ω-6, C20:4) induced histamine release from RBL-2H3 cells in a dose-dependent manner. We next examined the specificities of PUFAs with different ω-numbers. As shown in Table 1, 20–40 μM of LA (ω-6, C18:2) or γ-LN (ω-6, C18:3) also induced histamine release in a dose-dependent manner. The extents of histamine release induced by 40 μM of LA or γ-LN (23.28% and 28.35%, respectively) were slightly lower than that induced by AA (36.56%). Unlike the ω-6 PUFAs, none of the ω-3 PUFAs (α-LN, C18:3 or EPA, C20:5) induced histamine release from the RBL-2H3 cells. None of the PUFA concentrations used in the study were toxic to the RBL-2H3 cells, as determined using a Wako LDH-cytotoxic test kit (data not shown). In addition, a ω-9 fatty acid, oleic acid (ω-9, C18:1), produced little or no induction of histamine release, nor did the saturated fatty acids, stearic acid (C18:0) and arachidic acid (C20:0).

From the above results, we suggest the several trends as follows. (i) The position of the double bonds relative to the terminal methyl group is important, since the ω-6 series of fatty acids was more active than the ω-3 series. (ii) The effect on histamine release requires the presence of several double bonds in a *cis* configuration and increases with the degree of

unsaturation, since neither of the saturated fatty acids produced an effect.

3.2. *Effects of different PUFAs on calcium response in RBL-2H3 cells*

To further examine the effects of PUFAs on histamine release from RBL-2H3 cells, we examined the effects of PUFAs on calcium response. As shown in Fig. 1a, 40 μM of AA induced a rapid increase in $[Ca^{2+}]i$ shortly after the addition of AA, as shown by the increase in fura-2 fluorescence; this increase in $[Ca^{2+}]i$ was maintained for more than 300 s. As shown in Fig. 1b and c, the other ω-6 PUFAs (LA and γ-LN) also increased $[Ca^{2+}]i$. On the other hand, as shown in Fig. 1d and e, the ω-3 PUFAs (α-LN and EPA) did not induce an increase in $[Ca^{2+}]i$ in the RBL-2H3 cells. Furthermore, as shown in Fig. 1f and g, the saturated fatty acids, stearic acid and arachidic acid, also did not induce an increase in $[Ca^{2+}]i$. To determine the dose-dependent and external calcium-dependent effects of AA, the increase in $[Ca^{2+}]i$ induced by 10–40 μM of AA was examined as shown in Fig. 2 AA increased the $[Ca^{2+}]i$ in a dose-dependent manner, starting at a threshold of 10 μM; the $[Ca^{2+}]i$ induced by 20 μM of AA was about 70% of the $[Ca^{2+}]i$ induced by 40 μM. The $[Ca^{2+}]i$ increase was significantly smaller when the experiment was performed using a free-Ca^{2+}-depleted buffer. Therefore, the increase in $[Ca^{2+}]i$ induced by AA seems to be caused mainly by calcium influx from the external medium and partly by the mobilization of Ca^{2+} from intracellular stores.

Furthermore, since the increase in $[Ca^{2+}]i$ induced by 40 μM of AA was slightly or not inhibited by preincubation with a cyclooxygenase inhibitor (10 μM of indomethacin) or a lipoxygenase inhibitor (10 μM of nordihydroguaiaretic acid [NDGA]), a direct effect of AA on ion channels of RBL-2H3 cells was suggested (data not shown).

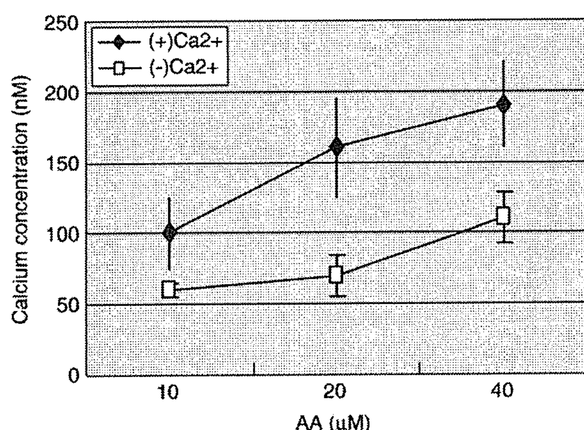

Fig. 2. Effects of various concentrations of AA on $[Ca^{2+}]i$ in RBL-2H3 cells. Cytosolic free-calcium concentration ($[Ca^{2+}]i$) in RBL-2H3 cells after 30 s of stimulation with 10–40 μM of AA in the presence (◆) or absence (□) of external calcium was determined. The values are the mean±S.D. for three different experiments. The $[Ca^{2+}]i$ before stimulation was 60 nM.

Table 2
Effect of PUFAs on IP_3 formation in RBL-2H3 cells

Drug	IP_3 formation (pmol/4×10^5 cells)[a]
40 μM AA	1.8±0.5
40 μM γ-LN	2.4±0.7
40 μM α-LN	1.8±0.7
40 μM EPA	1.9±0.6
0.4% EtOH	1.7±0.5

Each value represents the mean±S.D. of triplicate determinations. Similar results were obtained in two other experiments.

[a] Reaction was stopped at 20 min and IP_3 was extracted, and IP_3 concentration was measured by an IP_3 assay kit.

3.3. *Effects of PUFAs on IP_3 formation in RBL-2H3 cells*

Finally, we examined the effects of PUFAs on IP_3 formation in RBL-2H3 cells. As shown in Table 2, no changes in the IP_3 levels were observed after 20 min of incubation with 40 μM AA. Furthermore, no changes in the IP_3 levels were observed when the RBL-2H3 cells were treated with 40 μM γ-LN, 40 μM α-LN or 40 μM EPA. In addition, no significant changes were observed when the samples were treated with the ω-6 or ω-3 PUFAs. Therefore, the IP_3 level, which is probably determined by phosphatidylinostol turnover, and IP_3 formation are not involved in the ω-6 PUFAs-induced increase in $[Ca^{2+}]i$.

4. Discussion

In the present study, we showed that ω-6 PUFAs specifically induced histamine release and a rapid increase in $[Ca^{2+}]i$ in a cultured mast cell-like cell line, RBL-2H3. The specificity of the ω-6 PUFAs was confirmed, because neither the ω-3 PUFAs nor a ω-9 PUFA (OLE) were effective; two saturated fatty acids also did not have an effect.

Although some of the biological activities of PUFAs in the immune system are not distinguished among these groups of different ω-numbers [8], such as the triggering of cytosolic calcium mobilization in JURKAT T cells, some differences in their effects on the regulation of activated macrophage functions, like the production of interleukin 1 [9] or lymphocyte mitogenesis [10], have been observed. Furthermore, some differences in ω-6 and ω-3 fatty acids on the mastoparan-stimulated or IgE-mediated histamine release from a canine mastocytoma cell line (C2) or RBL-2H3 cell were reported [11–13]. In both cells, increase of degranulation by ω-6 fatty acid (AA) was higher than that by ω-3 fatty acid (EPA). The molecular mechanisms responsible for the regulation of mast cell functions by PUFAs are largely unknown. The greatest difference among PUFAs is their role as substrates for different series of prostaglandins (PGx); for example, ω-3 PUFAs are the sources of 1-series of PGx, like PGE_1 or PGA_1, whereas ω-6 PUFAs

are the sources of 2-series of PGx, like PGE_2 or PGA_2. In addition, before converting the AA to PGx, AA itself is released from RBL-2H3 cells in response to IgE or calcium ionophore stimulation [14]. Furthermore, in a previous paper, we showed that three days of pre-incubation with α-linolenic acid caused a reduction in AA levels and histamine release in RBL-2H3 cells [15]. We hypothesized that AA release itself, and not PGx, might have a biological effect on RBL-2H3 cells, leading to the regulation or even the initiation of subsequent reactions in the processes responsible for RBL-2H3 activation.

There was also an argument that fatty acids, especially PUFAs on both ω-3 and ω-6 series, regulate ion channels. In that report, AA was also suggested to act in an indirect way which involves cycloxygenase and/or lipoxygenase products derived from AA [16–18]. In other cases, the arachidonic acid-induced Ca^{2+} release or Ca^{2+} influx, which were not inhibited by either cyclooxygenase or lipoxygenase inhibitors, have been reported [19,20]. The results of our study also showed that cyclooxygenase or lipoxygenase inhibitors did not inhibit the increase in $[Ca^{2+}]i$ induced by AA. Thus, in RBL-2H3 cells, ω-6 PUFAs may regulate ion channels. So far as we examined, IP_3, a known mediator of $[Ca^{2+}]i$ mobilization, was not produced in response to the stimulation with ω-6 PUFAs. Therefore, AA seems to mobilize cytosolic calcium via the same IP_3-independent mechanisms previously described in JURKAT T cells [8] or in stimulus-secretion coupling in pancreatic cells [21].

The activation of protein kinase C (PKC) by fatty acid, especially AA, has been reported in several cell lines [22]. In this paper, we showed that ω-6 PUFAs induced histamine release from RBL-2H3 cells. We previously reported that increases in $[Ca^{2+}]i$ induced by a Ca^{2+}-ATPase inhibitor and PKC activation caused synergistic histamine release from RBL-2H3 cells [23]. Therefore, it is plausible that PKC activation by AA or other ω-6 PUFAs may be induced in RBL-2H3 cells.

In conclusion, we showed that ω-6 PUFAs increased $[Ca^{2+}]i$ and histamine release from RBL-2H3 cells, however, ω-3 PUFAs did not activate RBL-2H3 cells. These new findings are important for determining the mechanisms of the anti-allergic effects of ω-3 PUFAs.

Acknowledgement

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380 Primary Smallpox Vaccination Temporally Associated with New Onset Urticaria-Angioedema (UrtAng) Evolving to Chronic Disease

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RATIONALE: Urticaria following smallpox vaccination in adults has been described as a benign dermatologic reaction. (Clin Infect Dis 2004;38:958-65) The morbidity and disability associated with chronic urticaria, angioedema with anaphylactic features has not been described.

METHODS: A 23 year old healthy Marine received his primary smallpox vaccination with a major reaction but no initial systemic symptoms. On day 10, he developed total body hives and throat tightness requiring emergency treatment. He suffered repeated attacks with "fear of dying." His 10th episode with exercise included acute respiratory distress, emergency corticosteroids plus epinephrine, with resolution of symptoms. A year of follow up is described.

RESULTS: After 6 weeks corticosteroids, he reflared severely. His course was complicated by repeated heat and exercise triggered UrtAng with difficulty breathing. Despite extensive therapeutic interventions, symptoms occurred daily, were associated with disabling headaches, fatigue, unrefreshing sleep and pain severity 7-10/10 (visual-analogue-scale). Prolonged divided high dose steroids for months failed to suppress symptoms once tapered. At one year, on high dose H1, H2 and other blockers, the patient is profoundly disabled, unable to exercise without flares (headaches, fatigue, muscle aches, flushing). Normal tryptase and histamine noted with evidence of elevated circulating immune complexes.

CONCLUSIONS: Chronic urticaria, angioedema with multiple physical symptoms (chronic fatigue-like syndrome) associated with circulating immune complexes, as a sequel of smallpox vaccine reaction represents an unrecognized profoundly disabling autoimmune disease refractory to usual treatments. Although rare, the severity of this prolonged reaction merits further study to include a focus on improved treatment modalities.

381 Thrombocytopenia and Measles-Mumps-Rubella (MMR) Vaccine: Managing Loss Of Immunity to MMR

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RATIONALE: Thrombocytopenic purpura (TP) following vaccination with MMR occurs rarely and is listed as a compensable event (Vaccine Injury Table) by the National Vaccine Injury Compensation Program (VICP) at www.hrsa.gov/vaccinecompensation/. Strategies and outcomes for revaccination with MMR following loss of immunity have not been well defined.

METHODS: A 10 year old female presented for immunization recommendations due to loss of immune protection to rubella and rubeola but not mumps. Past medical history was unremarkable except for an episode of thrombocytopenic purpura at 16 months of age, 10 days following the receipt of her first dose of MMR. She developed generalized purpura and petechiae without a major bleeding event. Her platelet count was documented below 20,000/ cu mm. She was treated with immune globulin with subsequent normalization of her platelet count. The child's family was presented with four options and the associated benefit and risk of each. Options included no intervention, immune globulin prophylaxis every 6-12 months, administer MMR and follow platelet counts, or administer only the necessary vaccines (M,R) one at a time with more than a month between dosing.

RESULTS: The patient received the standard of care doses of rubella followed by measles monovalent vaccine with monitoring of platelet counts before and after each immunization. Both vaccines were tolerated with no laboratory or clinical evidence of platelet count reduction.

CONCLUSIONS: A single episode of MMR related thrombocytopenic purpura may not be predictive of recurrences with subsequent vaccine component challenges. The risk of recurrent TP remains to be defined in future surveillance studies.

382 Pre-transplant Cellular Alloimmunity as Assessed by a Panel of Reactive T-Cells (PRT) and Risk of Post-transplant Allograft Rejection

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RATIONALE: Organ transplantation is the optimal therapy for end-stage organ failure. Despite newer immunosuppressant agents, immune processes continue to cause morbidity/mortality in transplant patients. Noninvasive methods to assess post-transplant risk and guide individualized treatments are required. Our laboratory has developed a screening test, PRT, which assesses the strength of pre-transplant cellular alloimmunity analogous to the panel of reactive antibodies (PRA) evaluating risk of antibody mediated graft injury.

METHODS: Peripheral blood mononuclear cells from 28 kidney transplant candidates and 8 heart transplant candidates were tested against an HLA typed panel of allogeneic stimulator B cells. IFN-γ ELISPOT frequencies were measured. Positive assays were defined as a sample response to >75% of the test panel. Results were compared to PRA (positive >15% by flow beads) and when available, post-transplant outcomes.

RESULTS: 17 of 28 dialysis patients were PRT(-); none developed clinically recognized AR (acute rejection) within 6 months post-transplant. In contrast, 6/11 patients with PRT(+) assays pre-transplant, developed AR ($p=0.01$). There was no significant relationship between positive PRA and AR. Preliminary assessment of heart transplant candidates reveals 4/8 PRT(+) patients, 2 with positive PRA. 3 PRT(+) patients have thus far undergone heart transplantation; all 3 experienced grade 1A cellular rejection with negative C4d staining, a marker of antibody-mediated rejection.

CONCLUSIONS: The data strongly suggest pre-transplant PRT screening can non-invasively identify kidney and heart transplant patients at elevated risk for post-transplant cell mediated graft injury in the absence of detectable alloantibodies. Once confirmed by larger prospective trials, PRT screening could be used to guide clinical decision making.

Funding: National Institute of Health

383 Chimeric EGF Receptor That Detects IgE-binding/crosslinking

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RATIONALE: The crosslinking of high-affinity receptors for IgE (FceRI) with IgE and allergen is an essential step for activating mast cells. We have generated a chimeric receptor that can detect binding and crosslinking of IgE with high sensitivity.

METHODS: The cDNAs of human FceRI alpha and epidermal growth factor receptor (EGFR) were cloned. The fragments encoding the extracellular domain of FceRI alpha and the intracellular domain of EGFR were fused. The chimeric receptor was expressed on HLR-Elk1 cells, which are the HeLa cell-derived cell line stably expressing luciferase reporter gene that is transactivated by Elk-1. The binding and crosslinking of human IgE was measured by confocal fluorescence microscopy and luciferase assay, respectively.

RESULTS: The chimeric receptors were efficiently expressed on the plasma membrane of HLR-Elk1 cells. Confocal microscopic analysis revealed that the cells bound human IgE. Addition of human IgE and anti-IgE dramatically increased expression of the luciferase reporter gene.

CONCLUSIONS: The cells expressing FceRI-EGFR chimeric receptors could be a useful diagnostic tool that detects binding and crosslinking of IgE.

Funding: Health Labour Sciences Research Grant

SUNDAY