การศึกษาการกระตุ้นเบโซฟิลในการวินิจฉัยผู้ป่วยที่มีปฏิกิริยาภูมิแพ้เฉียบพลันจากสารทึบรังสีที่มีไอโอดีนเป็นส่วนประกอบ

นางสาวปานวาสน์ ปิ่นนพภัณฑ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

BASOPHIL ACTIVATION TEST IN DIAGNOSIS OF IMMEDIATE IODINATED CONTRAST MEDIA HYPERSENSITIVITY PATIENTS

Miss Panwas Pinnobphun

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Microbiology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2009

Thesis Title	BASOPHIL ACTIVATION TEST IN DIAGNOSIS OF IMMEDIATE IODINATED CONTRAST MEDIA HYPERSENSITIVITY PATIENTS
By	Miss Panwas Pinnobphun
Field of Study	Medical Microbiology
Thesis Advisor	Assistant Professor Jettanong Klaewsongkram, M.D.
Thesis Co-Advisor	Associate Professor Nattiya Hirankarn, M.D. Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Graduate School

(Associate Professor Pornpote Piumsomboon,Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor Ariya Chindamporn, Ph.D)

..... Thesis Advisor

(Assistant Professor Jettanong Klaewsongkram, M.D.)

..... Thesis Co-Advisor

(Associate Professor Nattiya Hirankarn, M.D. Ph.D.)

..... External Examiner

(Assistant Professor Wirach Maek-A-Nantawat, M.D.)

ปานวาสน์ ปิ่นนพภัณฑ์: การศึกษาการกระตุ้นเบโซฟิลในการวินิจฉัยผู้ป่วยที่มีปฏิกิริยาภูมิแพ้เฉียบพลันจากสารทึบรังสีที่มีไอโอดีนเป็นส่วนประกอบ. (BASOPHIL ACTIVATION TEST IN DIAGNOSIS OF IMMEDIATE IODINATED CONTRAST MEDIA HYPERSENSITIVITY PATIENTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.นพ. เจตทะนง แกล้วสงคราม, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. พญ. ดร.ณัฏฐิยา หิรัญกาญจน์, 75 หน้า.

ปฏิกิริยาภูมิแพ้เฉียบพลันจากสารทึบรังสียังคงเป็นปัญหาสำคัญในทางการแพทย์ ทั้งในแง่ของการวินิจฉัย และการป้องกัน ปัจจุบันวิธีการวินิจฉัยที่ดีที่สุดของโรคนี้คือ การติดตามจากประวัติการรักษาของผู้ป่วยและยังไม่มีวิธีการวินิจฉัยอื่นที่ดีพอในการวินิจฉัยโรคนี้ รายงานการศึกษาที่ผ่านมาแสดงให้เห็นว่าการทดสอบ การกระตุ้นเบโซฟิลโดยการประเมินระดับการแสดงออกของ CD63 บนผิวเซลล์ สามารถช่วยในการวินิจฉัยปฏิกิริยาภูมิแพ้จากยาบางชนิดได้ การศึกษานี้มีจุดประสงค์เพื่อวินิจฉัยปฏิกิริยาภูมิแพ้เฉียบพลันจากสารทึบรังสี ที่มีไอโอดีนเป็นส่วนประกอบ โดยอาศัยการทดสอบการกระตุ้นเบโซฟิลโดยมี CD63 เป็นเครื่องบ่งชี้ในการวินิจฉัย การทดลองนี้ทำการศึกษาในกลุ่มผู้ป่วยที่เคยมีประวัติการเกิดปฏิกิริยาภูมิแพ้เฉียบพลันจากสารทึบรังสี 24 คน และอาสาสมัครในคนปกติ 14 คน และทำการทดสอบการกระตุ้นเบโซฟิลโดยใช้ CD63 เป็นเครื่องบ่งชี้ของ Flow2-CAST Basophil activation test (Bühlmann Laboratories) ของสารทึบรังสีชนิดละ 2 ความเข้มข้นคือ 1:10 และ 1:100 ซึ่งพบว่าการทดสอบการกระตุ้นเบโซฟิลมีความไวประมาณร้อยละ 54.17 และมีค่าความจำเพาะประมาณร้อยละ 92.86 โดยไม่มีความแตกต่างกันระหว่างผู้ป่วยที่ให้ผลบวกและผลลบจากการทดสอบทางผิวหนัง นอกจากนี้เมื่อนำผลการทดสอบการกระตุ้นเบโซฟิลวินิจฉัยผู้ป่วยร่วมกันกับการทดสอบทางผิวหนังจะทำให้มีค่าความไวเพิ่มขึ้นเป็นร้อยละ 70.8 ดังนั้นการทดสอบการกระตุ้นเบโซฟิล ในห้องปฏิบัติการน่าจะมีประโยชน์ในการวินิจฉัยช่วยผู้ป่วยที่เกิดปฏิกิริยาภูมิแพ้เฉียบพลันจากสารทึบรังสี

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต ปานวาสน์ ปิ่นนพภัณฑ์

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก เจตทะนง แกล้วสงคราม

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม

4989132920 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: BASOPHIL ACTIVATION TEST / CONTRAST MEDIA / IMMEDIATE HYPERSENSITIVITY

PANWAS PINNOBPHUN : BASOPHIL ACTIVATION TEST IN DIAGNOSIS OF IMMEDIATE IODINATED CONTRAST MEDIA HYPERSENSITIVITY PATIENTS. THESIS ADVISOR: ASST. PROF. JETTANONG KLEWSONGKRAM, M.D., THESIS CO-ADVISOR: ASSOC. PROF. NATTIYA HIRANKARN, M.D. Ph.D., 75 pp.

The diagnosis and prevention of iodinated contrast media or radiocontrast media (RCM) hypersensitivity is still a major problem in clinical practice. At present, there are no alternative methods to diagnose RCM-induced hypersensitivity reaction other than history taking. Basophil activation test (BAT) by determining CD63 expression on basophil cell surface was demonstrated to correlate with hypersensitivity drug reactions. The purpose of this study, therefore, aims to explore the possibility whether BAT play a role in the diagnosis of patients with a history of RCM-induced immediate hypersensitivity reaction. Whole blood from 24 patients and 14 healthy donors were incubated in the presence or absence of 1:10 and 1:100 dilution concentrations of radiocontrast media. Levels of CD63 expressions on basophil surface were consequently determined by fluorescence staining and Flow cytometry analysis. The result from the study indicated that basophils from 13 out of 24 patients with a history of RCM reactions (54.17%) had increased CD63 expression and 11 out of 13 patients showed positive BAT result to both 1:10 and 1:100 concentrations of incubated RCM. Sensitivity and specificity of BAT were 54.17% and 92.86%, respectively. BAT results were not significantly different between skin test positive and skin test negative patients. In addition, BAT in combination with skin test results had increased sensitivity to 70.8%. In conclusion, basophil activation test (BAT) may be potentially useful in the diagnosis of patients with RCM-induced hypersensitivity reactions.

Field of Study : Medical Microbiology Student's Signature Panwas Pinnobphun

Academic Year : 2009 Advisor's Signature

Co-Advisor's Signature Nattiya Hirankarn

ACKNOWLEDGMENTS

I would like to express my deepest sense of gratitude to my advisor Assistant Professor Jettanong Klaewsongkram, M.D. and my co-advisor, Associate Professor Nattiya Hirankarn, M.D. Ph.D. for their great helps, guidance, encouragement, valuable suggestion and supports throughout my study.

My gratitude is also extended to Associate Professor Ariya Chindamporn, Ph.D, and Assistant Professor Wirach Maek-A-Nantawat, M.D. who serve as the committee of my thesis.

I would like to thank Miss Suprenee Buranapradithul for her kindness in guidance throughout the laboratories. Without her this work would not be accomplished.

Finally, I would like to express my deepest gratitude to my parents and members of my family for their love, understanding and encouragement extended throughout my study.

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LISTS OF ABBREVIATIONS

%	Percent
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre
µM	Micromolar
^{o}C	Degree Celsius
-ve	negative result
+ve	positive result
BBB	Blood brain barrier
BAT	Basophil activation test
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cm^{2}	Centimeter
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FcεRI	Fc epsilon recepter 1
fMLP	N-formyl-methionyl-leucyl-phenylalanine
IgG	Immunoglobulin G
IL-3	Interleukin-3

LISTS OF ABBREVIATIONS (cont.)

KCl	Potasium chloride
kDa	Kilodalton
ml	Millilitre
mg	Milligram
min	Minute
mM	Millimolar
NaCl	Sodium chloride
NSAIDS	Non steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
pH	Potential of Hydrogen ion
Rpm	Revolutions per minute
RCM	Radiocontrast media

CHAPTER I

INTRODUCTION

Since 1950, Iodinated contrast media (ICM) or Radiocontrast media (RCM) has been increasingly used in the clinic. It is found that the rate of using the ICM is around 75 millions times yearly worldwide (1). The RCM is generally considered safe. RCM allergy is significantly reduced caused by the use of non-ionic contrast media instead of ionic contrast media. Nevertheless, the adverse effect of using the RCM; especially immediate hypersensitivity reaction remains a significant problem for both patients and physicians since a number of patients need an examination by applying RCM. Currently, there are limited development of the predicted or diagnostic tools for the hypersensitivity reaction to RCM(2).

All iodinated contrast materials in current use are chemical modifications of a 2,4,6-tri-iodinated benzene ring with different side chains in the 1, 3, and 5 positions and different numbers of benzene rings. They are classified on the basis of their physical and chemical characteristics including osmolality, ionization in solution, and chemical structure. Currently, four classes of contrast material are commercially available: ionic monomers, nonionic monomers, ionic dimers, and nonionic dimers. Contrast materials are administered mostly in volumes of 50–150 mL; the compounds are rapidly distributed in the body and are recovered mainly unmetabolized in urine within 24 hours (3-5). The frequency of mild anaphylactic reactions ranges from 3.8% to 12.7% in patients receiving

high-osmolar ionic contrast material and 0.7% to 3.1% in patients receiving low-osmolar nonionic contrast material. The risk for serious or severe reactions—that is, anaphylaxis grade 3—has been estimated to be from 0.1% to 0.4% with ionic contrast material and 0.02% to 0.04% with nonionic contrast material (6-12).

The pathophysiology of hypersensitivity reactions to RCM is still unclear leading to the difficulty in diagnosis due to non-specific symptoms of the affected patients (13-15). The diagnosis is basically performed by personal judgment from patients' symptoms. Patients frequently already have congenital disease or received multiple types of drugs which could cause the symptoms similar to the finding in immediate hypersensitivity reaction by RCM. In addition, there is no current diagnostic method which is reliable enough. It is therefore necessary to develop new diagnostic method.

Basophil in the bloodstream and mast cell in the tissue can be considered as effecter cells which occur in the early stage of IgE meditated hypersensitivity, e.g., rhinitis, asthma and anaphylaxis. In addition, both types of cells are also related to the other process of allergic occurrence as hypersensitivity which is caused by complement activation. Normally, basophils represent less than 0.5% of the total leukocytes in peripheral blood. This is one of reasons why separation of basophil from white blood cell is difficult to be performed. However, basophil is significantly related to immediate hypersensitivity reaction. This leads to the development of Basophil activation experiment *in vitro*. Although the early developed test was the histamine release assay; this method could not be practically applied to clinical diagnosis due to the lack of accuracy. Next, the flow cytometry technology was used in basophil activation testing from specific antigen by analyzing the membrane surface markers, which are

upregulated upon activation. This method is called Basophil Activation Test (BAT) (16, 17).

BAT was developed by using the advantages of flow cytometry technology which could analyze difference between cellular types and can be used to identify specific cell populations, even those which are present in low amounts. It has proven to be useful in the study of allergen-induced activation. Therefore, this method is useful in research and experiment regarding allergen-induced activation.

The significant activation marker of basophil used in common research for BAT is the CD63 expression after activating Bbasophil with specific allergen. CD63 is 53 kDa tetraspan protein which could be found on basophil, mast cell, monocyte macrophage and platelet. Normally, CD63 is a protein in basophilic granule membrane covering histamine and others. CD63 expression has relation with deregulation process and histamine release therefore it could be use as activated basophil marker. A number of research performed experiment following BAT method by using CD63 as an activation marker of many kind of disease or allergen such as allergic to insect venom (18, 19) or latex (20). More recently, the CD203c marker (ecto-nucleotide pyrophosphatase/phosphodiesterase 3) was proposed as a valuable tool for the detection of activated basophils by flow cytometry. Indeed, this antigen, selectively expressed on basophils, mast cells and their progenitors, is rapidly up-regulated after activation of IgE sensitized basophils with the relevant allergen (21-23). These researches can specify that BAT method is practical, easy to use, less time, low cost and little patient's blood used for testing. Besides, this method is also effective in hypersensitivity diagnosis.

Therefore, the objective of our study was to study the role of basophil activation testing using CD63 and CD203c as activation marker for the diagnosis of immediate hypersensitivity reaction to RCM.

CHAPTER II

OBJECTIVES

The objectives of this study were:

1. To evaluate the sensitivity and specificity of BAT in the diagnosis of patients with a history of RCM-induced immediate hypersensitivity reaction compare with clinical history

2. To determine the correlation between BAT and skin tests in the diagnosis of immediate hypersensitivity reaction to RCM.

CHAPTER III

LITERATURE REVIEW

Radiocontrast media

Radiocontrast media (RCM) are compounds which are introduced into the organism by different routes. The RCM can be used to increase the sensitvity of radiographic images and resulted in higher accuracy in diagnostic imaging. The high-definition images include computerized tomography (CT), digital subtraction angiography (DSA), digestive sytem, biliary system, intravenous urography, phlebography of extremities, venography, arteriography, visualization of body cavities (e.g arthrography, hysterosalpingography, fistulography, dacrycistography), myelography, ventriculography, cisternography and other diagnostic procedures.

The first water soluble iodine contrast medium was used in 1920 and was discovered because patients with syphilis in those days were treated with sodium iodide. The sodium iodide was observed in an image of the abdomen as an "increased density" of the kidneys. Sodium iodide, however, had a high toxicity when used as contrast medium (24-26). The efforts to design less toxic contrast media were started in the 1920s and are still continuing. A major development occurred in the beginning of the 1950s when it was found that contrast media with three iodine atoms bound to a benzene ring had low toxicity (amidotrizoate Table 1, Fig. 2). A number of benzene ring with three iodine atoms is defined as a "mer". A monomer, for example, contains one such three-iodinated benzene ring, while a dimer contains two such structures. In the

1960s a radiologist, T. Almen, proposed the synthesis of monomers and oligomers of non-ionic, tri-iodinated contrast media (Fig. 1). The first non-ioinic monomer was produced by the Norwegian contrast medium company, Nyegaard & Co (Today Nycomed Imaging AS) (1, 27).

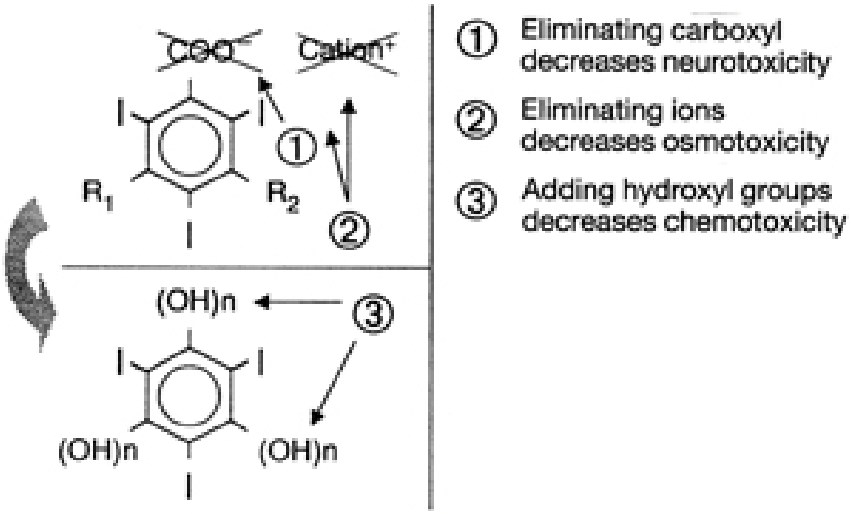


Figure 1. Transformation of an ionic monomer (above) to a non-ionic monomer (below)

Further factors that influence toxicity and water solubility are described below. Table 1 and Figures 2-5 show the most commonly used contrast media, their names, chemical structures, osmolality, viscosity and ratio between number of iodine atoms and number of contrast medium particles in an ideal solution.

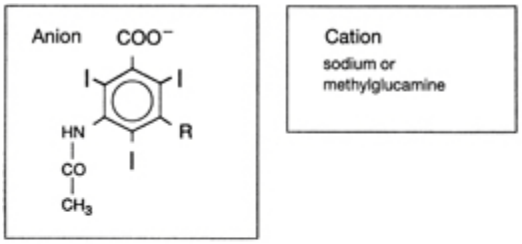
Table 1. Different contrast media - their structure, ratio, viscosity, osmolality and name

	Structure	**Ratio**	**Viscosity**		**Osmolality**	**Generic name**	**Trade name**
			20°	**37°**			
Figure 2	ionic monomer	3:2=1.5	5^{+} 9^{++}	3^{+} 5^{++}	1500-1600	iothalamate metrizoate amidotrizoate ioxithalamate	Conray Vasoray Isopaque Urografin Angiografin Gastrografin Telebrix
Figure 3	ionic dimer	6:2=3	12	6	600	ioxaglate	Hexabrix
Figure 4	non-ionic monomer	3:1=3	11	6	500-700	iohexol iopamidol iopromide ioversol	Omnipaque Iopamiro Ultravist Optiray
Figure 5	non-ionic dimer	6:1=6	25	10	300	iodixanol iotrolan	Visipaque Isovist

Values of viscosity (cP) and osmolality (mOsm/kg H_2O) have been approximated to an iodine concentration of 300 mg I/ml.

+ are viscosity values for sodium salts.

++ are viscosity values for meglumine salts.



Compound	R
Diatrizoate	– $NHCOCH_3$
Iothalamate	– $CONHCH_3$
Ioxithalamate	– $CONHCH_2CH_2OH$
Iodamide	– $CH_2NHCOCH_3$
Metrizoate	– $N(CH_3)COCH_3$

Figure 2. Ionic monomer

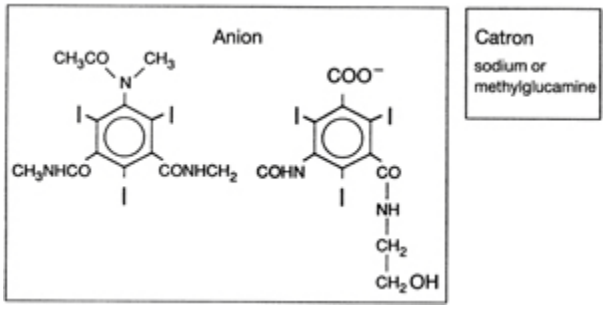


Figure 3. Ionic dimer

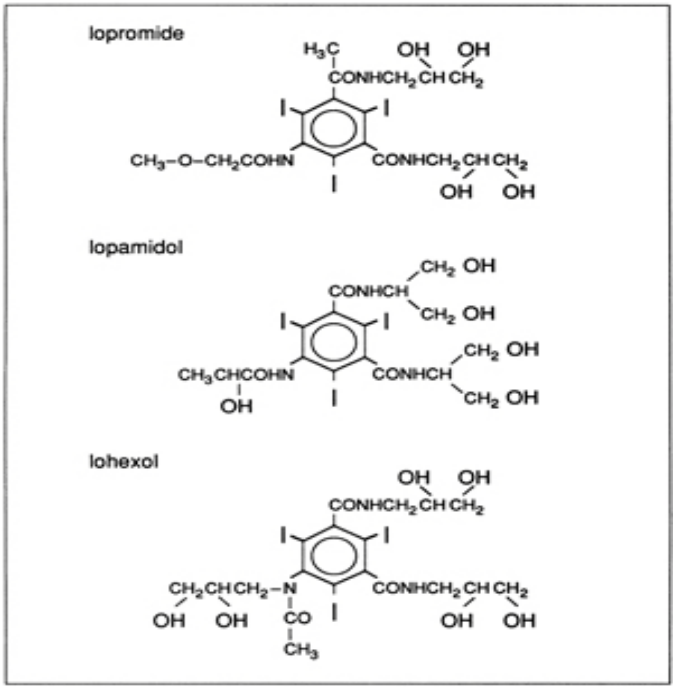


Figure 4. Non-ionic monomer

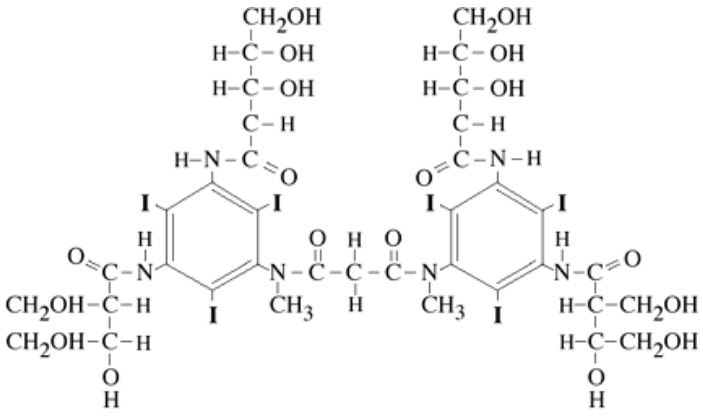


Figure 5. Non-ionic dimer

Water solubility and toxicology

Water is the most common molecule in the human body, both inside and outside the cells. In order to enable a high contrast medium concentration in extracellular water, high water solubility is necessary for contrast media in urography, angiography, etc. This water solubility is achieved in different ways by ionic and by non-ionic contrast media. Water is a polar solvent. The water molecules are electrically neutral (equal numbers of positive and negative unit charges within the water molecule), but the positive and negative charges are distributed so that there is a surplus of positive charges (lack of electrons) at the site of the hydrogen atoms (which form positive poles) and a surplus of negative charges (excess of electrons) around the oxygen atom (which forms a negative pole)(28-31).

lonic contrast media dissociate in water into electrically charged particles named ions. The positively charged ion may be a sodium ion or a meglumine ion. The negatively charged ion is the benzene derivative with three iodine atoms and a negatively charged carboxyl group. The ionic contrast media are water soluble because the positive and negative ions are attracted to the negative and positive poles of the water molecules(1).

Non-ionic contrast media are electrically neutral like the water molecules. The nonionic contrast media are water soluble because they contain polar groups (OH-groups, hydroxyl groups) which have an uneven distribution of electrical charges with excess electrons around the oxygen atoms (forming negative poles) and a deficit of electrons around the hydrogen atoms (forming positive poles). The electrical poles in the OH-groups of the contrast media are attracted to the electrical poles in the water molecules - thus achieving water solubility (1, 32).

The only desirable effect of a contrast medium is to attenuate radiation. All other effects of the contrast medium in the body, regardless whether they cause clinical symptoms or not, are not desired. When these effects cause changes observable in laboratory tests or clinical symptoms, they are deemed to be adverse effects. Different chemical structures have been designed to achieve high water solubility and this has resulted in contrast media with different toxicity.

The total toxicity of a contrast medium solution is the sum of the chemotoxicity of the contrast medium molecules, the osmotoxicity of the contrast medium solution and the ion toxicity - a surplus or deficit of various ions in the solution (33, 34):

1. The chemotoxicity of a contrast medium molecule may depend on its effects on proteins in the extracellular space and/or in the cell membrane, and effects on cell organelles and enzymes by the small numbers of contrast medium molecules which go intracellularly. (The carboxyl ion in ionic contrast media is an example of a chemical structure with high neurotoxicity in the subarachnoid space. Therefore, ionic contrast media must not be used in myelography.)

2. Osmotoxicity. Ionic contrast media have a high osmolality per amount of iodine, because the iodinated and negatively charged ions (diatrizote, iothalamate, metrizoate) are accompanied by the non- iodinated positively charged ions (sodium ions, meglumine ions) (see also the section: "Osmolality ratio, below). The hypertonicity of the contrast medium solution causes fluid shifts from erythrocytes, endothelial cells and other structures. This induces pain in arteriography, dilatation of blood vessels with a fall in blood pressure and viscosity changes of the blood.

3. Ion-imbalance. When contrast medium instead of blood flows through blood vessels, a too high or too low concentration of different ions produces side-effects (ventricular fibrillation at coronary arteriography, influence on plasma proteins).

Types of Radiocontrast Medium

They are classified on the basis of their physical and chemical characteristics including osmolality, ionization in solution, and chemical structure. Currently, four classes of contrast material are commercially available: ionic monomers, nonionic monomers, ionic dimers, and nonionic dimers (Figure 6).

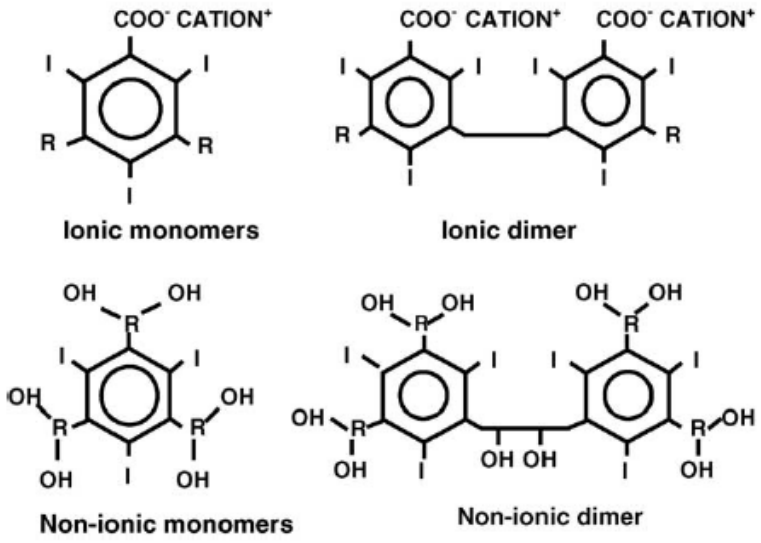


Figure 6. Structure of different contrast media classes

The more iodine, the more "dense" the x-ray effect. There are many different molecules. Some examples of organic iodine molecules are iohexol, iodixanol, ioversol. Iodine based contrast media are water soluble and as harmless as possible to the body. These contrast medium are sold as clear colorless water solutions, the concentration is usually expressed as mg I/ml. Modern iodinated contrast medium can be used almost anywhere in the body. Most often they are used intravenously, but for various purposes they can also be used intraarterially, intrathecally (the spine) and intraabdominally - just about any body cavity or potential space. The contras medium both ionic and non-ionic consist of monomer (1 benzoate acid ring) and dimmer (2 benzoate acid ring) (1, 34-36).

Radiocontrast media hypersensitivity

Radiocontrast media (RCM) are administered more than 75 million times per year for performing diagnosis and treatment of vascular disease and enhancement of radiographic contrast. Adverse reactions after RCM administration are common. Symptoms after RCM exposure may be regarded as hypersensitivity reactions or toxic reactions related to the well-defined toxicity of the compounds, or may be caused by factors unrelated to RCM, such as chronic idiopathic urticaria (Fig. 7). Hypersensitivity reactions to RCM may present clinically as anaphylaxis with the potential to result in fatalities or as delayed occurring exanthemas, not unlike those to other drugs (1, 5, 7, 34-38).

Epidemiology

The frequency and mechanisms of hypersensitivity reactions differ between the different types of RCM. Mild immediate reactions have been reported in 3.8% to 12.7% of patients receiving intravenous injections of ionic monomeric RCM and in 0.7% to 3.1% of patients receiving nonionic RCM. Severe immediate adverse reactions to ionic RCM have been reported in 0.1% to 0.4% of intravenous procedures, whereas reactions to nonionic RCM are less frequent (0.02% to 0.04%). Fatal hypersensitivity reactions may occur in 1 to 3 persons per 100,000 contrast media administrations and are not related to one particular type of RCM (6, 34, 39).

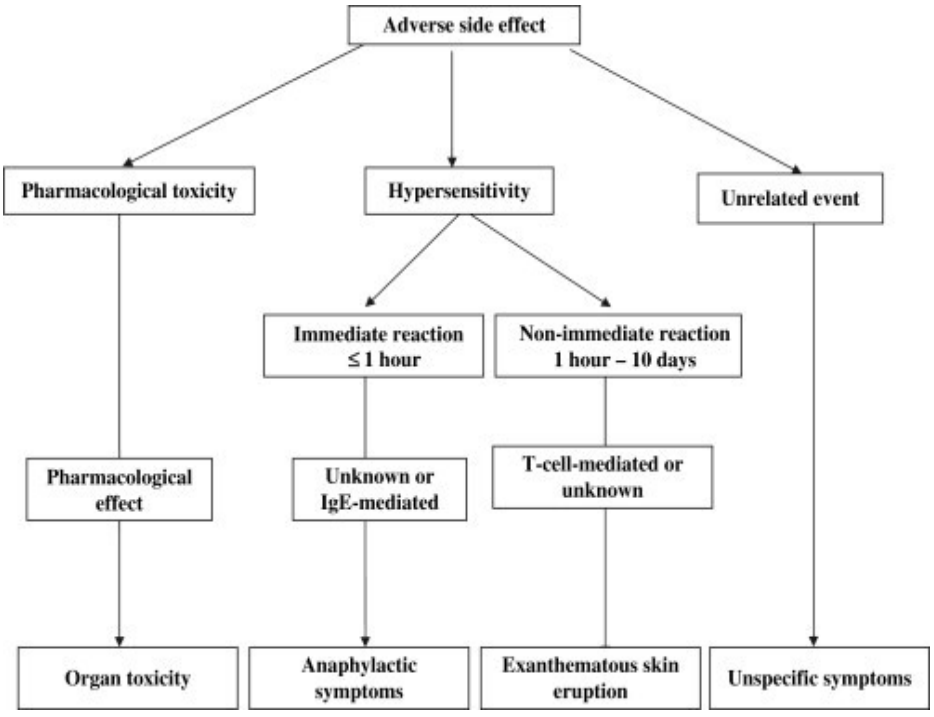


Figure 7. Classification of adverse side effects after RCM administration (34)

Clinical presentation of RCM Hypersensitivity

The symptomatology of anaphylaxis and anaphylactoid reactions is quite variable and can make diagnosis difficult at times. Systemic reactions include one or more forms of mucocutaneous reactions (pruritus, erythema or angio-oedema in severe cases). Pruritus and congestion of mucous membranes of the eyes, nose, and mouth are frequently observed. A more severe feature is laryngeal oedema and, consequently, upper-airway obstruction. In addition, respiratory decompensation involving bronchospasm may occur, leading to symptoms similar to those seen in asthma. Gastrointestinal events such as nausea and vomiting, abdominal pain, diarrhoea, etc. may also be part of the clinical picture of anaphylaxis (13, 30, 38, 40).

Anaphylactic and anaphylactoid reactions should be distinguished from other medical situations which are likely to occur in the context of medical imaging, such as vasovagal reactions (particularly with injectable drugs), functional vocal cord dysfunction, and panic attack. These situations may however be challenging as patients suffering real anaphylaxis do not always receive appropriate therapy (13, 30, 38, 40). However, in the case of panic attack, there is no urticaria, angio-oedema or hypotension. Table 2 show grading of the symptoms.

Table 2. Grading of anaphylactic and anaphylactoid reactions according to severity of clinical symptoms (34)

	Symptoms			
Grade	Skin	Abdomen	Respiratory tract	Cardiovascular system
I	Pruritus, flush, urticaria, angio-oedema			
II	Pruritus, flush, urticaria, angio-oedema (not mandatory)	Nausea, Cramping	Rhinorrhea, hoarseness, dyspnoea	Tachycardia (>20 beats/mn), BP change (>20 mmHg systolic), arrhythmia
III	Pruritus, flush, urticaria, angio-oedema (not mandatory)	Vomiting, defecation, diarrhoea	Laryngeal oedema, bronchospasm, cyanosis	Shock
IV	Pruritus, flush, urticaria, angio-oedema (not mandatory)	Vomiting, defecation, diarrhoea	Respiratory arrest	Cardiac arrest

Pathophysiology of immediate reactions to radiocontrast media

The mechanisms of the allergy-like reactions to RCM have been a matter of speculation for decades (Table 3). Anaphylaxis to RCM may be theoretically due to (14, 34, 41)

(1) a direct membrane effect possibly related to the osmolality of the contrast media solution or the chemical structure of the contrast media molecule (pseudo-allergy)

(2) an IgE mediated mechanism.

Table 3. Arguments for and against IgE mediated radiocontrast media allergy (34)

Con	Pro
No sensitization phase	Preclinical sensitization to cross-reactive substance possible
Repeated reactions do not always recur and do not always increase in severity	Previous reaction highest risk factor for subsequent reaction, case reports with increasing severity
No increase of plasma leukotrienes after RCM administration	Mast cell mediator release correlates with severity of reaction, positive basophil activation test to RCM in patients
Only anecdotal reports of RCM-specific lgE antibodies	Low levels of IgE antibodies to ioxaglic acid in one study
Low affinity of RCM	specific IgE to RCM higher in reactors than in controls in one study
RCM are not able to from haptens	Positive skin tests in patients but not in controls in optimal concentrations

Diagnosis of immediate RCM hypersensitivity

Immediately after the reaction, elevated plasma histamine and serum or plasma levels of histamine and tryptase have been found, especially in patients with severe or fatal immediate reactions. In cases in question for anaphylaxis, blood samples for histamine analysis should be drawn as soon as possible after the reaction and for tryptase 1 to 2 hours after the onset of symptoms.(42) Tryptase values should be compared with baseline levels. Further allergologic work-up is recommended between 2 and 6 months after the reaction because positive skin tests are more seldom found afterward. Patients are only rarely skin prick test (SPT) positive with undiluted RCM. Afterwards, IDTs with readings after 20 minutes are recommended with the RCM (300–320 mg I/mL) diluted 10-fold in sterile saline, because this concentration has been shown to give a low frequency of false-positive reactions in controls (0% to 4%)(43). Because cross-reactivity is frequent, a panel of several different RCMs should be tested in an attempt to find a skin test–negative product, which might be tolerated in future RCM examinations (14, 34, 41).

No commercial assay is available for routine measurement of serum levels of RCM specific IgE antibodies. The reliability of other in vitro tests, such as the basophil activation test, has not yet been established. Results from individual patients and the author's unpublished study indicate that the basophil activation test may be helpful; however, currently, it may only be used on an experimental scientific basis. Provocation is generally not recommended because intravenous applications of as low as 0.5 to 1 mL of RCM have led to severe anaphylaxis (44).

Basophil Activation Test

Peripheral blood basophils and tissue mast cells are primary effecter cells in IgE-mediated immediate allergic reactions such as rhinitis, asthma and anaphylaxis. They may also be involved in other kinds of allergic or pseudo-allergic reactions in which other activation mechanisms such as complement activation, non-IgE-mediated stimulation or non-immunological mechanisms are implicated (45-47)

Basophil activation induced by IgE-dependent (e.g. specific allergen, anti-IgE antibody) involves cross-linking of high-affinity IgE receptors which causes structural modification of the plasma membrane IgE receptor site. This causes activation of adenyl-cyclase which catalyses the transformation of ATP to cAMP. This in turn leads to an increase in the concentration of Ca^{2+} in the cytosol causing release of granule associated basic molecules such as histamine (Fig. 8).

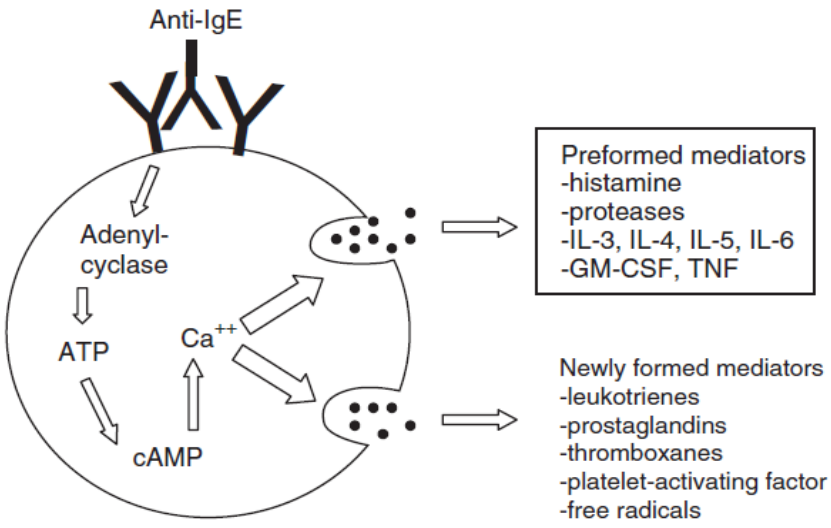


Figure 8. Simplified scheme of the intracellular events in anti-IgE induced basophil degranulation and the exocytosis of granules leading to release of various mediators (47)

These events cause the basophil to lose their staining affinity for basic dyes. If the reaction is intense, exocytosis of the granule occurs with the release of preformed (e.g. histamine and /or newly synthesised (e.g. LTC4) mediators, depending on the agonist used to activate the cells (48) . Microscopy has revealed two different pathways for basophil activation; anaphylactic degranulation, which is characterised by the fast morphological changes and exocytosis of intracellular granules or else by what is known as piecemeal degranulation, which involves slow morphological changes of the cytoplasmic granules that are packed into vesicles and transported to the plasma membrane with no intergranule or plasma membrane fusion.

Basophils represent less than 0.5% of the total leukocytes in peripheral blood, which makes their purification difficult. Since these cells play an important role in immediate allergic reactions, some functional *in vitro* tests have been developed which detect their activation. One of the first was the histamine release test, a technique that did not find extensive clinical application due to its insufficient sensitivity and specificity. This is why in the past few years several groups have taken advantage of flow cytometry and developed new tools to monitor basophil activation after antigen-specific stimulation using the expression of various membrane surface markers. Flow cytometry is a useful tool for the analysis of different cellular types and can be used to identify specific cell populations, even those which are present in low amounts. It has proven to be useful in the study of allergen-induced activation(19, 49, 50). In a first step the basis of these assays is the identification of basophils by specific fluorescent antibodies such as anti-IgE, anti-CCR3, and in a second step the demonstration of certain membrane phenotypes that appear after exposure to allergen such as CD63 and CD203c

CD63 as a Marker of Basophil Activation

CD63 is a 53 kDa lysosomal associated membrane protein (LAMP) and belongs to the tetraspanin transmembrane-4 super family (TM4SF) subfamily 1. It is a type 1 integral membrane glycoprotein of late endosomes and lysosomes. It is expressed primarily on the cytoplasmic granules of various resting cells and only weakly expressed on the cell membrane. It has been shown that activation of human basophils in vitro by allergen or anti-IgE antibody results in degranulation and high-density surface expression of CD63 (51-53) after the fusion of cytoplasmic granules with the cell surface membrane. The kinetics of the increased CD63 expression and of the accompanying histamine release are very similar, and there is a strong positive correlation between these two events (52, 53) Microscopic fluorimetry showed that the CD63 epitope was present intracellularly in resting basophils, and that CD63 expression was an all-or-nothing response with the level of response after stimulation varying from donor to donor. Apart from expression on activated basophils, CD63 is present on platelets, endothelial cells, monocytes and neutrophils(52, 54). CD63 has been shown to be predominantly expressed on basophils with little or no expression on B cells, T cells or monocytes and using anti-IgE (early activation marker) to firstly identify basophils followed by analysis of the upregulation of CD63 expression (later activation marker) has been recommended(55).

Many studies using CD63 have been carried out across a wide range of allergens, unfortunately also with a number of different experimental protocols. Flow cytometry has been used to investigate food allergy (56-58), venom allergy(48, 59, 60) , house dust mite (HDM)(61), pollen (62), latex(20, 63-65) , and allergy to various drugs including muscle relaxants (MR) (66) and betalactam antibiotics (53, 67) and non-

steroidal anti-inflammatory drugs (68-70) with varying degrees of success. The mechanism by which basophils are activated appears to have a bearing on whether an increase in CD63 expression occurs (71). A significant correlation has been reported between the flow cytometric method using CD63 and anti-IgE and histamine release assay after basophil activation by allergens or anti-IgE (17, 48, 55, 72) and the high level of sensitivity and specificity of the basophil activation test indicates that it can be a very reliable diagnostic tool.

CD203c as a Marker of Basophil Activation

In 1999, Bühring et al. showed that another marker, CD203c, is upregulated in response to IgE-dependent basophil activation and in recent years this has been suggested as a potential marker for basophil activation in allergy diagnosis. CD203c is also known as ectonucleotide pyrophosphatase/phosphodiesterase 3 (E-NPP3). It is a type II transmembrane protein, and cleaves a variety of phosphodiester and phosphosulphate bonds, and may be involved in the clearance of extracellular nucleotides. Compared to CD63, CD203c has been described as being selectively expressed by basophils, mast cells and by their CD34 + progenitors. It seems that the upregulation of CD63 and CD203c follow different kinetics, with rapid CD203c upregulation (reaching maximum levels at 5–15 min of stimulation) compared to CD63 (20–40 min) (73-76). In addition to their differing kinetics, CD63 expression is more similar to anaphylactic degradation (76). However, upregulation of CD203c is always accompanied by upregulation of CD63, and as is the case with CD63, upregulation of CD203c and degranulation occur simultaneously (74).

Basophil activation test in Radiocontrast media

Jiri Trcka et.al. (2008)(77) evaluated 96 patients with anaphylaxis symptoms after contrast material application using standardized intradermal skin testing. They found that there were four patients had positive skin tests, then they further evaluated *in vitro* Basophil activation tests using CD63 as a marker. In three patients with IgE-mediated contrast material allergy, CD63 upregulation in three patients with documented ICM anaphylaxis in correlation with skin tests The basophil activation test yielded maximally 15% activated basophils at 1 µg/mL, whereas activation of basophils was not induced at very low concentrations of contrast material (10^{-10} to 10^{-12} µg/mL). In contrast, in contrast material–stimulated samples of the controls, basophil activation was negative with all contrast material concentrations—that is, 1% to 2% activated basophils at 1 µg/mL.

Another case report from P. Dewachter ,et.al. (2009)(78) performed BAT using both CD63 and CD203c in a 75-year-old man who had history of amidotrizoate allergy. They found no upregulation of CD63 but instead detected an upregulation of CD203c in response to amidotrizoate only at 6 mg/ml. They also tested specificity of BAT in 2 healthy controls.

CHAPTER IV

MATERIALS AND METHODS

Patients

From Dr.Thatchai Kampitak's clinical study, skin test was performed in 63 patients with a history of immediate RCM hypersensitivity. Fifteen patients were skin test positive and 48 patients were skin test negative. In order to compare BAT in both groups all available skin test positive patients were invited to the study. Three patients in skin test positive group succumbed from the illnesses and one patient refused the study. As a result, 11 skin test positive patients and selected 15 skin test positive patients were recruited for BAT in this study.

Inclusion criteria

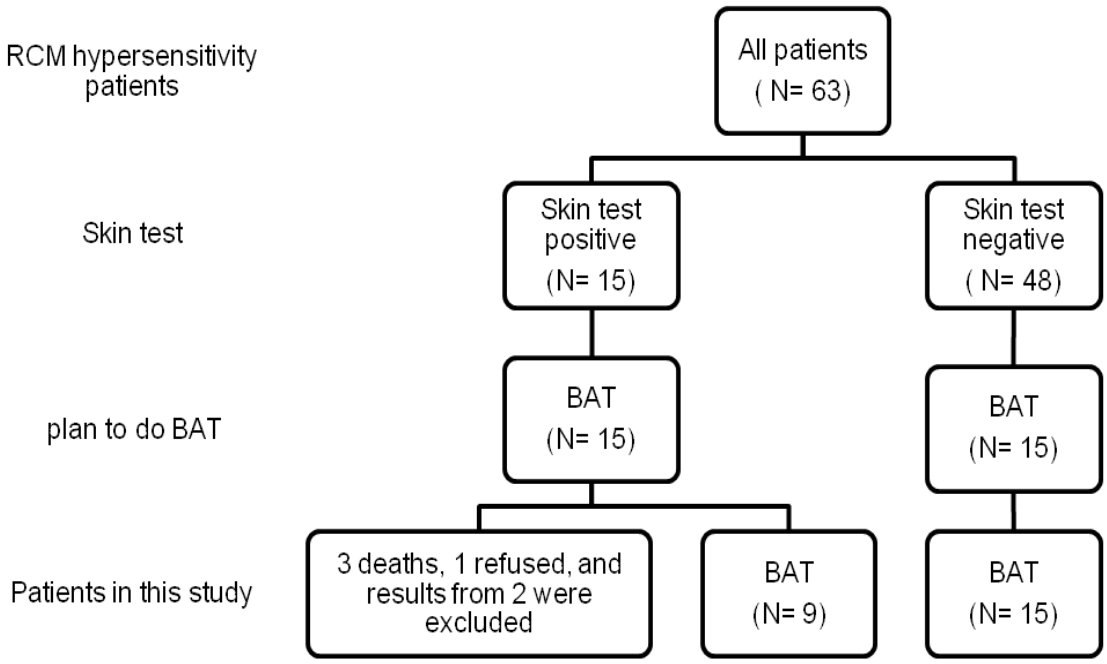
Adult patients with a history in immediate RCM hypersensitivity (at least 20 years old)

Exclusion criteria

Patients had not stopped taking oral antihistamines at least 5 days before blood sampling (6 weeks in case of astemizol)

A total of 26 patients with a history of immediate RCM hypersensitivity attending at King Chulalongkorn Memorial Hospital were included in the study. Eleven of them had positive intradermal skin test result to RCM and fifteen patients had negative skin test result. From the result there were 2 patients who had high background of CD63

expression. This is most likely due to technical error while processing the blood samples. Therefore, these 2 samples were not included in the further analysis.



Fourteen healthy blood donors never been exposed to RCM were recruited as negative controls. The study was approved by the ethics committee of the King Chulalongkorn University and all subjects gave their informed consent. Demographic data of the subjects were summarized in tables 4 and 5.

Table 4. Characteristics of the study population

Characteristics	Patients	Controls
Number of subject	24	14
Male/Female	9/15	7/7
Age, years (mean ± SD)	51.04 ± 14.66	42.3 ± 9.3
History of allergy		
airway allergy	1 (4.16%)	4 (28.57%)
food allergy	4 (16.67%)	2 (14.29%)
drug allergy	1 (4.16%)	1 (7.14%)

Table 5. Detail characteristics of each patient

No.	sex	age (year)	type of RCM	(first exposure) or (previous exposure)	BAT result	skin test result	Duration after RCM exposure until BAT	Grading of severity of reactions and Clinical manifestations	History of allergy (Y/N)	disease/Type of examinations	drug of patients before performed BAT
1	F	34	Ioxithalamate	f	-ve	+ve	27	1 urticaria	Y (shrimp)	endometriotic cyst, myoma uteri/ IVP	Acoxia (cox2)
2	F	56	Ioxithalamate	p	+ve	+ve	45	1 urticaria, angioedema	N	Hepatitis C, fatty liver, myoma uteri	Hepacap
3	M	38	Iopromide	p	-ve	+ve	16	1 urticaria (face and arm)	N	CBD stone/CT	No drug
4	M	70	Iopromide	p	+ve	+ve	18	1 urticaria (face)	N	CA stomach, HT/CT	Imatinib
5	F	65	Iohexol	f	-ve	+ve	13	1 MP rash	N	CA thyroid/CT	unavailable history
6	F	26	Ioxithalamate	f	+ve	+ve	36	3 hypotension, angioedema (swollen eye and mouth)	Y (shrimp, cat and house dust mite)	Ureteric stone/IVP	no drug
7	M	68	Iopromide	f	+ve	+ve	39	1 MP rash	N	CA stomach/CT	Plavix(75), Miracid(2),Atenolol(50), Moduretic,Zimmex(10), Folic acid, Dicloxacillin(250)
8	M	73	Iopromide	f	-ve	+ve	25	3 wheeze, hypotension anaphylactic shock	Y (Lumividine)	Hepatitis B virus, Cirrhosis, tubular adenoma/CT	B1-6-12

28

Table 5. Detail characteristics of each patient (Cont.)

No.	sex	age (year)	type of RCM	(first exposure) or (previous exposure)	BAT result	skin test result	Duration after RCM exposure until BAT	Grading of severity of reactions and Clinical manifestations	History of allergy (Y/N)	disease/Type of examinations	drug of patients before performed BAT
9	F	29	Iopromide	f	-ve	- ve	16	1 rash (face)	N	Vogt-Koyanagi-Harada (VKH) syndrome/IVP	Fluorometholone eye drop (FML)
10	F	28	Iopromide	f	-ve	- ve	15	1 rash (face)	N	pulmonary TB, gestational trophoblastic disease	INH (100),Rifampicin (300), B6
11	F	56	Iopamidol	p	-ve	- ve	21	1 rash (arm)	Y (spinach and pennicillin)	spondylothesis	Caltab1000,Tramadol50, Neurontin 300, Neotica balm
12	F	65	Iopromide	f	+ve	- ve	33	1 rash (arm)	N	DM, Hypertension, dyslipidemia	mixtard, enalapril
13	F	71	Iopromide	f	+ve	- ve	19	1 rash (arm, leg)	N	CA breast,	Hydrochlorothiazide, atenolol, manidipine, metformin, lorazepam
14	M	65	Iopromide	f	+ve	- ve	36	1 rash (neck, arm)	N	pituitary cushing disease S/P hypophysectomy	L-Thyroxin, Prednisolone, Conjugated estrogen, Medroxyprogesterone, DDAVP, Bestatin, Enaril
15	F	39	Iopromide	f	-ve	- ve	37	3 chills, low oxygen saturation	N	DM, Hypertension, dyslipidemia	cilostazol, simvastatin, folic acid, aspirin, amlodipine, mixtard, valsartan
16	M	37	Iopromide	f	+ve	- ve	19	1 rash (face, back)	N	carotid body tumor	Omeprazole, motilium

Table 5. Detail characteristics of each patient (Cont.)

No.	sex	age (year)	type of RCM	(first exposure) or (previous exposure)	BAT result	skin test result	Duration after RCM exposure until BAT	Grading of severity of reactions and Clinical manifestations	History of allergy (Y/N)	disease/Type of examinations	drug of patients before performed BAT
17	F	41	Iohexol	f	+ve	+ve	15	1 MP rash	N	stomach cancer	Chemo(FOLFIRI cycle 12); irinotecan, leucovorin, 5FU
18	M	64	Iopromide	f	-ve	- ve	14	1 urtifcaria (neck, back)	N	Abdominal aortic aneurysm, paroxysmal atrial fibrillation	no drug for a month (enalapril, digoxin, aspirin, furosemide, simvastatin, omeprazole)
19	F	43	Iopromide	f	+ve	- ve	19	1 urtifcaria (face)	N	hypertension, dyslipidemia, IV, Ct upper	simvastatin, addiK, diltiazem, prazosin
20	M	55	Iopromide	f	+ve	- ve	38	1 rash (neck)	N	CA colon with lung metastasis	no drug (previous xeloda, campto)
21	M	60	Iopromide	f	+ve	- ve	36	1 MP rash	N	Normal pressure hydrocephalus	Glipizide, metformin, atenolol, adalat, merislon, enalapril, baby aspirin, simvastatin, lorazepam
22	F	33	Iopromide	f	-ve	- ve	21	1 rash (opposite arm)	N	atrial septum defect S/P ASD closure with partial anomalous pulmonary venous return (PAPVR)	no drug
23	F	41	Iopromide	f	+ve	- ve	31	1 urticaria (face, trunk)	N	myasthenia gravis, hypertension	prednisolone (5mg/day), mestinon (50) 1x5, caltab, azathioprine (50) 2x1, enalapril
24	F	63	Iopromide	f	-ve	- ve	39	1 angioedema (swollen eye)	N	Hypertension	pantoprazole, air-x, merislon

Radio contrast media

This study used 5 RCMs. There were Ioxitalamate (Telebrix), Iobitridol (Xenetix), Iopamidol (Iopamiro), Iopromide (Ultravist) and Iohexol (Omnipaque). All of them were diluted in stimulation buffer for BAT test.

Cell viability assessment with Annexin V-FITC/PI staining

PBMCs were isolated from fresh blood in EDTA tube by Ficoll–Hypaque density gradient centrifugation. Briefly, fresh venous blood collected into EDTA anticoagulant treated tube was diluted with equal volume of RPMI-1640 and transferred to a 50 conical ml tube. Then, it was underlayed with 13 milliliters (ml) of IsoPrep in diluted blood. Next, the tube was centrifuged at 1,500 rpm for 30 minutes at 20ºC with no intervention. After centrifugation, Cells from lymphocyte layer were collected and washed two times with RPMI-1640 by centrifugation at 1,800 rpm for 10 minutes at room temperature.

3×10^5 cells of PBMC, which was adjusted to be fit in 100 µl of RPMI, incubated with 100 µl of RCM, which is various from undiluted to 10^{-5} concentration, for 20 minutes at 37ºC. In this experiment, the positive control is DMSO and the negative control is RPMI. Next, incubated cell is harvested by putting the tube into ice to stop reaction. After 5 minutes, wash PBMC two times with cool PBS by centrifugation at 1,800 rpm for 5 minutes at 4 c and suspended in binding buffer (10 mmol/l HEPES, pH 7.4, 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$). Aliquots of 100 µl suspension (3×10^5 cells) was incubated with 5 µl Annexin V-FITC and 5 µl PI (50 µg/ml) for 15 min at room temperature in the dark. Cell suspension was added with 400 µl of binding buffer, gently vortexed, and

analyzed within 1 h by flow cytometry a total 10,000 cells were counted apoptosis detection kit (BD Biosciences).

Basophil Activation Test

Basophil CD203c Expression was modified from the previous description (21, 79). All the tests were carried out within 2 h of blood sampling. Firstly, venous blood was collected in 10 mL EDTA tubes and stored at 4 ºC. Next, CD203c-induced expression was evaluated. Briefly, 100 µl of the patient's venous blood was incubated with 100µl of 1:10 and 1:100 dilution concentrations of RCM in polypropylene tubes. In order to evaluate the background basal values without stimulation (negative control), we added 10 µl of stimulation buffer (HEPES 20 mM , NaCl 133 mM , KCl 5 mM , CaCI 2 7 mM ,MgCI 2 3.5 mM , bovine serum albumin 1 mg/ml, pH 7.4) to whole blood in another tube. Regarding positive control, we used 10 µl of N-formyl-methionyl-leucyl-phenylalanine (fMLP) (2 mM).Then, tubes were incubated at 37°C for 20 min and reaction was stopped by incubating the samples on ice for 5 min. The basophils from the pellet were then double labeled by adding 2.5 µl of anti-CD203c PE-labeled antibody (Caltag Laboratories, Burlingame, Calif., USA) and 10 µl of anti-CCR3 PerCP-labeled antibody, and then incubated at 4 ºC for 45 min with protection from light exposure. Tubes were then vortexed gently and incubated at room temperature for another 10 min with 2 ml of pre-warmed 1xFACS lysing reagent. After that, the tubes were centrifuged 5 min, at 1000 g then supernatant was decanted and pellets were washed 2 times (centrifuged 5 min, at 1000 g) with PBS. Add 300 µL of fixing solution were added to each tube, gently shaking before flow cytometric analysis.

Basophil CD63 Expression was modified from the previous description (80, 81). All the tests were carried out within 2 h of blood sampling. Firstly, venous blood was collected in 10 mL EDTA tubes and stored at 4 ºC. CD63-induced expression was evaluated using the The Flow2 CASTs (Bühlmann Laboratories AG) was carried out according to the manufacturer's instruction. Briefly, the anti-coagulated blood samples were gently homogenized by inverting several times. For each patient and allergen, polystyrene tubes were prepared with 50 mL of allergen in 2 concentrations of RCM (1:10 and 1:100) diluted in stimulation buffer. Regarding positive controls, a monoclonal anti-FceRI antibody was used. In order to evaluate basal values without stimulation, 50 mL of stimulation buffer were applied to separate tubes. To each tube, 100 mL of stimulation buffer (containing calcium, heparin and IL-3 (2 ng/mL)), 50 mL of patients blood and 10 mL staining reagent containing a mix of anti-CD63-FITC and anti-CCR3-PE monoclonal antibodies were added.

The tubes were covered with an adhesive plastic sheet and incubated for 15 minutes at 37°C in a water bath. Stimulation was terminated by addition of 2 mL of the prewarmed (18-28°C) lysing reagent and after mixing tubes were incubated at room temperature for 5-10 minutes. After centrifugation (5 minutes at 500*g*), supernatants were decanted using blotting paper. Cell pellets were resuspended with 300 µL of wash buffer and gently vortexes. At least 500 basophils were acquired per sample using the FACScan flow cytometer (Becton-Dickinson Immunocytometry System, Heidelberg, Germany) equipped with a 488 mW argon ion laser, using Cell-QuestTM software.

Data were analyzed using CellQuest flow cytometry analysis software (BD Biosciences Pharmingen) according to instructions from Bühlmann Laboratories AG. In the first step, a gate (R1) was set by including the entire basophil population ($CCR3^{high}$)

with low side scatter (SSC^{low}). Eosinophils are located on the upper right and can be excluded due to their SSC^{high} position. In the second step, calculation of the percentage of CD63-positive cells (brightly fluorescent fluorescein isothiocyanate) was determined by comparing its number to the total amount gated in R1 (Figure 9).

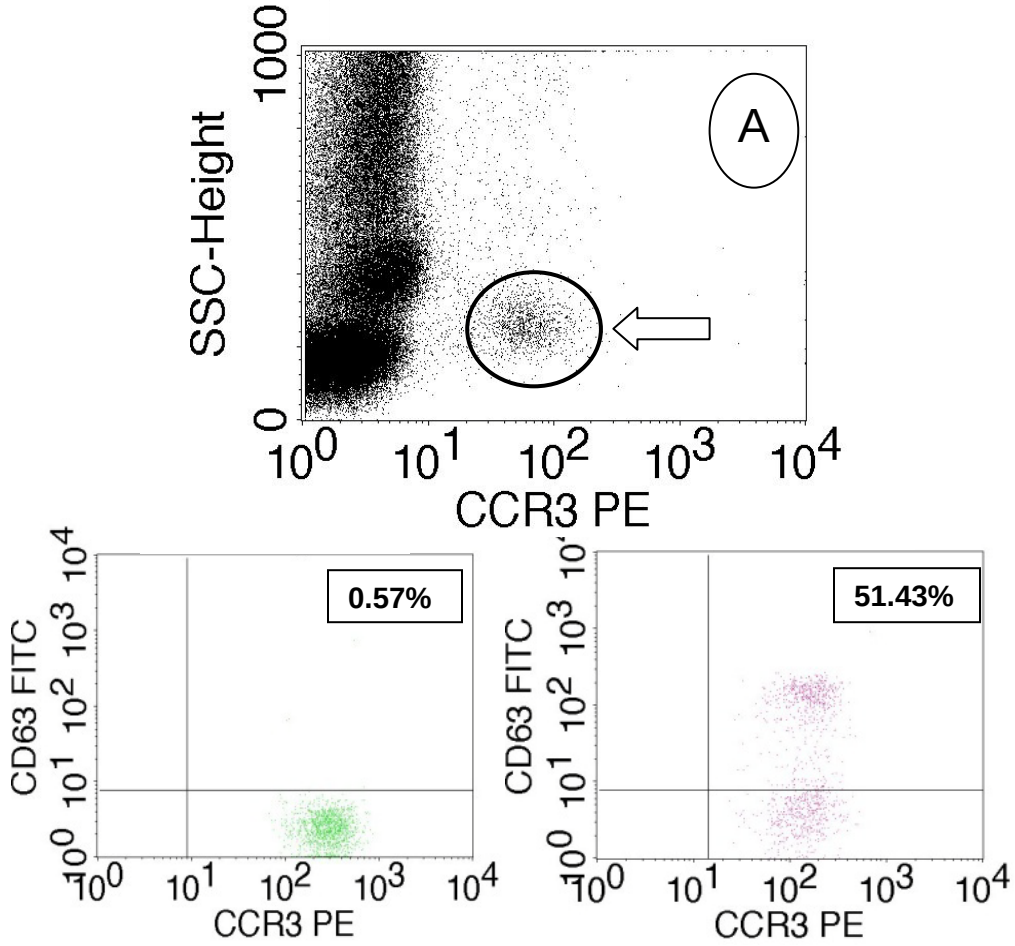


Figure 9. Gating strategy. Flow cytometric analysis of negative control (left) and positive control (FeεR1) (right)

In order to consider a result as positive, the percentage of basophils activated after incubation with allergen had to be ≥ 5%. Additionally the percentage of basophils activated after allergen stimulation should be at least double the percentage of spontaneously activated basophils (stimulation index ≥ 2) (82)

Statistics

Data were analyzed using the software SPSS, version 17 for Windows. Data distributions were checked for normality using the $\chi 2$ test. Depending on the results, a nonparametric Pearson Chi-Square test and Mann-Whitney U test were used for subgroup analysis. A p value of less than 0.05 was considered statistically significant.

CHAPTER V

RESULTS

Toxicity of Radiocontrast media to PBMCs

This experiment, varying concentrations of RCM were incubated with PBMCs for 20 min and cell viability was assayed with Annexin V-FITC and PI. The results showed that PBMC viability incubated with 4 RCM including Ultravist, Omnipraqe, Xenetix and Iopamiro in any concentration were about 95 - 97% compared to incubation PBMCs with normal saline (Figure 11-14). We can conclude that Ultravist, Omnipraqe, Xenetix and Iopamiro under our experimental conditions are not cytotoxic. However undiluted Telebrix displayed slight toxicity towards PBMCs as judged by a viability of 80% (Figure 10); therefore, the 1:10 and 1:100 concentrations of RCM were used in the following BAT.

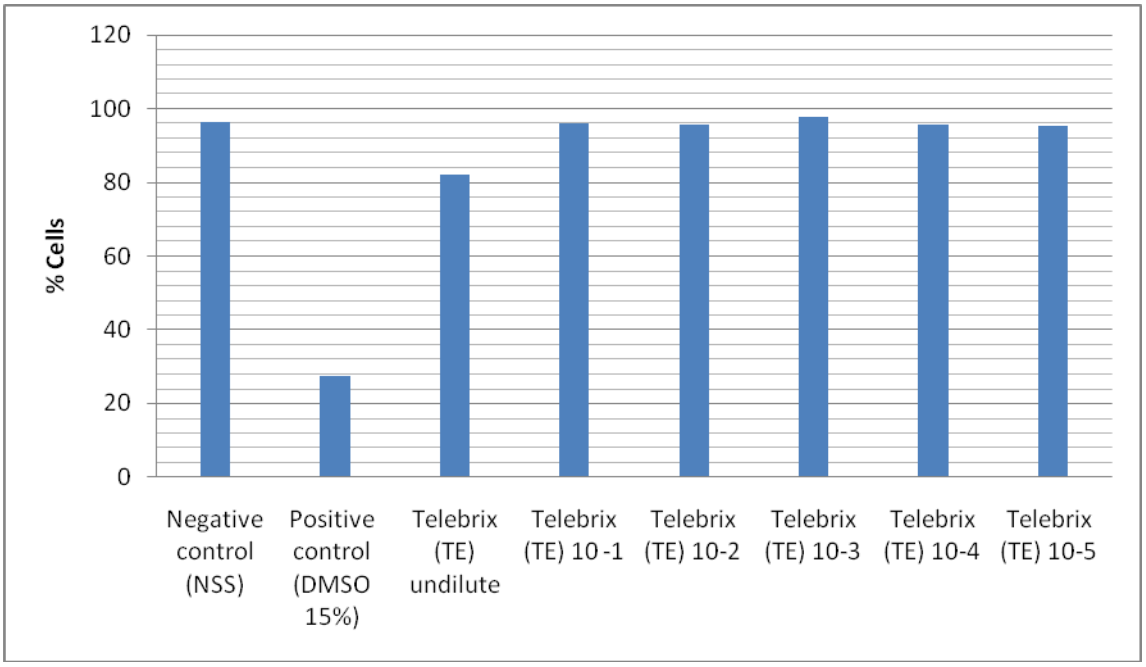


Figure 10. Effect of Telebrix (Ioxithalamate) on the cell viability of PBMCs

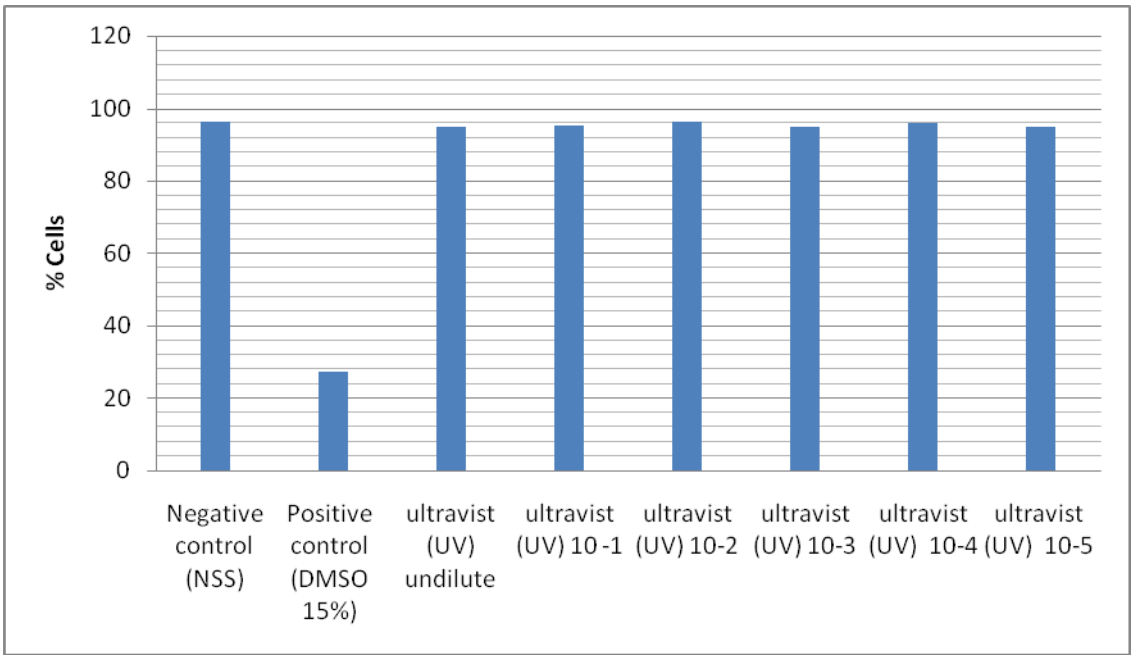


Figure 11. Effect of Ultravist (Iopromide) on the cell viability of PBMCs

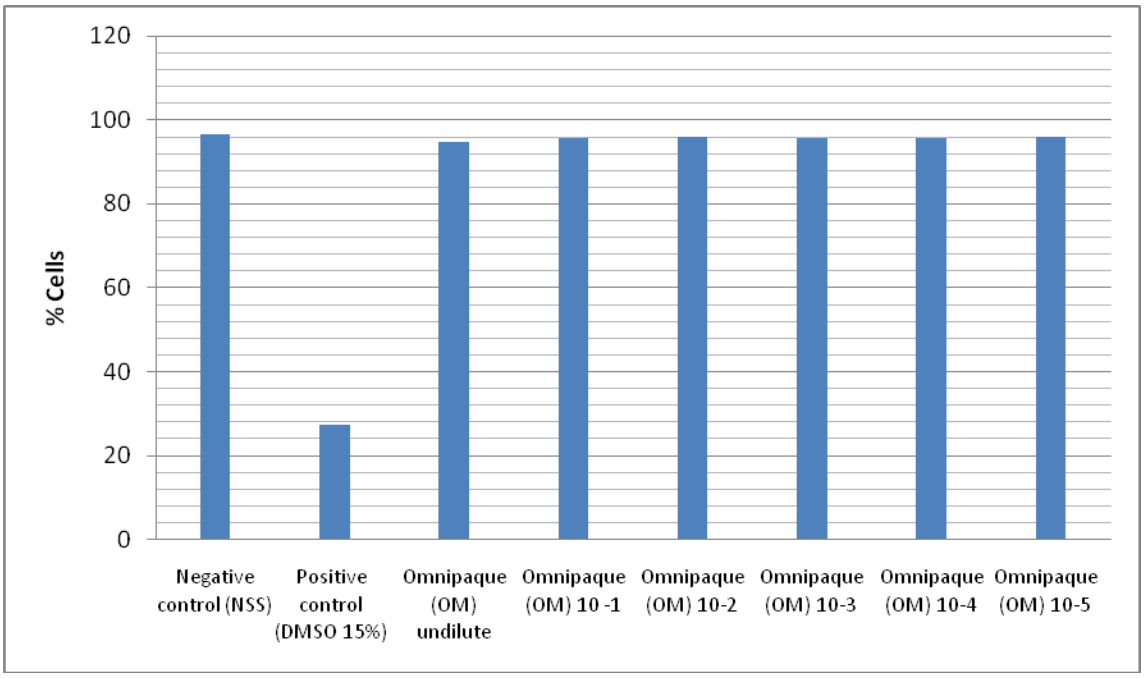


Figure 12. Effect of Omnipraqe (Iohexol) on the cell viability of PBMCs

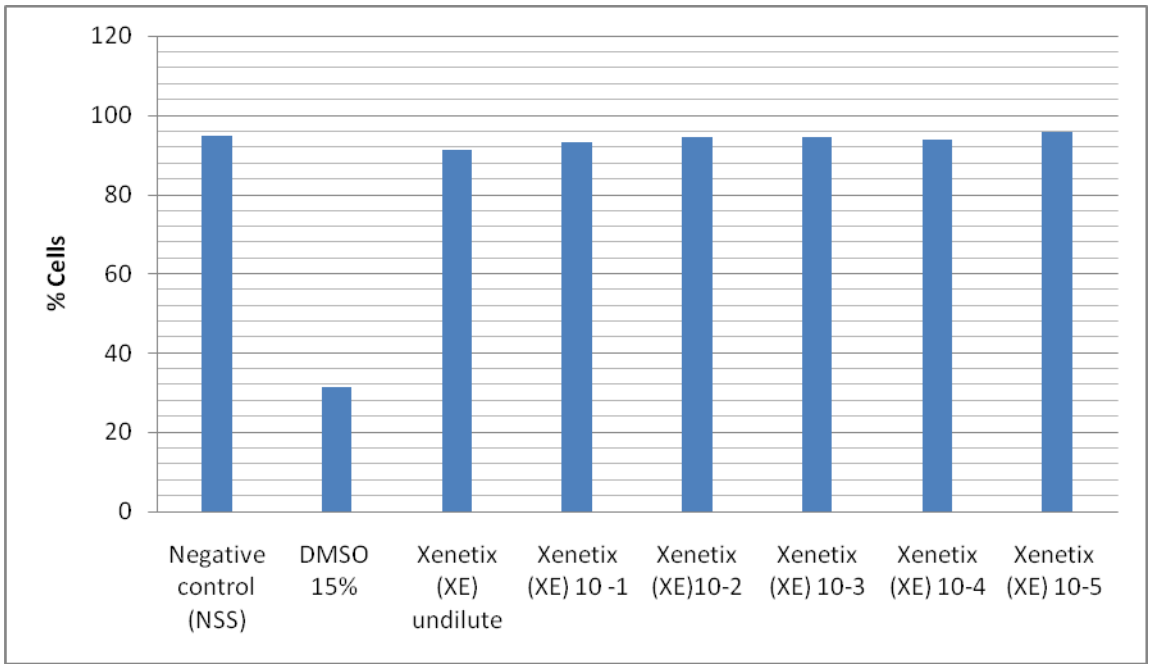


Figure 13. Effect of Xenetix (Iobitridol) on the cell viability of PBMCs

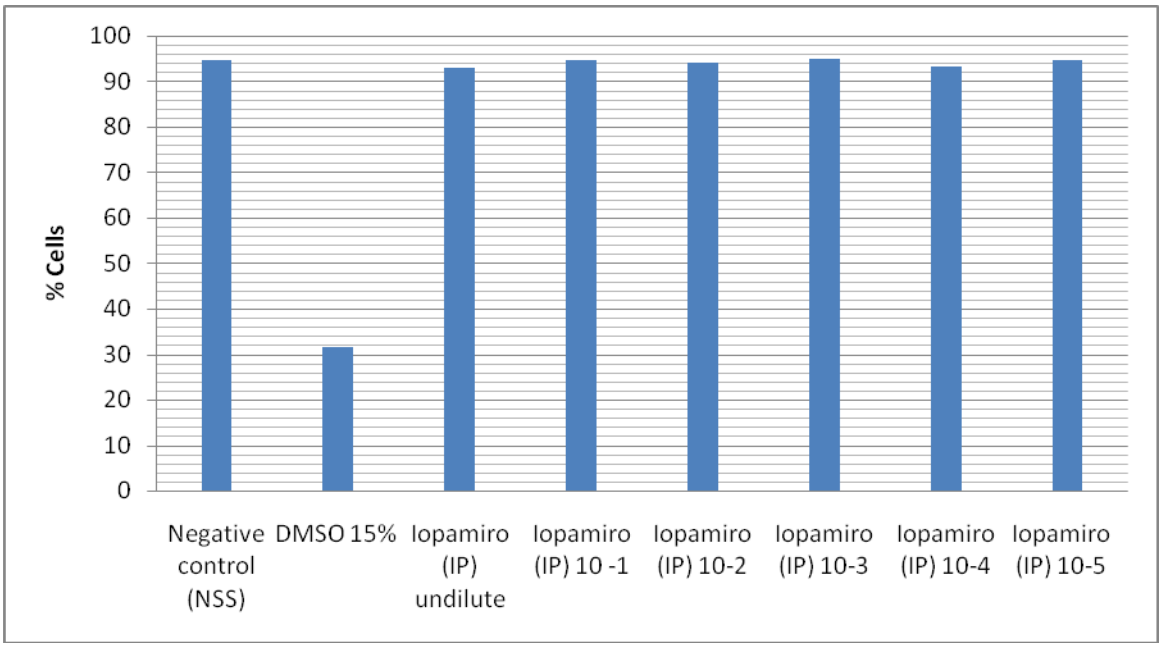


Figure 14. Effect of Iopamiro (Iopamidol) on the cell viability of PBMCs

Basophil activation test set up

We first set up protocol of BAT by using CD203c as an activation marker because many study reported that CD203c was a powerful marker.

In order to consider a result as positive, the percentage of basophils activated after incubation with allergen had to be $\geq$ 5%. Additionally the percentage of basophils activated after allergen stimulation should be at least double the percentage of spontaneously activated basophils (stimulation index $\geq$ 2) (82).

In this protocol, we used fMLP as positive control and stimulation buffer as negative control. In table 6, the results in 10 healthy controls showed that fMLP stimulation could increase the level of CD203c in 7 out of 10 samples. Three samples have very low CD203c expression not distinct from background.

Table 6. BAT CD203c expressions of negative and positive control using stimulation buffer as negative control

No.	BAT (% CD203c/CCR3)	
	Basal	**fMLP**
1	5.78	**32.83**
2	2.09	4.42
3	3.97	**14.12**
4	5.14	**11.02**
5	5.08	**33.74**
6	4.87	**11.25**
7	4.37	**24.2**
8	4.32	6.87
9	4.18	**14.81**
10	4.27	6.93

Note: Bold number indicated positive result of BAT test

We notice that the percentage of basophils positive for CD203c were lower than many previously reported studies (21, 73, 74) because they had very high expression background (figure 15A and B). Therefore, we try using stopping buffer to the negative control comparing to using stimulation buffer. In figure 15C and D and table 7, the results showed that the use of stopping buffer resulted in a higher percent of CD203c positive basophils between 35.51% - 87.59% suggested that the use of stopping buffer can decrease spontaneous CD203c expression in the negative control sample.

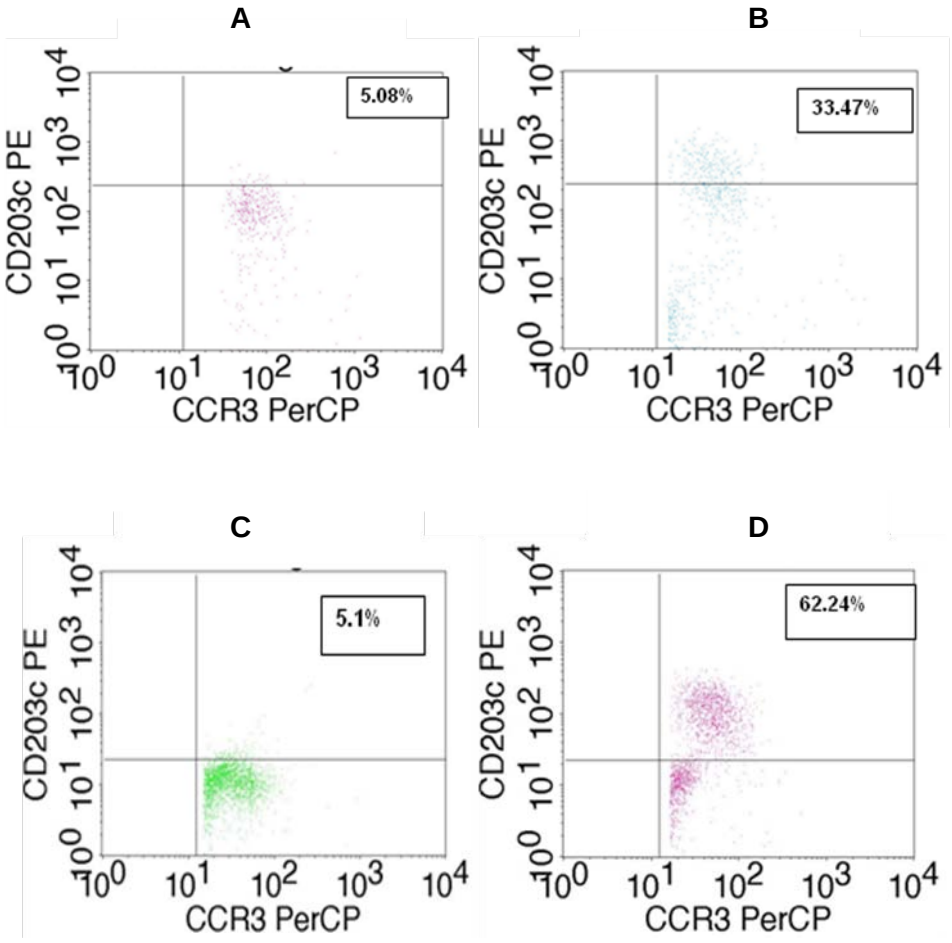


Figure 15. Flow cytometric analysis of negative control (A, C) and positive control (B, D). Gating result of BAT CD203c expression condition using stimulation buffer (A, B) were higher than the condition using stopping buffer (C, D)

Table 7. BAT CD203c expressions of negative and positive control using stop buffer as negative control

No.	BAT (% CD203c/CCR3)	
	Basal	**fMLP**
1	4.65	**80.73**
2	4.86	**80.29**
3	4.59	**66.42**
4	4.17	**63.89**
5	4.48	**35.51**
6	5.1	**62.24**
7	4.61	**81.77**
8	4.82	**87.59**
9	4.73	**76.95**
10	4.8	**73.79**

Note: Bold number indicated positive result of BAT test

Basophil activation test using CD203c expression

We performed BAT by using CD203c as an activation marker in 8 RCM hypersensitivity patients (table 8) and 7 healthy controls (table 9) as a pilot study. We found that all 8 patients with history of RCM hypersensitivity gave positive BAT result to the specific RCM that they have been exposed to. However, when testing in healthy control, we found that 3 out of 7 (42.85%) also gave positive BAT result to at least one type of RCM. The sensitivity of BAT using CD203c is 100% and the specificity is 57.14% which was quite low in contrary to previous reports which suggested that the BAT is very specific test. Therefore, we stoped testing the patients sample with CD203c and change the system to use another basophil marker, CD63.

Table 8. BAT CD203c expressions in immediate RCM hypersensitivity patients using stopping buffer as negative control

No.	Sex	Age	RCM	BAT (% CD203c/CCR3)				skin test
				Basal	fMLP	RCM concentrations		
						1:10	1:100	
1	F	26	Ioxithalamate	4.65	80.73	**20.66**	**28.16**	+ ve
2	M	68	Iopromide	4.86	80.29	**65.43**	**50.29**	+ ve
3	M	73	Iopromide	4.59	66.42	**22.24**	**15.76**	+ ve
4	F	65	Iopromide	4.17	63.89	**30.16**	**15.25**	- ve
5	F	71	Iopromide	4.13	79.6	**51.81**	**45.01**	- ve
6	M	65	Iopromide	4.66	78.16	**44.74**	**40.09**	- ve
7	F	39	Iopromide	4.63	88.95	**66.25**	**61.38**	- ve
8	M	37	Iopromide	5	71.78	**50**	**45.32**	- ve

Note: Bold number indicated positive result of BAT test

Table 9. BAT CD203c expressions in healthy controls using stopping buffer as negative control

No.	Sex	Age	Basal	fMLP	RCM									
					Iopromide		Ioxithalamate		Iohexol		Iopamidol		Iobitridol	
					1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100
1	F	45	4.62	81.1	2.92	8.7	0.38	**12.68**	3.87	6.81	0.91	5.1	0.21	3.99
2	M	45	4.8	73.8	2.28	6.74	0.53	8.14	1.79	4.22	0.32	1.21	0.72	0.65
3	M	49	4.48	35.5	2.12	2.7	1.52	4.38	ND	ND	ND	ND	ND	ND
4	F	34	5.1	62.2	8.25	9.76	6.91	8.94	ND	ND	ND	ND	ND	ND
5	F	36	4.61	81.8	**15.79**	4.61	4.64	**24.5**	ND	ND	ND	ND	**18.8**	**21.91**
6	M	42	4.82	87.6	**15.3**	**26.52**	4.17	**27.03**	ND	ND	ND	ND	ND	ND
7	M	28	4.73	77	5.18	8.98	3.13	7.65	ND	ND	ND	ND	ND	ND

Note: Bold number indicated positive result of BAT test

Basophil activation test using CD63 expression

We used another activation marker of basophil, CD63 in patients who have history of immediate RCM hypersensitivity. The percentages of basophils expressing CD63 to negative control stimulation varied between 0.2 and 5.25 % in healthy controls (table 11) and 0.09 and 4.17% in the patients (table 10) with the exception of 2 patients who had high background of 15.33% and 42.47% expressing CD63. This is most likely due to technical error while processing the blood samples. Therefore, these 2 samples were not included in the further analysis. For the positive control, in this assay we use anti-FcεR antibody to stimulate basophil. For patients and healthy controls, all of them gave positive BAT after stimulating with anti-FcεR antibody. The percentages of basophils expressing CD63 to positive control stimulation varied between 12.96 and 92.19% (patients), and 9.3 and 86.12% (controls).

The table 10 shows Basophil activation test comparing with skin test result of immediate hypersensitivity reaction to radio contrast media (RCM).

Twenty four patients (9 male, 15 female) were tested for BAT using CD63 marker. Average ages were 57.4 years (range: 37-73 years) for men and 47.1 years (range: 26-71 years) for women. 19 out of 24 patients (79.17 %) had allergic to Iopromide while 3 patients (12.5%) had a Ioxithalamate allergy. There were one patients (4.17%) had allergic Iopamidol and two patients (8.33%) for Iohexol.

The results of CD63 expression, 13 patients showed positive results to RCM, 11 out of 13 were positive in both 1:10 and 1:100 concentrations of RCM but 2 out of 13 patients was positive only 1:10 dilute concentration of RCM (table 10). In this protocol we also found 1 control had positive in this test (table 11). According to the positivity

criteria defined, the results of CD63 measurements were compared between patients and healthy controls, and the sensitivity and specificity of these assays were calculated using their clinical history to RCM hypersensitivity as gold standard. The result from the study indicated that basophils from 13 out of 24 patients with a history of RCM reactions (54.17%) had increased CD63 expression. The sensitivity of BAT is 54.17%.

The table 11 illustrates Basophil activation test of health control sample patients with five radio contrast media (RCM) types. Fourteen patients (7 male, 7 female) were attended in BAT. Average ages were 42 years (range: 28-49 years) for men and 43 years (range: 33-65 years) for women.

BAT was performed with the five types of RCM, Ioxitalamate , Iobitridol , Iopamidol, Iopromide and Iohexol. The test shows that only one control (male) demonstrated a positive result in 1:10 concentration of Telebix. Consequently, the experiment of this RCM illustrated a specificity value approximately 92.86%. However, the health control sample patients had never been received the five types of RCM before. Therefore, they are possible not a good control sample for the test and the positive reaction might reflect the possibility that the particular healthy control may have reaction to RCM if he/she expose to the reagent.

Table 10. BAT CD63 expressions in immediate RCM hypersensitivity patients

No.	Sex	Age	RCM	BAT (% CD63/CCR3)				skin test	Time delay (month)
				Basal	FceR	RCM concentrations			
						1:10	1:100		
1	F	34	Ioxithalamate	0.35	62.32	1.1	1.91	+ve	27
2	F	56	Ioxithalamate	4.17	88.45	**20.1**	**14.16**	+ve	45
3	M	38	Iopromide	0.92	90.78	4.31	1.53	+ve	16
4	M	70	Iopromide	2.99	70	**12.24**	4.23	+ve	18
5	F	65	Iohexol	0.57	51.43	2.17	3.21	+ve	13
6	F	26	Ioxithalamate	0.56	88.22	**60**	**18.53**	+ve	36
7	M	68	Iopromide	0.53	89.87	**26.96**	**34.53**	+ve	39
8	M	73	Iopromide	1.53	61.61	3.46	2.08	+ve	25
9	F	39	Iopromide	0.94	86.82	5.61	3.18	-ve	16
10	F	27	Iopromide	0.09	92.19	3.47	3.78	-ve	15
11	F	56	Iopamidol	0.25	78.37	0.91	0.7	-ve	21
12	F	65	Iopromide	0.22	79.32	**24.07**	**14.61**	-ve	33
13	F	71	Iopromide	0.28	55.18	**19.96**	**7.01**	-ve	19
14	M	65	Iopromide	0.27	49.28	**13.12**	**7.5**	-ve	36
15	F	39	Iopromide	0.59	25.54	1.68	0.96	-ve	37
16	M	37	Iopromide	0.42	92.19	**20.45**	4.87	-ve	19
17	F	41	Iohexol	3.77	43.78	**47.52**	**33.74**	+ve	15
18	F	54	Iopamidol	42.47	85.9	28.18	16.39	+ve	33
19	M	44	Iobitridol	15.33	60.12	13.97	8.95	+ve	21
20	M	64	Iopromide	0.97	92.19	1.04	1.18	-ve	14
21	F	43	Iopromide	3.73	83.6	**32.74**	**21.81**	-ve	19
22	M	55	Iopromide	1.38	15.75	**12.8**	**14.16**	-ve	38
23	M	60	Iopromide	1.09	87.99	**93.91**	**93.31**	-ve	36
24	F	33	Iopromide	0.82	12.96	0.76	1.34	-ve	21
25	F	41	Iopromide	0.5	72.41	**43.17**	**28.46**	-ve	37
26	F	63	Iopromide	0.91	49.59	3.79	2.31	-ve	39

Note: 1.Bold number indicated positive result of BAT test

2. Patients number 18 and 19 were eliminated from the analysis due to high background (highlighted rows).

Table 11. BAT CD63 expressions in RCM Healthy controls

No.	Sex	Age	Basal	FcεR	RCM									
					Iopromide		Ioxithalamate		Iohexol		Iopamidol		Iobitridol	
					1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100
1	F	33	0.56	85.96	0.4	0.56	0.93	0.83	1.06	0.5	2.01	1.49	ND	ND
2	M	48	0.42	54.09	2.44	1.44	**14.35**	1.04	5.38	3.43	2.74	3.53	3.87	2.32
3	F	65	1.21	63.14	3.81	1.44	2.13	0.73	2.58	1.56	2.06	1.41	4.26	4.81
4	F	45	1.87	85.45	3.49	2.05	4.04	6.11	1.46	1.94	4.04	6.32	3.94	4.8
5	M	45	1.73	83.07	5.53	4.88	4.97	3.76	5.28	4.16	4.04	4.29	5.15	4.14
6	M	49	1.7	53.95	5.17	3.49	5.1	5.38	ND	ND	ND	ND	ND	ND
7	F	34	1.46	86.12	4.82	1.71	5.16	1.9	ND	ND	ND	ND	ND	ND
8	F	36	1.92	23.17	3.41	5.2	6.39	3.53	ND	ND	ND	ND	3.7	2.5
9	M	42	0.2	82.94	1.39	1.13	3.07	1.97	ND	ND	ND	ND	ND	ND
10	M	28	2.12	75.35	1.6	1.07	5.76	1.55	ND	ND	ND	ND	ND	ND
11	M	38	3.97	9.3	2.91	2.89	3.81	2.37	ND	ND	ND	ND	ND	ND
12	F	36	2.3	37.09	2.24	2.25	1.49	2.54	ND	ND	ND	ND	ND	ND
13	F	50	5.25	32.78	6.06	3.74	5.35	4.1	ND	ND	ND	ND	ND	ND
14	M	43	3.2	47.53	1.18	0.91	1.9	0.86	ND	ND	ND	ND	ND	ND

Note: Bold number indicated positive result of BAT test

Comparison of results in the basophil activation tests to skin test results

The next experiment is for comparing a prior skin test result with Basophil activation test. This experiment used allergic sample patients (24 patients) same as the prior skin test. In skin test positive group, 5 out of 9 patients (55.56%) were positive in BAT test. There were 8 out of 15 patients (53.33%) who had negative skin test that also positive in BAT test. After thoroughly analysis between BAT and the prior skin test results, it could be seen that the positive results by BAT were not significantly (p = 0.691) different between skin test positive and skin test negative patients. Besides, BAT in combination with skin test results can increase sensitivity to 70.8%

The comparison of patient characteristics classified by BAT positivity

There were no differences in terms of gender, age, a history of drug or food allergy, previous exposure to RCM, previous reaction to RCM, immunosupressive drug, severity of reaction, RCM type (Iopromide or other drugs), and skin test positivity between patients with positive and negative BAT results. BAT positivity was not associated with elapsed time between the reactions and basophil activation testing. Median times between the reactions and basophil activation testing were 34.14 months (range 18-45) and 16 months (range 15-39) in the patients who had positive and negative BAT, respectively (table 12).

Table 12. Comparison of patient characteristics between patients with positive and negative BAT

	Positive BAT patients	Negative BAT patients	P value
Number of patients	13 (39%)	11 (45.83%)	-
Female gender	7 (53.84%)	8 (72.72%)	NS
Mean age (year)	53.69 ± 14.59	48.27 ±16.07	NS
History of drug, airway or food allergy	1 (7.69%)	3 (27.27%)	NS
Previous exposure to ICM	2 (15.38%)	2 (18.19%)	NS
Median time from reaction to BAT (month)	34.14	16	NS
Skin test positive	5 (38.46%)	4 (36.36%)	NS
Immunosupressive drug	4 (30.77%)	-	NS
Severe reaction (grade III)	1 (7.69%)	2 (18.18%)	NS
Kind of RCM (Iopromide)	10 (76.92%)	8 (72.72%)	NS
Kind of RCM (non Iopromide)	3 (23.08%)	3 (27.27%)	NS

NS = nonsignificant

CHAPTER VI

DISCUSSION

Radiocontrast media (RCM) are administered more than 75 million times per year for performing diagnosis and treatment of vascular disease and enhancement of radiographic contrast(1). Adverse reactions after RCM administration are common. Symptoms after RCM exposure may be regarded as hypersensitivity reactions or toxic reactions related to the well-defined toxicity of the compounds, or may be caused by factors unrelated to RCM, such as chronic idiopathic urticaria. Hypersensitivity reactions to RCM may present clinically as anaphylaxis with the potential to result in fatalities or as delayed occurring exanthemas, not unlike those to other drugs (14).

The adverse effect of using the RCM; especially immediate hypersensitivity reaction remains a significant problem for both patients and physicians since a number of patients need an examination by applying RCM. Currently, there are limited developments of the predicted or diagnostic tools for the hypersensitivity reaction to RCM. Most studies so far have focused in skin test for RCM hypersensitivity. However, the skin tests have very low sensitivity ranging between 4-73 % (43, 77, 83). This is likely due to the limitation that the skin test can only detect IgE-mediated hypersensitivity.

Turning to other laboratory-based assays, measurement of plasma histamine or tryptase levels can indicate that a reaction has taken place. However, often more than one agent is given at the same time. These assays are also unable to be used when investigating cross-reactivity to other agents. For a laboratory-based assay to be accepted for clinical diagnosis ideally it should offer good sensitivity, specificity and positive and negative predictive values which have been established in well-conducted clinical trials. It must be reproducible and technically straightforward and cost-effective. Since the initial description in 1994, assessing basophil activation using CD63 and/or CD203c upregulation, flow cytometric methods have proven interesting to allergists (45, 84).

Many studies show BAT were a useful tool that can diagnosis both IgE and non-IgE immediate mechanism summarize in table 13 (82). A large number of studies using CD63 and/or CD203c have been carried out across a wide range of allergens, unfortunately also with a number of different experimental protocols. Flow cytometry has been used to investigate food allergy, venom allergy, house dust mite (HDM), pollen, latex, and allergy to various drugs including muscle relaxants (MR), betalactam antibiotics and non-steroidal anti-inflammatory drugs with varying degrees of success. The mechanism by which basophils are activated appears to have a bearing on whether an increase in CD63 expression occurs. A significant correlation has been reported between the flow cytometric method using CD63 and anti-IgE and histamine release assay after basophil activation by allergens or anti-IgE and the high level of sensitivity and specificity of the basophil activation test indicates that it can be a very reliable diagnostic tool.

Table 13 Summary of BAT for diagnosis allergy and their sensitivity and specificity (82)

Allergen	Cut-off	Sensitivity	Specificity	Test
HDM	10% (no ROC curves)	56–70%	91–100%	IgE/CD63
HDM, timothy pollen	15% (ROC curves)	93.3%	98.4%	IgE/CD63
HDM	18%(16 µg/ml)	96%	96%	CD123+/HLA-DR-/CD63+
	8% (1.6µg/ml) (ROC curves)	82%	100%	
Cypress pollen	>15% (according to manufacturers instructions)	91.2%	100%	Basotest®
Grass pollen	> 9.5% (ROC curves)	85%	100%	Basotest®
		72%	92%	FastImmune (CD123+/ HLA-DR-/CD63+)
Latex	17% (ROC curves)	93.1%	91.7%	IgE/CD63
Latex	At least 2 sequential dilutions = >10% positive basophils	50%	100%	IgE/CD45/CD63
		75%	100%	IgE/CD45/CD203c
Latex	10% (ROC curves)	93%	100%	FAST
Latex	22% (ROC curves)	79.3%	96.7%	Basotest®
Bee/wasp/paper wasp venom	5% (no ROC curves)	91%	90%	IgE/CD45/CD63
Wasp/Bee venom	>15% (according to manufacturers instructions)	Wasp 0.45 µg/ml–78.6% 0.045 µg/ml–25.6% 0.0045 µg/ml–7.1 % Bee 0.45 µg/ml–50% 0.045 µg/ml–35.7% 0.0045 µg/ml–0%	100%	Basotest®
Wasp venom	15% (ROC curves)	92%	80%	IgE/CD63

Allergen	Cut-off	Sensitivity	Specificity	Test
Bee venom	25% (2.5-fold increase in number activated cells)	91.3%	90%	CD123+/HLA-DR-/CD63+
Wasp venom		85.3%	83.3%	
Bee/wasp venom	15% (CD63)	89[a], 88, 83%	100%	Basotest®
	14% (CD203c) (ROC curves)	97[a], 73, 66%	89%	IgE/CD203c
Anisakis	33% (15 µg)	97%	78%	CD123+/HLA-DR-/CD63+
	21% (1.5 µg)	100%	96%	
	8% (0.12 µg) (ROC curves)	91%	89%	
Food allergies	≥5% for ≥ 2 successive concentrations	58%	97%	IgE/CD63
Carrot	8.9%	85%	85%	IgE/CD63
Celery	6.3%	85%	80%	
Hazelnut	6.7% (ROC curves)	85%	80%	
Apple	10%[b]	100%	100%	IgE/CD63
	17%[c] (ROC curves)	88%	75%	
Apple	10% (mean basophil activation + 4SD)	75%	64%	IgE/CD63
Celery		65%	86%	
Carrot		75%	82%	
Neuromuscular blocking agents	15% (no ROC curves)	64%	93%	IgE/CD45/CD63
Neuromuscular blocking agents	>10% for ≥ 2 successive concentrations	54%	100%	IgE/CD45/CD63 OR CCR3/CD24/CD63
Neuromuscular blocking agents	>10% (according to manufacturers instructions)	79%	100%	IgE/CD45/CD63
		36%	100%	IgE/CD45/CD203c
Rocuronium bromide	4% (ROC curves)	91.7%	100%	CD123+/HLA-DR-/CD63+
Metamizol	5% SI ≥ 5 (for any two concentrations)	42.3%	100%	FAST

Aspirin & other NSAIDs	5% (ROC curves) plus SI >2	15–55%	74–100%	FAST
NSAIDs	SI ≥ 2	42.85%	100%	Basotest®
Betalactams	5% (ROC)	50%	93.3%	FAST
Betalactams	SI ≥ 2 @ any 2 concentrations, plus activation ≥ 5%	39.1%	93.3%	FAST
Betalactams	≥ 5% above basal level plus SI >2	25%	100%	IgE/CD63
Betalactams	SI ≥ 2 @ at least 1 concentration	49%	91%	Basotest®
Betalactams (amoxicillin anaphylaxis)	>2 SD above	20%	79%	IgE/CD45/CD63
	controls	60%	100%	IgE/CD45/CD203c
Cat	8.1% (mean +2SD control)	100%	95%	IgE/CD45/CD63
			95%	IgE/CD45/CD203c
	1.38 SI (mean + 2SD control)	95%		

So far there were only 2 previous reports about BAT in RCM hypersentivity with the total of only 4 patients (77, 78). In this study, we investigate the role of BAT using CD203c and CD63 in 26 RCM hypersensitivity patients and 14 healthy controls. There are several issues to be discussed as following.

First, in this study, CCR3 was used as a basophil marker instead of anti-IgE antibody which was mostly used in other studies. We found that the use of CCD3 is better because 1) CCR3 is constitutively over-expressed in basophils, 2) CCR3 is the main receptor of eotaxin that is clearly involved in various allergic processes. In addition, because Fcε expression is known to vary on basophil surfaces from one patient to another; therefore, basophil identification using anti-IgE sometimes is difficult. Basophils express about 20,000 copies of CCR3 on a single cell surface, representing a considerably higher density than other leucocytes. The study of Guillaume M., et al. (2002) could show, that fMLP-induced up-regulation of CD63 was higher (31.2±4.9%) in the CCR3 protocol than in the anti-IgE protocol (14.5-3.4%) in allergy to muscle relaxant drugs(85)

Second, CD63 is expressed on the membrane of basophil granules, and is therefore not detectable on the surface of resting basophils. In contrast, CD203c is constitutively and exclusively expressed on the surface of basophils, mast cells and their progenitors. Following allergen challenge, CD203c is rapidly upregulated in sensitized subjects, and thus a promising target molecule for flow-cytometry-based evaluations. Therefore, the percentage of CD203c expression level in negative control showed higher data than CD63 expression. In addition, CD203c upregulation was very sensitive molecule that could be induced by shaking or by any other force.

Third, in the effort to develop BAT using CD203c as a marker, we got 100% BAT positive in the patient group (n = 8). However, we concerned about the low specificity (42.8%) in the healthy control group (3 out of 7 samples). When these 3 BAT positive healthy controls were re-tested using CD63 BAT system, we found that none of them show positive result. It seems that the BAT system using CD203c in our study still needed further modification to improve the specificity. Therefore, we didn't further analyze the result from CD203c BAT.

Forth, only one healthy control (female) demonstrated a positive CD63 BAT result in 1:10 concentration of Telebix. Consequently, the experiment of this RCM illustrated a specificity value approximately 92.86%. However, the health control sample has never received the five types of RCM before. As a result, they are not a perfect control group for the test and it is possible that he/she can develop hypersensitivity to RCM upon exposure in the future.

Fifth, our main results using CD63 BAT system (n = 24) showed that the sensitivity was 54.17% which was higher than the sensitivity of skin test previously performed in 96 patients about 23.6%. Our results supported finding from Trcka J. in 2008 in which BAT was positive for CD63 in three patients who had anaphylaxis reaction to radio contrast media (RCM) (77). Similarly, the positive results were shown in the skin test as well. Furthermore, in 2009 (78), a research group of Dewachter P. performed BAT in patients who had Amidotrizoate allergy. The outcome illustrated that there was one patient who had anaphylaxis reaction to radio contrast media and his skin test was positive. However, his BAT was negative for CD63 but positive for CD203c. Unfortunately, we cannot compare our BAT result using CD63 and CD203c. In addition,

this study was performed BAT by using only RCM that patients had allergic reactions because of budget limitation.

Sixth, in addition, this study performed BAT in 24 patients (2 patients were excluded due to high background) who had skin test positive and negative. We found that CD63 BAT were positive not only in the patients who had positive skin test but also in patients with negative skin test as well. In the case of patients who had negative skin test but positive in BAT might suggested that the patients had a non- IgE mediated mechanism of RCM hypersensitivity.

Seventh, when analyzing the association between BAT results to clinical parameter, we could not detect any positive association. However, our study has some limitations. Firstly, the data regarding prevalence and patient characteristics of the reactions were obtained by retrospective method, therefore the complete data could not be retrieved and the exact incidence of the reactions could not be demonstrated. Secondly, the diagnosis of the reactions which relied mainly on a clinical history was made by radiologists, so the assessment biases might occur due to difference in diagnostic criteria. Thirdly, there were no supportive data from *in vitro* tests such as specific IgE to RCM due to unavailability of these tests. Finally, provocation test by implicated ICM and negative skin test ICM could not be done due to ethical reasons.

Eighth, since we could not find any correlation between skin test and BAT, we try combine the two test together for the diagnosis of immediate RCM hypersensitivity RCM, and the sensitivity was increased to 70.8%. And since the specificity of the test is very high, we suggested that anyone who shows a positive BAT result in a RCM type might be at risk to immediate hypersensitivity reaction to that RCM type in the future. Therefore, this information will be useful for those people who need to receive the RCM

type because they can protect themselves by taking the RCM preventive medicine. In another way, they can receive the other types of RCM instead to prevent to immediate hypersensitivity reaction to RCM.

Ninth, there were some confounding factors which could affect BAT such as blood collection. Samples should be mix gently or kept cool because vigorous mixing could activate spontaneous basophil activation can cause false negative result. In addition, the amount of EDTA in blood could influence basophil activation ability because too much EDTA decreases Ca^{2+}, an important ion for basophil activation, and can deviate BAT result.

In conclusion, basophil activation test may be potentially useful in the diagnosis of patients with RCM-induced hypersensitivity reactions.

CHAPTER VII

CONCLUSION

This result of this study showed the sensitivity and specificity of BAT were 54.17% and 92.86%, respectively. BAT in combination with skin test results had increased sensitivity to 70.8%. In addition, the comparison of patient characteristics between positive and negative BAT patients demonstrated no differences in terms of gender, age, a history of drug or food allergy, previous exposure to RCM, Immunosupressive drug use, severity of reaction to RCM, RCM type (Iopromide or other drugs), severity of reaction to RCM, and skin test positivity. In conclusion, basophil activation test (BAT) may be potentially useful in the diagnosis of patients with RCM-induced hypersensitivity reactions.

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APPENDIX

APPENDIX

REAGENTS PREPARATION

1. Sitmulation buffer

HEPES	20 mM,
NaCl	133 mM
KCl	5 mM
$CaCl_2$	7 mM
$MgCl_2$	3.5 mM
Bovine serum albumin (BSA)	1 mg/mL

Adjust volume to 1 liter with distilled. Adjust pH to 7.4. The solution was mixed and sterilizers by autoclaving at 121 C for 15 min. Keep refrigerated.

2. Stop buffer

HEPES	20 mM,
NaCl	133 mM
KCl	5 mM
EDTA	0.27 mM

Adjust volume to 1 liter with distilled. Adjust pH to 7.3. The solution was mixed and sterilizers by autoclaving at 121 C for 15 min.

BIOGRAPHY

Miss Panwas Pinnpobphun was born on September 29, 1984, in Nonthaburi province. She received her Bachelor's Degree of Science (Biology) from the Department of Biology, Faculty of Science, Mahidol University in 2005. She has enrolled a Master degree program at the Program in Medical Microbiology, Graduate School, Chulalongkorn University since 2009.