basophils perform important functions in anaphylaxis however, more recently it became clear that mast cells and basophils have quite different mechanisms and chemical mediators (Figure 1)[23].

(1) Classical pathway of anaphylaxis by mast cells and IgE

Mast cells are prevalent in the skin, mucous membrane, and the circumvascular, and they become sensitized to an allergen when allergen-IgE complexes bind the high-affinity IgE receptor FcεRI expressed on their cell surface. When re-exposed to the same allergen, the allergen cross-links the IgE/FcεRI complex on the surface of the mast cell, and an activation signal is transmitted that induces mast cell degranulation and the extracellular release of secretory granules that contain histamine and other chemical mediators[24, 25]. As a result, vascular permeability increases, bronchus smooth muscles contract, etc., leading to the characteristic anaphylactic symptoms such as rapid hypotension and dyspnea[26].

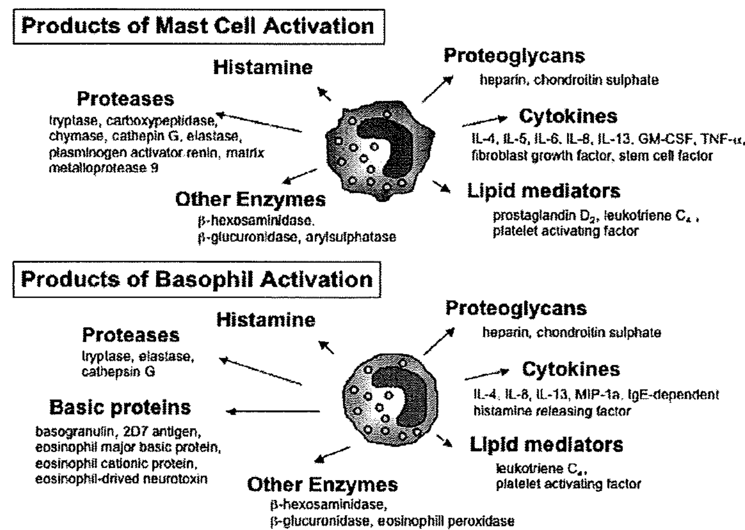

Figure 1. A. Mast cells and their activation products. B. Basophils and their activation products. Figure courtesy of Dr. A.F. Walls (Reference 23). IL, Interleukin; GM-CSF, Granulocyte-macrophage colony stimulating factor; TNF, Tumor necrosis factor; MIP, Macrophage inflammatory protein.

It was recently shown that anaphylactic pathosis cannot be completely attributed to the classical pathway based on analyses of a mouse model of the 'classical' pathway, which consists of IgE, FcεRI, mast cells, and histamine. For example, mast cell-deficient, IgE-deficient, or FcεRI-deficient mice still exhibited generalized anaphylaxis when they were sensitized to an allergen and then re-exposed to the same allergen[27-31]. These findings strongly suggest that there is another anaphylactic route that is distinct from the classical pathway (Figure 2)[32].

(2) A new anaphylaxis pathway that involves basophils and IgG

Mice lacking the FcRγ chain common to FcεRI, which is the IgE receptor, and FcγRI/III, which is the IgG receptor, do not experience anaphylactic reactions, suggesting that there is an IgG-mediated anaphylactic response in addition to an IgE-mediated reaction. After these mice were given antigen-specific IgG_1 monoclonal antibodies and then challenged with intravenous allergen, systemic anaphylaxis with tachycardia was induced[29]. Furthermore, it was ascertained that IgG_1-mediated anaphylaxis is induced in mast cell-deficient mice, which strongly suggest that a type of IgG_1-mediated, mast cell-independent anaphylaxis exists[27, 29]. Macrophages were considered a potential cell type that is responsible for IgG_1-mediated anaphylaxis[31], but there has been no conclusive proof establishing their role in this response. Because the high-affinity IgE receptor, FcεRI, is expressed on both mast cells and basophils in mice, it is conceivable that basophils are also are responsible for IgE-mediated anaphylaxis[33].

Tsujimura et al. developed a mouse monoclonal antibody that can deplete basophils in vivo. Using this antibody, they showed that basophils were dispensable for IgE-mediated anaphylaxis, while mast cells were critical for the "classical" anaphylactic pathway mediated by IgE and histamine. They injected allergen-specific IgE into basophil-depleted mice, and then challenged these mice with allergen. The anaphylactic response occurred in the basophil-depleted mouse, while mast cell-deficient mice exhibited no anaphylactic responses[34].

They also developed a penicillin G–specific mouse monoclonal IgG_1 antibody, and injected this IgG1 antibody into basophil-depleted mice. While an anaphylactic reaction occurred in control mice that were injected with penicillin G combined with bovine serum albumin, basophil-depleted mice did not have an anaphylactic response. Interestingly, this IgG-dependent anaphylactic response was more severe than the IgE-dependent response. Furthermore, these responses occurred in both normal and mast cell-deficient mice. Collectively, these

experiments provided seminal evidence that basophils play an important role in IgG-dependent anaphylaxis [34]. The allergen-specific IgG_1 and allergen complexes were found mainly on the surface of basophils, but also on macrophages, neutrophils, and natural killer cells by flow cytometry analysis. However, if these cell types except basophils were depleted from the mice prior to injecting the antibody and allergen, there was still an IgG-dependent anaphylactic response[34]. These results strongly indicate that basophils are a key component of IgG-dependent anaphylaxis.

(3) Platelet-activating factor and basophil-mediated anaphylaxis

Unlike IgG-dependent anaphylaxis, IgE-dependent anaphylaxis in mice is prevented by pre-treating with an anti-histamine drug. Based on these findings, the intervention of chemical mediators other than histamine likely contributes to IgG-dependent anaphylaxis[34]. Platelet-activating factor (PAF) is also known to act on the vascular endothelium and facilitate its permeability. When mice were injected with a PAF antagonist, IgG-dependent, but not IgE-dependent, anaphylaxis was almost completely inhibited. The stimulation by allergen and allergen-specific IgG_1 caused a significant elevation in PAF production, only in basophils. Furthermore, when conditioned medium from basophils that were stimulated with immune complexes was added to human vascular endothelial cells (normal human umbilical vein endothelium cells: HUVEC), their intercellular space expanded as an indication of vascular permeability; furthermore, this phenotype was inhibited with a PAF antagonist. From these results, it became clear that basophils quickly combine the immune complexes that are formed in the blood through the IgG receptor, which induces the production and release of factors such as PAF that ultimately lead to systemic anaphylaxis[34]. Although basophils are a minor population of cells in the blood and occupy only 0.5% of peripheral leukocytes, when they are activated by immune complexes they induce strong systemic anaphylaxis by secreting PAF, which induces vascular permeability 1,000-10,000 times higher than histamine.

(4) The role of basophils in human anaphylaxis

The experiments described above analyzed passive anaphylaxis, which is induced by challenging with an allergen after administering allergen-specific IgE or IgG. To examine anaphylaxis reactions that are more similar to human allergic conditions, Tsujimura et al. examined the role of basophils in active anaphylaxis by immunizing mice with an allergen two weeks prior to an intravenous injection of the allergen. This active anaphylaxis is more serious than passive anaphylaxis and caused fatal anaphylactic shock not only in normal mice but also in mast cell-

deficient mice. However, death from anaphylactic shock was prevented in mast cell-deficient mice when they were pretreated with the basophil-depleting antibody. This finding provided clear evidence that basophils have a decisive role in active anaphylaxis. Interestingly, fatal anaphylactic shock was not prevented in basophil-depleted normal mice that have mast cells. Therefore, these results indicate that both the classical pathway caused by mast cells and the new pathway caused by basophils equally contribute to anaphylactic shock[34].

Although it is not clear whether these results in mice apply to humans, there are many human reports that suggest there is an alternative pathway in addition to the classical pathway. There are reports of clinical anaphylactic cases, especially due to drug allergies, where allergen-specific IgE was not detected[35], mast cell tryptase was not elevated[36], and allergen-specific IgG antibody was elevated[37-39]. Recently, Vadas P. et al. showed that serum PAF levels were directly correlated and serum PAF acetylhydrolase activity was inversely correlated with the severity of anaphylaxis, and that the failure of PAF acetylhydrolase to inactivate PAF may contribute to the severity of anaphylaxis[40].

Based on analyses in mice, the new basophil-mediated anaphylaxis pathway is thought to require more allergens and antibodies than the classical pathway[34]. Therefore, when large amounts of soluble material are introduced into the body, such as antibody therapy, which has received recent attention as a molecular therapy, both the classical pathway and the new pathway may contribute to intense anaphylactic shock. Moreover, when we use peptide-specific immune therapy for autoimmune diseases or hyposensitization treatment for allergic diseases to induce immunologic tolerance, there is a danger that these therapies will lead to IgG-mediated anaphylaxis. Therefore, high-risk patients should be monitored not only for IgE levels but also for allergen-specific IgG, basophil function and/or serum PAF levels.

Roles of Basophils in Chronic Allergic Reactions

Basophils are often recruited to the site of allergic inflammation. However, for a long period of time, there was no definitive evidence that basophils were crucially involved in the pathogenesis of chronic allergic disorders. Mukai et al. showed that basophils are responsible for the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells[41]. Using a chronic cutaneous allergy mouse model they showed that basophils act as an initiator cell rather than an effector cell in chronic allergic inflammation.

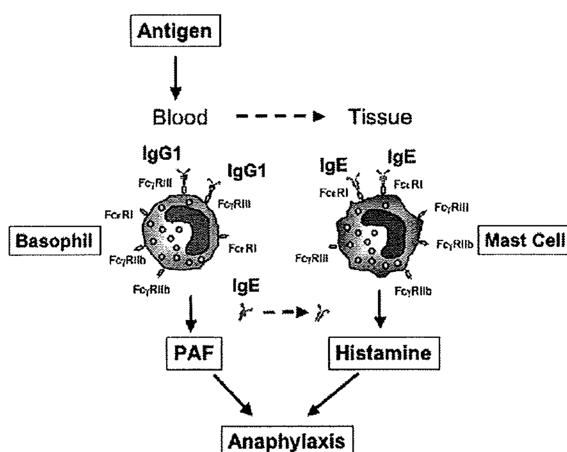

Figure 2. Schematic of the two anaphylaxis pathways. The classical pathway involving mast cells is initiated when antigen-IgE immune complexes bind FcεRI on the cell surface. The anaphylactic reaction is caused by the release of histamine from mast cells. The new pathway involving basophils begins when antigen-IgG_1 immune complexes bind to FcγRIII surface receptors, and platelet-activating factor (PAF) is released from basophils.

(1) Roles of IgE in the chronic allergic reaction

Matsuoka et al. established transgenic mice that carry genes encoding the heavy and light chains of hapten trinitrophenol (TNP)-specific IgE to generate a model system that would help elucidate both the pathological roles of IgE in the acute and chronic phases of allergic inflammation and the immunobiological roles in vivo. These mice produced high titers of TNP-specific IgE and their mast cells were heavily loaded with IgE[42]. According to their colleague's report, the immediate-type allergic ear swelling response of the biphasic response appeared after the ear of the mouse was intracutaneously challenged with the homologous TNP-binding ovalbumin[43]. Furthermore, on the second day of the antigenic challenge the ear began to swell again and more than doubled in skin thickness on the fourth day. Pathologic histology was used to show the detailed aspects of chronic allergic inflammation; for example, there was a robust infiltration of cells including basophils, and cornification was recognized as hyperplasia of the epidermis. The third phase of ear swelling was shown to be antigen-specific and IgE-dependent and it could be induced when antigen-specific IgE was given to normal mice (passive sensitization) prior to the antigen challenge. This

phenomenon was seen both in transgenic and wild-type mice. It was proven that IgE contributed not only to the immediate-type allergic response but also to the chronic allergic inflammatory response[41].

(2) The role of basophils in the IgE-dependent chronic allergic inflammatory response

When mast cell-deficient mice were challenged with the antigen after passive sensitization of IgE, the first and second phases of ear swelling, which are the immediate-type allergy response, were not observed, while the third-phase of ear swelling was observed both in these mice and in normal mice[41]. Based on this phenomenon, it is thought that mast cells are dispensable for the IgE-dependent chronic allergic response and that the immediate-type allergic response is required to induce the chronic response. Cyclosporine A almost completely inhibited the third phase of ear swelling and cellular infiltration, whereas an anti-histamine, cyproheptadine, did not have any significant effects on the third phase of the reaction. Given the delayed time of ear swelling, T cells were thought to contribute to this response. However, the third phase of ear swelling was also observed in T cell-deficient mice and T cells were not essential for the initiation of IgE-dependent chronic allergic inflammation[43].

Neither immediate ear swelling nor the third phase of ear swelling was observed in mice deficient for FcεRI, which is a high-affinity IgE receptor. This result indicates that the third phase of ear swelling requires a cell type that expresses FcεRI on its surface. To identify this cell type, Mukai et al. transferred various cells from normal mice into FcεRI-deficient mice and studied whether the third phase of ear swelling was restored. When FcεRI-expressing basophils that also express the natural killer cell marker DX5 (CD49b) were transferred into FcεRI-deficient mice, the third phase of ear swelling was restored. These findings indicate that this novel mechanism that leads to the development of chronic allergic inflammation is induced by basophils through the interaction of antigen, IgE, and FcεRI[41].

(3) Basophils as potential therapeutic targets for chronic allergic inflammation

When Mukai et al. examined the cells that infiltrated into the skin during the third phase of ear swelling skin, basophils comprised only 1-2% of the infiltrate and most of the cells were eosinophils and neutrophils. Therefore, they wanted to determine how this minor basophil population could cause chronic allergic inflammation[41].

Using the basophil-depleting monoclonal antibody established by their colleagues, they confirmed that this antibody could markedly decrease the number of basophils and further showed that mice pretreated with this antibody did not exhibit the third phase of ear swelling[44, 45]. This result proves that basophils are responsible for IgE-dependent chronic allergic inflammation. Furthermore, when this basophil-depleting antibody was given 2 or 3 days after allergen injection and the third-phase ear swelling had already occurred, the ear swelling and inflammation were inhibited and eosinophil and neutrophil infiltration was decreased markedly[44]. This result suggests that basophils function more as an initiator cell than as an effector cell and indicates the possibility that chronic allergic inflammation could be treated by targeting basophils. Once activated by allergen-mediated cross-linking of IgE/FcεRI, basophils secrete humoral factors such as a cytokines and chemokines that may directly or indirectly contribute to the infiltration of eosinophils and neutrophils.

The Role of Basophils in the Control of T Cell Differentiation

(1) T cell development and IL-4

CD4 positive T cells are functionally divided into four types; Th1, Th2, and Th17 are helper T cells[46-49], while Tregs are regulatory T cells[50]. Naive T cells differentiate into these functional T cells in response to different cytokines. IL-4 plays a crucial role in the development of Th2-type immune responses and the regulation of immunoglobulin isotype switching to IgE[51-53]. The IL-4-producing cell that produces sufficient levels of IL-4 to differentiate naive cells into Th2 cells in the lymph node is still unknown. Because Th2 cells produce variable amounts of Il-4, T cells themselves may be the predominant IL-4-producing cell that stimulates naive T cells to differentiate into Th2 cells[51, 54]. On the other hand, dendritic cells (DCs) and macrophages are required for CD4+ T cells to develop into Th1 cells. DCs are generally divided into three types; DC1 cells express the highest levels of MHC class I, class II, CD40, B7.1 and B7.2 compared to DC0 and DC2 cells. In terms of IL-12 production, DC1 cells have enhanced production, while DC2 cells produce lower levels than DC0 cells. Both DC0 and DC1 supported the differentiation of IFNγ-producing Th1 cells, but not IL-4-producing Th2 cells from TCR-transgenic naïve mouse Th cells. However, DC2 cells selectively enhanced the differentiation of IL-4-producing Th2 cells[55].

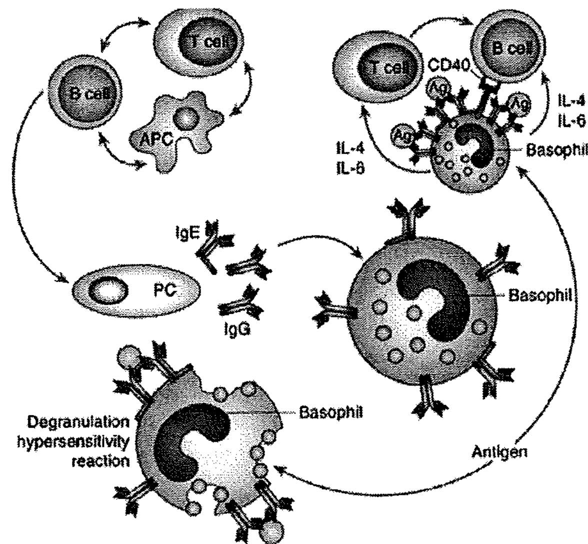

Figure 3. Basophils are mediators of the immune response. Upper left: Classical primary response by CD4+ T cells, B cells, and APCs, Ag-presenting cell (APC); PC, plasma cell. After IgG and IgE are produced by plasma cells (PC), basophils bind these circulating Abs. Upper right: A second presentation with Ag results in Ag-loaded basophils that begin producing the cytokines IL-4 and IL-6. Ag presentation and cytokine production by basophils enhance the secondary humoral immune response (upper right). The hypersensitivity reaction is caused by basophil degranulation and the release of lipid mediators (lower left). (Reference 64)

(2) The roles of basophils in Th2 cell development

Based on previous studies, basophils along with mast cells were thought to be cell types that produce chemical mediators such as the histamine and leukotoluene and cause allergic reactions. However, recently, basophils have received much attention because they have been shown to produce massive amounts of IL-4 when activated[56-59]. A basophil produces ten-fold more IL-4 than a Th2 cell. When naïve T-cells were cultivated with activated basophils in vitro, the naïve T cells differentiated into Th2 cells as a direct result of the IL-4 that was secreted by basophils[60, 61]. In addition, it has been established that parasitic infected mice have an abundance of differentiated Th2 cells. It was also reported that basophils produced IL-4 under such conditions[58, 59]. However, it is still unknown whether the IL-4 produced by basophils initiates Th2 cell differentiation or

maintains the Th2 cell dominant status. Furthermore, it was also unknown whether basophils interact with the naïve T cells in vivo. Naïve T cells differentiate into Th2 cells in the peripheral lymph node, and it is thought that basophils, unlike lymphocytes, circulate in the peripheral blood and never enter the peripheral lymph nodes. However, Sokol et al. recently elucidated a novel role of basophils in immunized mice[62]. They noted that many allergens have protease activity; therefore, they injected the protease papain into mice and then examined the secretion of IL-4 and other Th2-inducing cytokines. They showed that basophils transiently entered the regional lymph nodes soon after papain administration and that basophils secreted IL-4 after direct stimulation with papain. These results suggest that basophils also have important roles in the initial stages of immunization.

Roles of Basophils in Immunological Memory Responses

The cellular basis of immunological memory remains controversial. The classical primary immune response by CD4+ T cells, B cells, and antigen–presenting cells (APC) is initiated when antigen is presented to the host. Following IgG and IgE production by plasma cells (PC), basophils bind circulating antibody of either class (Figure 3). Denzel et al. showed that basophils bound large amounts of intact antigens on their surface and were the main source of IL-4 and IL-6 in the spleen and bone marrow after restimulation with a soluble antigen[63]. They also showed that basophil depletion resulted in a much lower humoral memory response and greater susceptibility of immunized mice to *Streptococcus pneumoniae*-induced sepsis. Adoptive transfer of antigen-reactive basophils significantly increased specific antibody production, and activated basophils, together with CD4+ T cells, profoundly enhanced B cell proliferation and immunoglobulin production. These basophil-dependent effects on B cells required IL-4 and IL-6 and increased the capacity of CD4+ T cells to provide B cell help (Figure 3. upper right). The circumstances that differentiate this participatory role of basophils from its traditional mast cell-like reaction, consisting of full degranulation, lipid mediator release, and cytokine release and subsequent anaphylactic reaction, remain unknown (Figure 3)[64].

Although basophils are not an abundant cell type and have been previously overlooked, they have recently emerged as highly important immune cells. Because anaphylactic shock and other serious reactions that involve basophils are closely related and can cause serious conditions, it is possible that basophils are maintained at low numbers to avoid these conditions. However, because they are usually low in number, they may be easy to target for treatment. Because brilliant

research in recent years has greatly contributed to our understanding of the importance of basophils, further research on the function of basophils will be more forthcoming in the future. As for the development of new treatments, basophils can now be viewed as essential components of immune responses and potential therapeutic targets.

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Antiproliferative Effect of Cucurbitacin B Extracted from *Trichosanthes cucumerina* L. on Human Cancer Cell Lines

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ABSTRACT

Objective: To determine the antiproliferative effect of cucurbitacin B extracted from *Trichosanthes cucumerina* L. on human cancer cell lines.

Methods: Two human lung non-small cell (adenocarcinoma) cancer cell lines i.e., LK87, and QG95, two human colon adenocarcinoma cell lines i.e., HCT15, and HT29, including one renal cancer cell line, A498, and one pancreatic cancer cell line, NOR-P, were used in this study. The viability of cells was assessed by using WST-8 which is based on detection of LDH released from damaged cells and reacts with WST-8 to form a yellow color. Cells were treated with the compound at various concentration from 1 through 100 μg/ml.

Results: The ED50 values (effective doses that are required for 50% inhibition growth of tumor cells) of the compound on human cancer cell lines ranged from approximately 69 μg/ml in HCT15 cells up to 231 μg/ml in QG95 cells. The inhibition of proliferation of this compound on these human cancer cell lines was observed to be in a dose dependent manner.

Conclusion: It could be concluded from this observation that this compound has a modest direct toxic effect to these cell lines with the highest toxic effect on human colon cancer cells.

Keywords: *Trichosanthes cucumerina*, Buap-Khom, cucurbitacin B, human cancer cell lines

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Trichosanthes cucumerina L., named in Thai "Buap- Khom", is a plant in the Cucurbitaceae family which is commonly found in Southeast Asia and Australia. From several reports, plants in this family are composed of many genera accounting for 110 genera and about 640 species which are mostly woody or herbaceous with climbing or trailing stems bearing tendrils and often arising from a tuberous rootstock.[1,2] In Thailand, this plant wildly grows along the river in some provinces such as Kanjanaburi. For Thai folk medicine, some of them have been used for the properties of antihelmintic, antidiabetic, and antiinflammatory effects except for *Trichosanthes cucumerina* which is inedible because of the bitter taste inducing nausea and vomiting symptoms.[3] In addition, in other countries, such as India, these special group of plants especially their seeds and fruit have been prescribed to treat various diseases i.e., infections and malignancies.[4] Several investigations have demonstrated the striking cytotoxic activities of these familial plants isolated compounds, cucurbitacins which are the major component, against several human cancer cell lines such as breast and lung cancer cells.[5-7]

In this study, cucurbitacin B was isolated from the juice of the fruit of *Trichosanthes cucumerina* and purified. This compound was tested against several human lung, colon, pancreatic, and renal cancer cell lines which have not previously been investigated.

MATERIALS AND METHODS

Plant extraction process

30 kg of fresh fruit of *Trichosanthes cucumerina* were collected from Kanjanaburi Province. They were immediately pressed and filtered to get the juice. After

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getting the juice (5 liters), it was shaken in ether in the separatory funnel and 1 gram of crystalline mixture was obtained. The crystalline mixture was purified by reversed phase column (RP-18) with the solvent system of water and acetonitril (55:45).[8] The major component which comprises cucurbitacin B (mixtured with dihydrocucurbitacin B) was gained from this extraction and purification procedure.

Extract preparation

For preparation, 0.0015 gm of the compound was dissolved in 50 μl of 95% ethanol and added up with 950 μl media to get the 1 ml stock. For all the experiment, the stock was diluted with media to get the concentrations at 10, 50, 100, 500, and 1,000 μg/ml. For the final concentrations from 1-100 μg/ml, 10 μl of each prepared diluted compound was added to each well in 96 well plates.

Cell lines and culture

Two human lung non-small cell (adenocarcinoma) cancer cell lines i.e., LK87, and QG95, two human colon adenocarcinoma cell lines i.e., HCT15, and HT29, one renal cancer cell line, A498, and one pancreatic cancer cell line, NOR-P, were used in this study. Human lung cancer cell lines were kindly provided by Dr.Koichi Takayama and Dr.Hiroyuki Inoue, Research Institute of Diseases of the Chest, Kyushu University, Japan. The other cell lines were kindly provided by Dr.Kenzaburo Tani, Medical Institute of Bioregulation, Kyushu University, Japan. All human lung and colon cancer cells were maintained in RPMI1640 (Nacalai Tesque, Kyoto, Japan) except for HT29 colon cancer cell line which was maintained in McCoy's 5A (Gibco, USA). Human renal carcinoma cell line was maintained in MEMalpha (Gibco, USA) and pancreatic cancer cell line was maintained in DMEM (Nacalai Tesque, Kyoto, Japan). All medium were supplemented with 10% fetal bovine serum (Japan Bioserum, Japan) and 1% antibiotic plus antimycotic agent (Nacalai Tesque, Kyoto, Japan). All cell lines were incubated at 37°C with 5% CO_2 and humidified atmosphere.

Cell viability assay

Viability of cells was assessed by using WST-8 (Nacalai Tesque, Kyoto, Japan) asssay (2-2(methoxy-4-nitrophenyl)3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt), which is based on detection of LDH releasing from damaged cells and reacts with WST-8 to form a yellow color.[9] In brief, cells were seeded at a density of $1x10^4$ cells/well in 96 well plates and treated with the compound at various concentrations from 1 through 100 μg/ml. Doxorubicin was used as a positive control. After a 48-hour incubation, 10 μl of WST-8 was added to each well and incubated at 37°C with a 5% CO_2 incubator for an additional 4 hours. The absorbance at 450 nm. of the dissolved solution was measured by using an Elisa plate reader (ThermoLabsystem, Japan). Data was calculated by using the formula as followed[10]:

Cell death (%) = [(control O.D.-sample O.D.)/ control O.D.] x100

Cell viability = 100-(% Cell death)

Statistic analysis

All experiments were performed in triplicate with three experiments. Data were expressed as the mean ± standard deviation. The R square equation was used to calculate the ED50 value. A P-value less than 0.05 were considered statistically significant.

RESULTS

Antiproliferative effects of compound on human cancer cell lines.

The ED50 values (effective doses that are required for 50% inhibition growth of tumor cells) of the compound on human cancer cell lines were summarized in Table 1 ranging from approximately 69 μg/ml in HCT15 cells up to 231 μg/ml in QG95 cells.

The antiproliferative effect, expressed as cell viability, of colon cancer cell lines, HCT15 and HT29, renal cancer cell, A498, and pancreatic cancer cell, NOR-P, showed the modest inhibition by the compound with the ED50 values at 69.391 ± 18.382, 106.431 ± 20.756, 105.912 ± 3.057, and 87.396 ± 1.950 μg/ml respectively as shown in Fig. 1A and 1C. The compound inhi-bited growth of lung cancer cells, LK87 and QG95, in the modest to low activity with the ED50 values at 99.517 ± 6.116 and 231.830 ± 11.182 μg/ml respectively as illu strated in Fig. 1B. All inhibition of cancer cells by the compound on the proliferation of these human cancer cell lines was observed to be in a dose dependent manner.

DISCUSSION

This investigation of the antiproliferative effects of cucurbitacin B was performed on several human cancer cell lines which have never been previously reported. The biological inhibitory effect of the compound depends on not only healthy or malignant cells but also the difference in the type of cell lines. Comparing in all these cancer cells, this compound has a modest to low activity on human lung cancer cell lines, whereas the inhibitory activity of the compound on other cell lines was in a modest pattern. Therefore, it could be concluded from this observation that this compound has a modest direct toxic effect to these cell lines.

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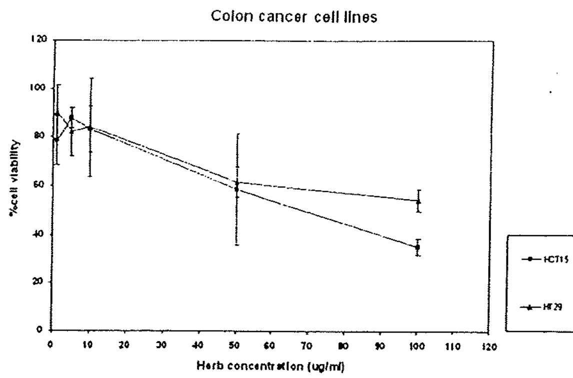
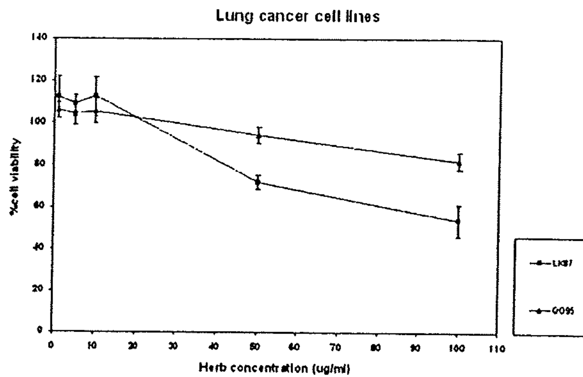
TABLE 1. ED50 values of human cancer cell lines were demonstrated as mean ± standard deviation.

Type of cancer cell line	ED50 value (mean SD)
Colon cancer: HCT15	69.391 ± 18.382 μg/ml
HT29	106.431 ± 20.756 μg/ml
Renal cancer: A498	105.912 ± 3.057 μg/ml
Pancreatic cancer: NOR-P	87.396 ± 1.950 μg/ml
Lung cancer: LK87	99.517 ± 6.116 μg/ml
QG95	231.830 ± 11.182 μg/ml



1A



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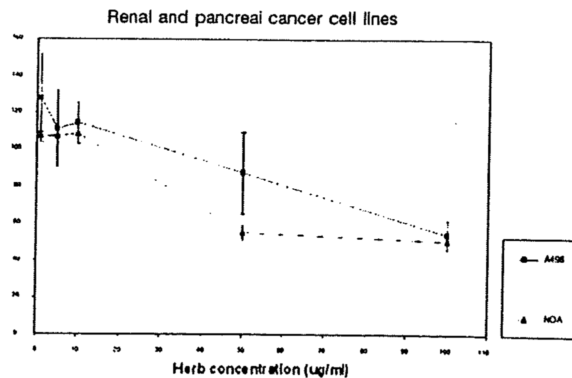

1C

Fig 1. Proliferative inhibition of cucurbitacin B on two colon cancer cell lines, two lung cancer cell lines, one renal cancer cell line and one pancreatic cancer cell line. The cells were seeded at $1x10^4$ cell/well in 96 well plates for 48 hours. HCT15 and HT29 colon cancer cell lines (1A), LK87 and QG95 lung cancer cell lines (1B) A498 renal cancer cell line and NOR-P pancreatic cancer cell line (1C) at different concentrations of 1, 5, 10, 50, 100 and 150 μg/ml for 48 hours. The cell viabilities were determined by MTT assay.

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Development of monoclonal antibodies for analyzing immune and hematopoietic systems of common marmoset

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Objective. **Common marmosets are considered experimental animals of primates useful for medical research. We developed several monoclonal antibodies (mAbs) directed to CD molecules to gain initial insight into the immune and hematopoietic systems of this organism, and analyzed the basic cellularity and characters of marmoset lymphocytes.**

Materials and Methods. **Anti-marmoset CD antigen mAbs were prepared using marmoset antigen-expressing transfectants and used for flow cytometric analyses and cell fractionation. Expression of T-cell−related cytokine gene transcripts was examined in response to T-cell receptor stimulation by reverse transcription polymerase chain reaction analyses. Hematopoietic progenitor activities of marmoset bone marrow cells were examined in fractionated cells by mAbs against CD117 (c-kit) and CD34.**

Results. **CD4 and CD8 expression profiles in T-cell subsets of marmoset were essentially similar to those in mouse and human. CD4$^+$ and CD8$^+$ subsets were isolated from marmoset spleens. Detected transcripts after stimulation of T cells included Th1-, Th2-, and Th17-related cytokines in CD4$^+$ cells and cytotoxic proteases in CD8$^+$ cells, respectively. Colony-forming abilities were detected mainly in CD117 (c-kit)$^+$ cells, irrespective of CD34 expression.**

Conclusions. **Marmoset immune system was basically similar to human and mouse systems.** **© 2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Mice are the most commonly used experimental animals as an alternative for humans, and many disease models have been generated using these animals. However, because of the evolutional distance between the two species, there are remarkable differences recognized in their genetic and physiological functions [1–5]. In particular, humans and mice exhibit differences in various aspects of immunity, such as T-cell subset differentiation and cytokine signal transduction. For example, for Th17 differentiation, both interleukin (IL)-1 and IL-6 are necessary in humans, whereas transforming growth factor−β (TGF-β) and IL-6 are important in mice. Thus, mice are not always versatile as a human alternative, despite their ease in gene manipulation.

One of the approaches for overcoming this species specificity is to establish a human immune system in immunodeficient mice. For generating such humanized mice, severe combine immunodeficient (SCID), nonobese diabetic (NOD)-SCID, RAG2-null, and NOD/shi-SCID/IL-2Rg^{null} (NOG) mice have been used as recipients because they each lack their own immunity, to varying degrees, because of gene mutation/deficiency [6–8]. On the other hand, human cord blood cells, lymphoid tissues/cells, or leukemic cells have been engrafted as donors into such mice [9–11]. Reconstitution of the human immune system in mice has been successful to varying extents, depending on both the type of recipient mouse and donor cells. In a combination

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of human cord blood cells and NOG mice; T and B cells of human origin emerge in peripheral blood, but the produced antigen-specific human antibody is mainly immunoglobulin (Ig) M but not IgG [12–16]. This suggests that the interaction of T and B cells does not occur properly in these xenografted mice [17]. Thus, reconstitution of human immunity is not necessarily complete. Furthermore, use of human tissues may involve an ethical issue, precluding the possibility of preclinical use.

The common marmoset, a new world monkey, and humans are in the same evolutional entity of anthropoidea. The common marmoset is the only experimental animal among primates that has been artificially bred and maintained as a closed colony for >20 years. This species was identified as one of the most useful primates because of its size, availability, and widespread use in biomedical research [18,19]. Disease models of neurological disorders and human-specific virus infections have been established with this animal [20–24]. In particular, models of autoimmune diseases, such as experimental allergic encephalomyelitis, which resembles clinical and pathological features of human multiple sclerosis, compared to other animal models including other monkeys, have been generated and found to be useful for drug screening and evaluation [22,25,26]. Thus, compared to mice, common marmosets appear to emerge as a closer alternative to humans. Recently, we succeeded in the development of green fluorescent protein (GFP)–transgenic marmoset [27]. This might open another approach to human immunity in addition to the aforementioned human-to-mouse xenograft.

However, our knowledge of the immune system of the common marmoset is still not enough to establish it as an experimental animal and use as an alternative for human immunity and its disorders. One major obstacle to overcome initially is the lack of various fundamental molecular resources necessary for marmoset research. This led us to cloning and determining the primary structures of 30 of the most representative immune system–related genes of the marmoset [28]. In addition to the gene resources, antibodies directed toward immune-system–related molecules also have to be developed. Although anti-human antibodies have been screened for their cross-reactivity with marmoset immune cells, it is not certain whether cross-reacting antibodies, if any, authentically recognize the corresponding marmoset antigens [29,30].

In this study, we established several anti-marmoset monoclonal antibodies (mAbs) that were directed against CD4, CD8, CD25, CD45, and CD117 (c-kit). By using these mAbs, we have preliminarily characterized T-cell subsets and hematopoietic progenitors of the common marmoset, and we also analyzed expression profiles of various cytokine genes in T-cell subsets. The present findings are expected to serve as a basis for further development of the common marmoset as a useful experimental animal concerning primate immunology.

Materials and methods

Animals

Common marmosets were obtained through CLEA Japan (Tokyo, Japan) and kept at the Central Institute for Experimental Animals (Kawasaki, Japan) during the experiments. Experiments using common marmosets were approved by the Institutional Committee for Animal Care and Use and performed at Central Institute for Experimental Animals according to institutional guidelines. The marmoset age (1 to 4 years old) and sex were arbitrary. In some cases, marmosets were injected subcutaneously with 10 mg/kg/d of human granulocyte colony-stimulating factor (G-CSF; Neutrogin, Chugai Co. Ltd, Tokyo, Japan) for 5 successive days. Female 5- to 6-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and kept in specific pathogen-free conditions in the animal facility at Juntendo University School of Medicine (Tokyo, Japan). NOD/Shi-scid, common gc-null (NOD/SCID/γc-null; NOG) mice were provided from the Central Institute for Experimental Animals (Kawasaki, Japan). Experiments using mice were performed following the guidelines set by the university.

Reverse transcription polymerase chain reaction, cDNA cloning, and sequence analyses

RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). RNA at 50 ng was reverse-transcribed to cDNA and amplified using the primers and OneStep RT-PCR kit (Qiagen). Reverse transcription polymerase chain reaction (RT-PCR) amplification was performed under the following conditions: reverse transcription was at 50°C for 30 minutes, polymerase activation was at 95°C for 15 minutes with 33 cycles of PCR, each cycle consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. PCR products were subjected to agarose gel electrophoresis. Primers used for PCR were as follows: for *IL-2*, 5′-ATGTACAGCATGCAGCTCGC-3′ and 5′-GCTTTGACAGAAGGCTATCC-3′; for *IL-4*, 5′-TGTCCACGGACACAAGTGCGA-3′ and 5′-CATGATCGTCTTTAGCCTTTCC-3′; for *IL-5*, 5′-GCCAAAGGCAAACGCAGAACGTTTCAGAGC-3′ and 5′-AATCTTTGGCTGCAACAAACCAGTTTAGTC-3′; for *IL-6*, 5′-ATGAACTCCTTCTCCACAAGCGC-3′ and 5′-GAAGAGCCCTCAGGCTGGACTG-3′; for *IL-10*, 5′-GGTTACCTGGGTTGCCAAGCCT-3′ and 5′-CTTCTATGTAGTTGATGAAGATGTC-3′; for *IL-17A*, 5′-CTCCTGGGAAGACCTCATTG-3′ and 5′-CAGACGGATATCTCTCAGGG-3′; for *IL-17F*, 5′-CAAAGCAAGCATCCAGCGCA-3′ and 5′-CATTGGGCCTGTACAACTTCTG-3′; for *IFN-γ*, 5′-CTGTTACTGCCAGGACCCAT-3′ and 5′-CGTCTGACTCCTTCTTCGCTT-3′; for *TGF-β*, 5′-GCCCTGGACACCAACTACTGC-3′ and 5′-GTCGCATTTGCAGGAGCGCAC-3′; for *TNF-α*, 5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3′ and 5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′; for *granzyme B*, 5′-ATATGAGGCCAAGCCCCACT-3′ and 5′-TCTCCAGCTGCAGTAGCATA-3′; for *perforin-1*, 5′-GGCCTGTGAGGAGAAGAAA-3′ and 5′-GCCCATCAGGTACTGACTCA-3′; for *HPRT*, 5′-TGACCAGTCAACAGGGGAC-3′ and 5′-GCTCTACTAAGCAGATGGC-3′. The procedure of cDNA cloning was the same as reported by us previously [28]. The primers used were as follows: for *granzyme B*, 5′-CCAAGAGCTAAAAGAGAGTAAGGGGGAAAC-3′ and 5′-AGCGGGGGCTTAGTTTGCTTCCTGTAGTTA-3′; for *perforin 1*, 5′-GTGTAGCCGCTTCTCTATACGGGA