From Production Skills to Scientific Knowledge,
and onward to Fusion with Medical Science

Indirect Composite Resin

Safety Evaluation Report

Biological Evaluation of "Luna-Wing"

Vol.4 in a Series on Safety Reports

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Biological Science Safety Laboratory

Table of Contents

Introduction

Metals, ceramics, and resin are used for dental materials, but only after fulfilling strict conditions. They must demonstrate stability in the demanding intraoral environment, safety in their contact with the human body, and functionality in use. As technological innovation continues apace, the advanced development of new materials for use with the human body offers medical professionals and patients many new advantages. However, when advancing product development with new chemical substances and new technology, safety obviously is and must remain the single most important concern. In fact, the revised Japanese Pharmaceutical Affairs Law of April 2005 places stronger emphasis than ever before on the evaluation of medical devices, including dental materials, in terms of their safety and effectiveness.

At Yamamoto Precious Metals Co., Ltd. (hereafter Yamakin), we have performed extensive fundamental research on indirect composite resin for the purpose of new materials development. We have also conducted a wealth of research on the physical and chemical properties of monomers and inorganic fillers, their mechanical properties and other related topics. Moreover, in order to progress to product development in a way that will guarantee product safety along with excellent mechanical and physical properties, since 2003, we have conducted joint research with the Oral Tumor Control Science Tumor Pathology Course at Kochi Medical School, Kochi University. In 2005, The Biological Science Safety Laboratory was established in the Department of Oral and Maxillofacial Surgery, Kochi University. Yamakin has undertaken these initiatives because we see ourselves as a manufacturer which provides safety to both patients and the medical professionals who serve them. For evaluation of biological safety, The Biological Science Safety Laboratory carries out the “Biological Evaluation of Medical Devices” conforming to ISO 10993, the international standard, and also our own examinations based on cell, cell tissue, and genetic engineering.

Even though cytotoxicity has been reported for the main components of indirect composite resin – monomers, urethane dimethacrylate and triethylene glycol dimethacrylate – they are still used as main components in most products on the market because of their longstanding results in clinical practice. In addition, endocrine disruptor action has been reported in some of the polymers used in indirect composite resin. Therefore, it is necessary to fully consider potential impacts on the human body when developing new products.

The indirect composite resin which Yamakin has developed based on the results of our fundamental research is a nano-composite resin that takes the safety concerns mentioned above into full consideration. At the same time, nano-technology is exploited to the utmost, and our product has the highest filling rate among the composite resins covered by the Japanese national health insurance system. This composite resin is compounded of 20-nm spherical fillers in the resin matrix and is improved greatly in its strength and abrasion resistance by being compounded with 100-nm spherical fillers; as a result, it can achieve the requirements demanded of a basic indirect composite resin.

With “Indirect Composite Resin for Biological Safety”as its overriding development concept, a wide range of tests on the safety of basic indirect composite resin, has been conducted based on the stipulations of ISO 10993. These tests include: 1) Acute Toxicity Test on mice 2) Colony-Forming Assay with Cultured Cells (*In Vitro* Cytotoxicity Test) 3) Reverse Mutation Test on bacteria (Mutagenicity Test) 4) Skin Sensitization Test on guinea pigs, and 5) Oral Mucosa Irritation Test on hamsters. Through this testing process, safety for human usage is confirmed.

Luna-Wing, designed as an indirect composite resin, has become the final product in this process of development, adding to the basic indirect composite resin a small amount of color pigment along with a fluorescent pigment compound for color adjustment with natural teeth. There are various kinds of additives, depending on their properties, but it remains a fact that there are no reported cases of their having undergone biological evaluation. Even if basic indirect composite resin is considered to be safe for the human body, if the relevant additive displays any physical impact, however slight, the resin itself is not safe.

To purse safety even further, Yamakin has carried out tests conforming to ISO 10993, along with its own testing, based on cells, cell tissue and genetic engineering. Testing was undertaken in collaboration with Kochi Medical School, Kochi University, centering on color pigments, fluorescent pigments, and primers. We now bring together the results of this collaborative research testing, and report the data as Indirect Composite Resin Safety Evaluation Report: Biological Evaluation of “Luna-Wing” (Vol. 4 in a Series on Safety Reports). We are confident that the safety information provided here can help to ensure security for medical professionals and patients.

June 2006
Yamamoto Precious Metal Co., Ltd.
Biological Science Safety Laboratory

《 Outline 》

1. Acute Toxicity Test

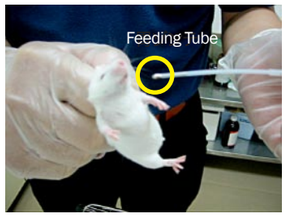
Purpose of the Test
The Systemic Toxicity Test is carried out in order to predict the safety of a sample in an environment that closely resembles that of the human body. There are different appearances for toxicity: Acute (toxicity shows quickly) and Chronic (toxicity shows over a long time); this test is for Acute.

Summary of the Test
Ⅰ. Four-week-old mice are prepared and kept for 1 week.


Mice in a Cage


Cages

Ⅱ. Normally, a single suspension of a sample is forcibly administered (when necessary, multiple doses) to mice using a feeding tube.

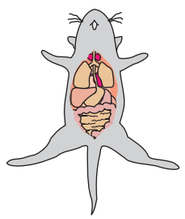


Forced Oral Dosage Using a Feeding Tube

Ⅲ. Fourteen days after dosing, weight and other health conditions are observed. If toxicity is shown, weight reduction or weakening will be observed. Death may be observed when toxicity is strong.


Observing Health Condition

Ⅳ. After observation, all of the mice used in the examination are vivisected and shape change to the organs is ascertained along with mass change.


Observation of Shape Change to the Organs after Vivisection

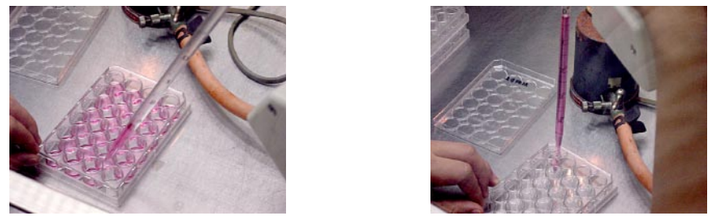
2. Colony-Forming Assay with Cultured Cells (*In Vitro* Cytotoxicity Test)

Purpose of the Test
The test is performed to evaluate toxicity at the cell level. For this test, a Colony-Forming Assay with Cultured Cells is selected from among possible evaluation methods using cells. One of the characteristics of this test is that it can show severe reactivity to toxicity. It is also recommended in ISO 10933-5.

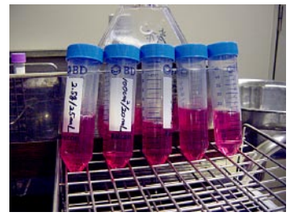
Summary of the Test
Ⅰ. V79 (Chinese Hamster Lung) cells are incubated and grown.


Incubation of Cells

Ⅱ. Approximately 100 cells are placed in each hole on culture plates.


Seeding Cell Suspension on Culture Plates

Ⅲ. Solution composition is extracted for 24 hours from a sample using culture fluid. The extract is added on the cells and incubated for 6 days.


Extraction from Samples


6-day Incubation

Ⅳ. The number of colonies is counted. If the number of colonies formed is close to the original number of cells, there is no toxicity.

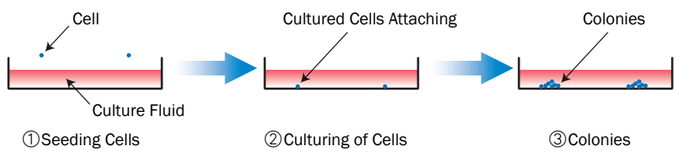
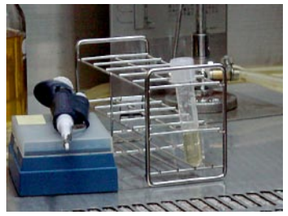


Fig.1: Flow from Addition of Cells to Colony Formation

3. Reverse Mutation Test (Mutagenicity Test)

Purpose of the Test
If a component or components of products have an influence on DNA, it will cause various problems. It may cause cancer in cases where DNA is impacted after birth, and in cases where DNA in general somatic cells is impacted, it will cause deformation in descendant cells or even in further generations. Therefore, it is absolutely essential to ascertain the influence on DNA of products component(s). This test is carried out in order to predict such risks.

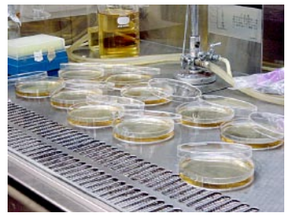
Summary of the Test
Ⅰ. Bacteria (salmonella and colon bacteria) are incubated and soaked for 8 hours at 37℃.


Culture Fluid with Bacteria

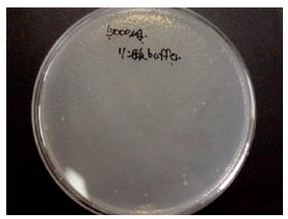

Automatic Thermostat Shaker

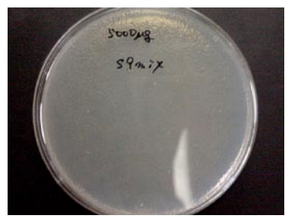
Ⅱ. The incubated bacteria and materials are compounded and added into an agar medium which has almost no nutritional ingredients. It is then left for 48 hours.


Compound of Bacteria and Materials


Seeding Bacteria into Agar Medium

Ⅲ. After 48 hours, the number of bacterial colonies is counted. If the sample produces abnormality in DNA, several hundred to more than 1,000 colonies will have formed.

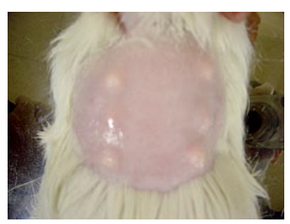



Seeding Cell Suspension on Culture Plates

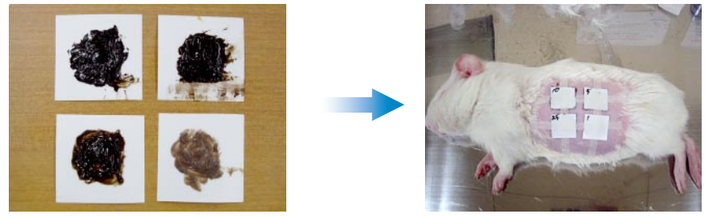
4. Skin Sensitization Test

Purpose of the Test
The test is performed to measure the level of allergenic risk a sample exhibits.

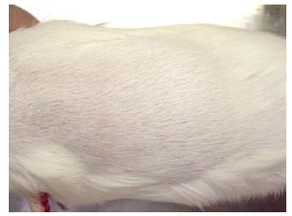
Summary of the Test
Ⅰ. A guinea pig is subcutaneously injected with a reagent in order to make it physically sensitive to allergy.

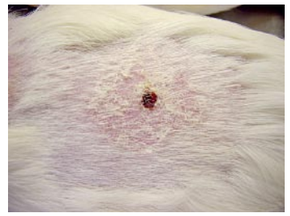

After Subcutaneous Injection

Ⅱ. Test samples are attached to the flank of the guinea pig as in a skin patch test and left for 48 hours.

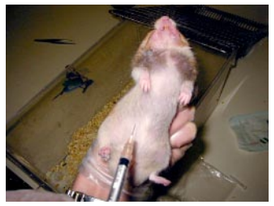

Application of Samples

Ⅲ. The attached samples are removed after 48 hours, and the presence or absence of an allergic reaction is confirmed. If there is no allergic reaction, the skin remains the same as shown in the picture on the bottom left (the reddened area is caused by surgical tape), and if there is an allergic reaction, it will cause inflammation and form a scab as shown in the picture on the bottom right.


No Allergic Reaction


Allergic Reaction

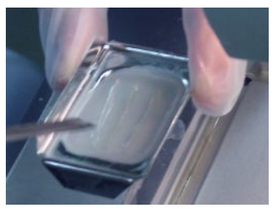
5. Oral Mucosa Irritation Test


Anesthetization

Purpose of the Test
A wide range of toxic substances adversely impact the human body by being absorbed into it. However, other toxic substances cause abnormality on the surface texture, as with cigarette tar. This test is to analyze impact on surface texture in the mouth.

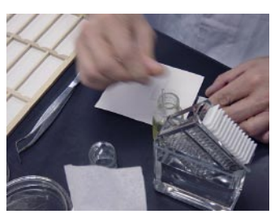
Summary of the Test
Ⅰ. Samples are placed into the cheek pouches of a hamster and left for 2 weeks. They are then removed.

Ⅱ. A permanent preparation of the cheek pouches is made.


Cheek Pouches are Wrapped to make Paraffin Blocks

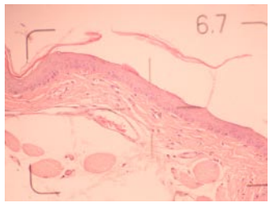

A Permanent Preparation is Made

Ⅲ. The permanent preparation is dyed.


A Glass Cover is Installed to Complete a Permanent Preparation

The Permanent Preparation is Dyed

Ⅳ. The structure is observed. If the sample is not a substance that exhibits adverse impact in the mouth, the tissue sequence will be in order; however if adverse impact has occurred, the structure is thickened and a mass of neutrophil, eosinophile, basophile leucocyte and macrophage will appear.



Normal Tissue

6. Cell Growth Inhibition Test

Purpose of the Test
As with the Colony-Forming Assay with Cultured Cells, this test is performed to evaluate toxicity at the cell level. Among the characteristics of the test is that it is the most popular of *in vitro* Cytotoxicity tests and that the evaluation is conducted based on the cell reproduction rate.

Summary of the Test
Ⅰ. Cells are placed on a culture plate.
Ⅱ. The extract is placed on the cells from a sample. Incubation is for 48 hours.
Ⅲ. The cell reproduction rate is measured.
The evaluation method is comparing the number of cells reproducing before and after adding the extract.

7. DNA Synthesis Test

Purpose of the Test
The cells composing the human body perform cytokinesis repeatedly every day, and the old cells are discharged outside the body. The test is performed to ascertain whether there is toxicity in the sample or not by observing if the reaction is performed normally, paying attention to reproduction and composition of DNA, which always performs the cytokinesis.

Summary of the Test
Ⅰ. Cells are placed on a culture plate.
Ⅱ. The extract is placed on the cells from a sample. Incubation is for 42 hours.
Ⅲ. After 42 hours, a reagent that reacts with the site of synthesis when DNA is compounding is added.
Ⅳ. Incubation takes place for another 6 hours.
Ⅴ. The quantity of the reacted reagent is measured. With the quantity of compounded DNA without adding the extract serving as a base, evaluation is conducted by observing how much the quantity of compounded DNA changes after adding the extract.

8. Cytotoxicity Test

Purpose of the Test
Cell death takes place in two major ways. One is apoptosis, and the other is necrosis. Apoptosis is DNA-programmed cell death, for example, the disappearance of cells between the fingers and toes in a developing human embryo. Necrosis is cell death caused by external factors, such as burns. This test is performed in order to ascertain whether or not both forms of cell death have occurred.

Summary of the Test
Ⅰ. Cells are placed onto a culture plate. Incubation is for 6 hours.
Ⅱ. Culture fluid is removed after incubation.
Ⅲ. The extract is placed on the cells from a sample. Incubation is for 48 hours.
Ⅳ. Coloring liquid is added and left for 10 minutes in order to confirm apoptosis and necrosis.
Ⅴ. Appropriate equipment is used to count the number of normal cells and dead cells (flow cytometry).

9. DNA Fragmentation Test

Purpose of the Test
The test is performed in order to ascertain whether or not apoptosis (programmed cell death) has occurred, by analyzing DNA fragmentation.

Summary of the Test
Ⅰ. Cells are placed on a culture plate. Incubation is for 6 hours.
Ⅱ. Sample solution is added to the incubated cells. Incubation takes place for 48 hours.
Ⅲ. The DNA is removed.
Ⅳ. The DNA is dyed.
Ⅴ. The DNA is electronically isolated.
Ⅵ. The presence or absence of apoptosis is confirmed by analyzing the DNA in its isolated condition.

10. Protein Synthesis Test

Purpose of the Test
The test is carried out in order to evaluate toxicity by observing increase or decrease of the synthesis quantity after RNA-synthesized cells proteins have been dyed.

Summary of the Test
Ⅰ. Cells are placed onto a culture plate. Incubation is for 6 hours.
Ⅱ. Culture fluid is removed after incubation.
Ⅲ. The extract is added to the cells from a sample. Incubation takes place for 48 hours.
Ⅳ. Protein is extracted.
Ⅴ. The protein is dyed.
Ⅵ. The absorptivity is measured.

《 Methods 》

1. Materials

The materials used in the tests are shown in the table below.

Materials	Main Components or Characteristics
Fluorescent Pigment	Europium Alkaline-Earth Phosphate
Color Pigment	Inorganic Pigment (TiO_2, Fe_2O_3, ZrO_2, etc.) Organic Pigment
Primer	Thiol Primer
Resin (Test Model)	Dentine (A3 Shade) Hardened Material
UDMA	Urethane Dimethacrylate
TEGDMA	Triethylene Glycol Dimethacrylate

Note: The basic indirect composite resin (Test Model) has already been confirmed as ISO10993-compliant by testing; it was therefore evaluated under company-internal criteria. UDMA and TEGDMA are added for purposes of comparison.

2. Acute Toxicity Test

2-1 Test Animals
Four-week-old male and female ICR-type mice (10 males and females for both test and control groups) are prepared -and kept for approximately one week. They are used for testing after the absence of abnormalities is confirmed. Five of the test animals are kept in once polycarbonate cage, with room temperature set at 23°C±2°C with the lighting time at 14 hours per day.

2-2 Preparation of Sample Solution
Fluorescent pigment, color pigment, and primer are suspended in pure water and sample solution is prepared at 100mg/ml.

2-3 Evaluation Method
Test animals fast for approximately 4 hours before administering. After measuring their weight, 2g/kg of a single dose of sample solution is forcibly administered to both male and female test groups using a feeding tube. For the control group, 0.6ml of pure water for female and 0.7ml for male is also administered. The observation term is 14 days; administration is performed frequently and observed once a day from the day following administration. Weights are measured from 7 to 14 days after administration, and t-testing is used to compare groups with a 5%-significant level of difference. The test animals are dissected after the observation term.

3. Colony-Forming Assay (*In Vitro* Cytotoxicity Test)

3-1 Performing Cells
V79 Cells (Chinese Hamster Lung)

3-2 Preparation of Sample Solution
Fluorescent Pigment, Color Pigment
The sample solution (100%) is made by adding 1mL of MO5 culture medium to 0.1g of a sample and extracting for 24 hours at 37°C. It is then sterilized with a 0.22μm filter and diluted with MO5 culture medium.
Fluorescent Pigment : 3.13, 6.25, 12.5, 25, 50 and 100%
Color Pigment : 3.13, 6.25, 12.5, 25, 50 and 100%

Primer
For primer, the sample solution（1000μg/mL-100%）is made by diluting with MO5 culture medium. It is then sterilized with a 0.22μm filter and diluted with MO5 culture medium.
Primer : 3.13, 6.25, 12.5, 25, 50 and 100%

3-3 Method
V79 cells (Chinese Hamster Lung) propagated in a single layer by trypsinization are exfoliated and 200 pieces/mL of cell suspensions is prepared using MO5 culture medium. The cell suspensions are scattered with 0.5m/L over each well on a tissue culture plate and incubated for approximately 6 hours at 5% CO_2 at 37°C. After incubation, attachment of the cells to the bottom of the wells is confirmed and the culture medium is removed. 0.5m/L each of sample solution at each respective density, negative control sample solution, and the extract is placed in each of four wells , and incubation takes place for 6 days at 5% CO_2 at 37°C. After the incubation, each well is dipped into a 10% neutral phosphoric acid buffer formalin solution for 30 minutes and the wells are dyed with 0.1% of methylene blue solution for 15 minutes. The number of colonies is then counted. For the samples, the test is repeated twice.

4. Reverse Mutation Test (Mutagenicity Test)

4-1 Performing Bacterial Strains
The bacterial strains used for the test are mutated salmonella strains（TA100, TA98, TA1535, TA1537） and colon bacterium (WP2uvrA).

4-2 Preparation of Sample Solution
Fluorescent pigment, color pigment, and primer are compounded with dimethyl sulfoxide (DMSO) in a centrifuge tube at 50mg/mL and sample solution (100%) is created by mixing with a test-tube mixer. This sample solution is then diluted using DMSO. Fluorescent pigment, color pigment, primer : 3130, 6250, 12500, 25000 and 50000μg/mL DMSO is used as a negative control group.

4-3 Method
Preincubation assay (with and without metabolic activation for the sample) is performed for the test. 0.1mL of the sample solution diluted at a set density, S9Mix or 0.5mL of 0.1mol/L of sodium phosphate buffer solution (pH704), and 0.1mL of bacteria suspension respectively is added into a sterilized tube. After 20 minutes of pre-incubation in a thermostat inspection chamber at 37°C, 2mL of top agar is added and mixed in, spread evenly and solidified in minimal glucose agar plate culture medium. Incubation takes place for 48 hours at 37°C and the number of colonies which have appeared through reverse mutation is counted.

5. Skin Sensitization Test

5-1 Test Animals
The test group is composed of nine guinea pigs; six more are used for each negative control group and positive control group (prompt sanitization material treatment group).

5-2 Method
On 2cm × 2cm filter paper at 0.1m/L each, a 10%, 1%, and 0.1% Vaseline mixture of fluorescent pigment, color pigment, and primer for the test group and a 10%, 1%, and 0.1% Vaseline mixture of Vaseline and a sample for the control group is applied. (The same test under the same testing conditions as the sample is performed for the negative control group in order to confirm that the result of the sample is not an error.). The preparations are then applied on the sheared and shaved flank of the guinea pig. For the positive control group, apply 0.01% (w/v) of acetone solution of DNCB (2,4-dinitrochlorobenzen) at 0.01m/L each.

6. Oral Mucosa Irritation Test

6-1 Test Animals
Five-week-old male Syrian hamsters (golden hamsters) are prepared and kept for approximately 2 weeks; 15 hamsters per sample are used for the tests after confirming the absence of abnormalities. Two to three of the test animals are put into one polycarbonate cage and kept at a room temperature of 23°C±2°C with the lighting time set for 14 hours per day.

6-2 Method
After measuring weights of the test animals, 40mg/kg of pentobarbital sodium is administered in the abdominal cavity to produce general anesthesia. The mucous membrane of the cheek pouches is removed carefully so as to avoid scratches and it is confirmed if there is any damage, such as inflammation, on the mucous membrane after thorough cleaning with buffered saline solution. Excess water is removed with paper; the cheek pouches are restored to their original condition. The samples are applied on one side of all of the check pouches and solvent control on the other. After application, the cheek pouches are sewn together in order to maintain the sample and the solvent control. Application is set for 2 weeks, and the test animals are anesthetized with ether and euthanized by bleeding. The cheek pouches are removed after the application term. The cheek pouches are visually checked and solidified with formalin liquid. Following the usual directions, the tissue is dehydrated, penetrated, embedded in paraffin and dyed with hematoxylin eosin (H.E.) after cutting thinly. The permanent preparation created here is observed histopathologically with an optical microscope.

7. Cell Growth Inhibition Test

7-1 Performing Cells
3T3 Cells (mouse embryonic fibroblast cells)

7-2 Preparation of Sample Solution
Fluorescent Pigment, Color Pigment
The sample solution is made by adding 1mL of culture medium per 0.1g and extracting it at 37°C for24, 48, and 168 hours.

Primer, UDMA, TEGDMA
Because it is difficult to dissolve them into culture medium for the primer, UDMA and TEGDMA are dissolved in DMSO in advance and diluted step by step as shown below.
Primer : 62.5, 125, 500, 1,000μg/mL
UDMA : 470 (1,000) , 235 (500) , 117.5 (250) , 58.8 (125) μg/mL (μM)
TEGDMA: 286 (1,000) , 143 (500) , 71.5 (250) , 35.75 (125) μg/mL (μM)
The density of DMSO in the sample solution here is prepared at less than 1% without toxicity for cultured cells. For the sample solution, DMSO-diluted solution of the same density as diluted is used as a reference value.

Resin (Test Model)
After washing resin (test model) with tap water and distilled water for injection of the Japanese pharmacopeia (from HIKARI PHARMACEUTICAL CO., LTD.) and drying, ultraviolet sterilization is performed on both sides for 30 minutes on a clean bench. 1mL of culture medium is added to each 5 cm^2 of surface area of the sample and extracted to make a sample solution in incubation at 5% CO_2 for 24, 48, and 168 hours at 37°C. All of the sample solution is then sterilized with a 0.22μm filter and used for the test.

7-3 Method
3T3 cells propagated in a single layer by trypsinization are exfoliated and 5×10^4pieces/mL of cell suspensions are prepared. 1mL of cell suspensions is scattered over each well on a tissue culture plate and incubated for approximately six hours at 5% CO_2 at 37°C. After incubation, attachment of the cells to the bottom of the wells is confirmed and the culture medium is removed. 1mL of sample solution for each sample is added and the samples are incubated or 48 hours at 5% CO_2 at 37°C. The culture medium on each well is removed and 100μL of MTT solution is added. The samples are then matured for 2 hours at 37°C, 100μL of DMSO is added, and the samples are matured at room temperature for 5 minutes. Viability is calculated by measuring absorptivity at 570nm with a micro plate reader (THERMOmax: Molecular Devices, LLC.) and absorptivity rate from a reference value of 100%. All tests are repeated three times.

8. DNA Synthesis Test

8-1 Performing Cells
3T3 Cells (mouse embryonic fibroblast cells)

8-2 Preparation of Sample Solution
Fluorescent Pigment, Color Pigment
The sample solution is made by adding 1mL of culture medium per 0.1g and extracting at 37°C for 24 hours.

Primer, UDMA, TEGDMA
Because it is difficult to dissolve them into culture medium for the primer, UDMA and TEGDMA are dissolved in DMSO in advance, with the density prepared as shown below.
Primer : 500μg/mL
UDMA : 286 (1) μg/mL (mM)
TEGDMA: 470 (1) μg/mL (mM)
The density of DMSO in the sample solution here is prepared at less than 1% without toxicity for cultured cells. For the sample solution, DMSO-diluted solution of the same density as diluted is used as a reference value.

Resin (Test Model)
After washing resin (test model) with tap water and distilled water for injection of the Japanese pharmacopeia (from HIKARI PHARMACEUTICAL CO., LTD.) and drying, ultraviolet sterilization is performed on both sides for 30 minutes on a clean bench. 1mL of culture medium is added to each 5 cm^2 of surface area of the sample and extracted to make sample solution in incubation at 5% CO_2 for 24 hours at 37°C. All of the sample solution is then sterilized with a 0.22μm filter and used for the test.

8-3 Method
3T3 cells propagated in a single layer by trypsinization are exfoliated and 2×10^5 pieces/mL of cell suspensions are prepared. 1mL of cell suspensions is scattered at 100μL over each well on a tissue culture plate and incubated for approximately 6 hours at 5% CO_2 at 37°C. After incubation, attachment of the cells to the bottom of the wells is confirmed and the culture medium is removed. 100μL of sample solution for each sample is added and incubated for 48 hours at 5% CO_2 at 37°C. Six hours before the incubation, 1μCi of 3H-Tymidine per well is added and the radioactivity of extracted 3H is measured with a liquid scintillation counter.

9. Cytotoxicity Test

9-1 Performing Cells
3T3 Cells (mouse embryonic fibroblast cells)

9-2 Preparation of Sample Solution
Fluorescent Pigment, Color Pigment
The sample solution is made by adding 1mL of culture medium per 0.1g and extracting at 37°C for 24 hours.

Primer, UDMA, TEGDMA
Because it is difficult to dissolve them into culture medium for the primer, UDMA and TEGDMA are dissolved in DMSO in advance, with the density prepared as shown below.
Primer : 500μg/mL
UDMA : 286 (1) μg/mL (mM)
TEGDMA: 470 (1) μg/mL (mM)
The density of DMSO in the sample solution here is prepared at less than 1% without toxicity for cultured cells. For the sample solution, DMSO-diluted solution of the same density as diluted is used as a reference value.

9-3 Method
As with the Cell Growth Inhibition Test, a cell suspension is prepared, 3mL is scattered on a culture medium on a tissue culture plate and incubated for approximately 6 hours at 5% CO_2 at 37°C. After incubation, attachment of the cells to the bottom of the wells is confirmed and the culture medium is removed. 3mL of sample solution is added to each test sample (100%) and incubated for 28 hours at 5% CO_2 at 37°C. After incubation, the culture fluid including floating cells is collected into a centrifugal tube (15mL), the plate is washed with 5mL PBS (–), and the PBS (–) is collected into the same tube. Trypsin solution is added to the plate and kept for 5 minutes at 37°C. After the cells are released, the fluid is put into a centrifugal tube and centrifuged for 5 minutes at 1,200rpm to perform aspiration for supernatant. The cells density is set at 1×10^6pieces/mL with 1 × Binder buffer diluted at 10 times with distilled water. 500μL of cell-suspension liquid at the appropriate density is then placed into a flow cytometry tube, adding 5μL. The fluid is mixed gently, adding 5μL of Annexin V-FITC undiluted solution and 10μL of Propidium iodide solution in iced water and incubating out of sunlight as a material for flow cytometry. Besides the materials, a tube sample with only 5μL of Annexin V-FITCT and 10μL of Propidium Iodide Solution added is prepared using -cell-suspension liquid as a reference value for sensitivity adjustment and fluorescence correction of flow cytometry. The materials are analyzed with a flow cytometry meter.

10. DNA Fragmentation Test

10-1 Performing Cells
3T3 Cells (mouse embryonic fibroblast cells)

10-2 Preparation of Sample Solution
Fluorescent Pigment, Color Pigment
The sample solution is made by adding 1mL of culture medium per 0.1g and extracting at 37°C for 24 hours.

Primer, UDMA, TEGDMA
Because it is difficult to dissolve them into culture medium for the primer, UDMA and TEGDMA are dissolved in DMSO in advance, with the density prepared as shown below.
Primer : 500μg/mL
UDMA : 286 (1) μg/mL (mM)
TEGDMA: 470 (1) μg/mL (mM)
The density of DMSO in the sample solution here is prepared at less than 1% without toxicity for cultured cells. For the sample solution, DMSO-diluted solution of the same density as diluted is used as a reference value.

Resin (Test Model)
After washing resin (test model) with tap water and distilled water for injection of the Japanese pharmacopeia (from HIKARI PHARMACEUTICAL CO., LTD.) and drying, ultraviolet sterilization is performed on both sides for 30 minutes on a clean bench. 1mL of culture medium is added to each 5 cm^2 of surface area of the sample and extracted to make sample solution in incubation at 5% CO_2 for 24 hours at 37°C. All of the sample solution is then sterilized with a 0.22μm filter and used for the test.

10-3 Method
Exfoliate 3T3cells (Chinese Hamster Lung) propagated in a single layer by trypsinization are exfoliated and 1×15 pieces/mL of cell suspension is prepared using culture medium. Cell suspension is prepared, 3mL is scattered on culture medium on a tissue culture plate and incubated for approximately 6 hours at 5% CO_2 at 37°C. After incubation, attachment of the cells to the bottom of the wells is confirmed and the culture medium is removed. 3mL of sample solution is added to each sample and incubated for 48 hours at 5% CO_2 at 37°C. Using a cell scraper, the culture cells are collected into an Eppendorf tube, with sedimentation for 5 minutes; 1mL of TORIZOL Reagent is then added to dissolve the cells. 0.2mL of chloroform is added to the cell lysate, it is stirred for 15 seconds, and sedimentation takes place for 15 minutes (4°C) at 12,000rpm; it is then left for 2-3 minutes at room temperature. Aspiration of clear supernatant is performed, 300μL of 100% ethanol is added to the rest of the suspension and it is left for 2-3 minutes; sedimentation is then performed for 5 minutes at 2,000rpm. After performing aspiration of the supernatant, 500μL of 0.1M sodium citrate (in 10% ethanol) is added, it is left for 30 minutes at room temperature, and sedimentation is performed for 5 minutes at 2,000rpm to re-perform aspiration of the sedimentation. 500μL of 0.1M sodium citrate (in 10% ethanol) is again added and aspiration performed on the supernatant also, adding 1mL of 75% ethanol. This is left for 20 minutes and DNA is extracted by performing 5-minute sedimentation at 2,000 rpm and aspiration of supernatant. The fixed quantity of DNA is calculated from 260mm absorptivity measured by adding 20μL of TE buffer after dissolving the DNA. Based on the fixed quantity result, DNA solution is collected and compounded with electrophoretic buffer (DNA Solution: Electrophoretic-6) to set the quantity at 0.05μg. SeaKem ME agarose is used as electrophoretic agarose gel, diluted with TAE buffer at 1.8%. Electrophoresis is performed for 1 hour with Mupid-2Plus at a constant voltage of 100V.

11. Protein Synthesis Test

11-1 Performing Cells
3T3 Cells (mouse embryonic fibroblast cells)

11-2 Preparation of Testing Liquid
Fluorescent Pigment, Color Pigment
The sample solution is made by adding 1mL of culture medium per 0.1g and extracting at 37°C for 24 hours.

Primer, UDMA, TEGDMA
Because it is difficult to dissolve them into culture medium for the primer, UDMA and TEGDMA are dissolved in DMSO in advance, with the density prepared as shown below.
Primer : 500μg/mL
UDMA : 286 (1) μg/mL (mM)
TEGDMA: 470 (1) μg/mL (mM)
The density of DMSO in the sample solution here is prepared at less than 1% without toxicity for cultured cells. For the sample solution, DMSO-diluted solution of the same density as diluted is used as a reference value.

Resin (Test Model)
After washing resin (test model) with tap water and distilled water for injection of the Japanese pharmacopeia (from HIKARI PHARMACEUTICAL CO., LTD.) and drying, ultraviolet sterilization is performed on both sides for 30 minutes on a clean bench. 1mL of culture medium is added to each 5 cm^2 of surface area of the sample and extracted to make sample solution in incubation at 5%CO_2 for 24 hours at 37°C. All of the sample solution is then sterilized with a 0.22μm filter and used for the test.

11-3 Method
As performed in DNA Synthesis Test, cell suspensions are prepared; 1mL is put in place and incubated for approximately 6 hours at 5% CO_2 at 37°C. After incubation, attachment of the cells to the bottom of the wells is confirmed and the culture medium is removed. 1mL of sample solution (100%) is added to each sample and incubated for 48 hours at 5% CO_2 at 37°C. After the incubation, the sample solution is collected from each well into an Eppendorf tube; sedimentation is performed for 5 minutes at 2,000rpm and aspiration of the supernatant is conducted. 5μL of 2-propanol solution (0.2mM) of Phenyl methyl sulfunyl fluoride and 100μL of mixed solution of 1mL TNE buffer are added, the content fluid of each original well is replaced; the samples are steeped in iced water for 15 minutes after thorough pipetting. The cells of each well are propagated by pipetting and collected into a tube, and sedimentation is then performed for 15 minutes at 12,000rpm, after which the supernatant is transferred into a tube. 2.5μL of the supernatant is compounded with 200μL of BCA Protein Assay Reagent (PIECE); absorptivity is measured at 570nm and protein determination is performed.

《 Results 》

1. Acute Toxicity Test

1-1 Incidence of Death
No incidence of death has been confirmed for either male or female during observation.

1-2 General Condition
No abnormality has been confirmed for either male or female during observation.

1-3 Weight Change
No weight change has been confirmed for either male or female or between control groups in any test group after either 7 or 14 days from administration.

1-4 Autopsy Findings
No abnormality has been confirmed in main organs for either male or female test animals at autopsy after the period of observation.

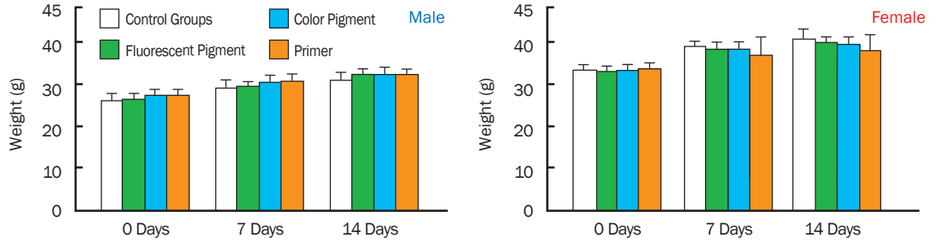


Fig. 1: Result of Acute Toxicity Test (Weight Change)

2. The Colony-Forming Assay

No reduction in the number of colonies at fluorescence pigment extraction at any density (Fig.2). No reduction in the colony-formation rates for negative control group sample solution against empty extract was confirmed at this time. For color pigment extract, a reduction in the number of colonies appeared with an increase in the sample solution density, and a 50% inhibitory density is approximately 39%. For primer, a reduction in the number of colonies appeared with an increase in the testing liquid density, but the value for IC50 did not increase.

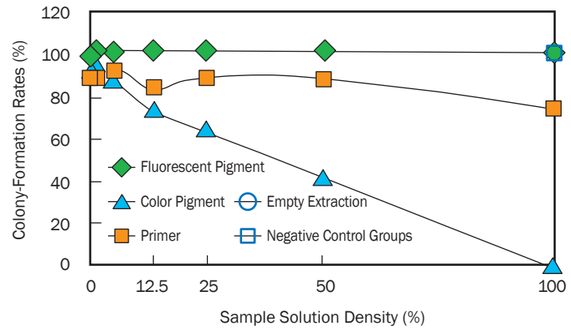


Fig. 2: Result of the Colony-Forming Assay

3. Reverse Mutation Test

No dose-dependent change and no more than a double increase or decrease in the number of reverse mutation colonies was confirmed compared to negative control groups for all samples.

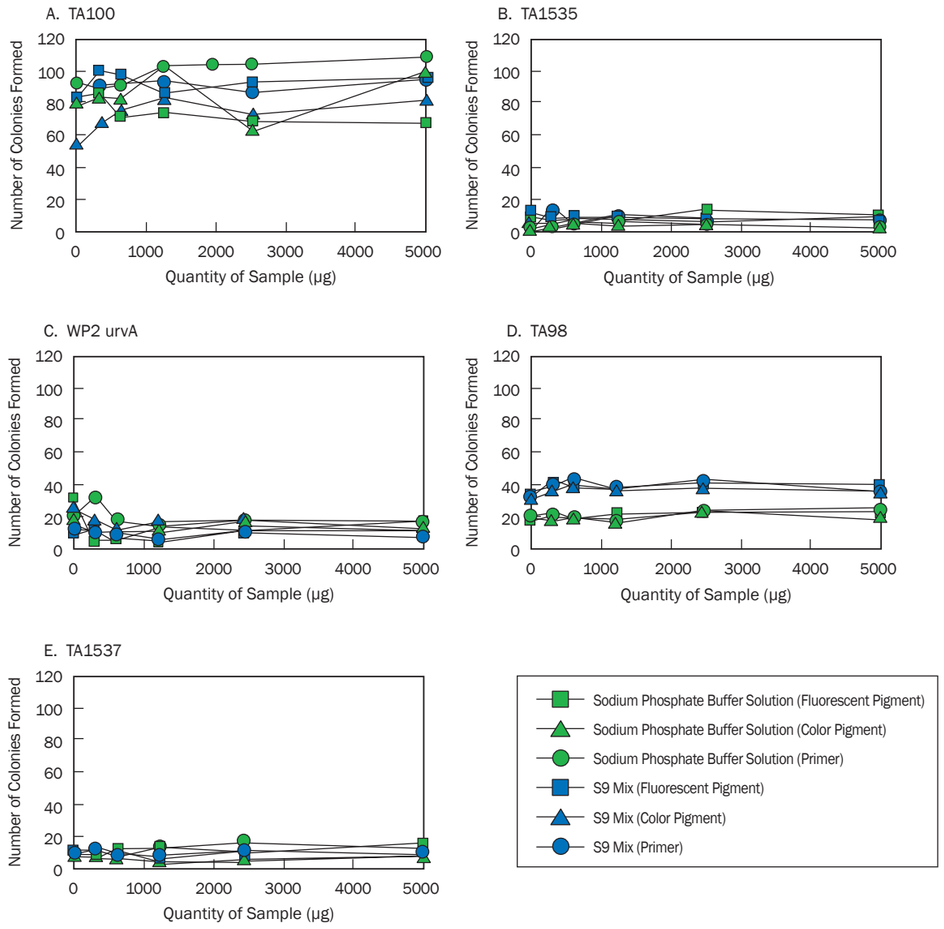


Fig. 3: Result of Reverse Mutation Test using Bacteria

4. Skin Sensitization Test

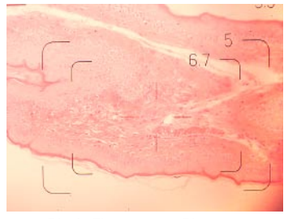
No skin reaction was confirmed on any application site 48 and 72 hours after application in test groups; sensitivity was 0% both 48 hours and 72 hours after induction (Table 3). For negative control groups, no skin reaction was confirmed on any application site both 48 and 72 hours after application in test groups. On the other hand, for positive control groups, clear red spots were confirmed within 24 hours from induction and medium to severe inflammation appeared within 48 hours; necrosis and scabbing were confirmed within 72 hours. Sensitivity was 100% at 24, 48, and 72 hours.

Table.3: Result of Skin-Sensitization Test

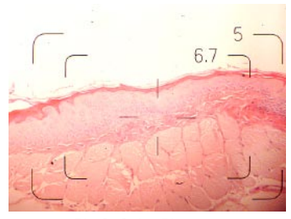
Test Group	The Number of Test Animals	Density	Observation Duration (hrs)	Positive Rate (%)
Fluorescent Pigment	9	10%	48	0
			72	0
		1%	48	0
			72	0
		0.1%	48	0
			72	0
Color Pigment	9	10%	48	0
			72	0
		1%	48	0
			72	0
		0.1%	48	0
			72	0
Primer	9	10%	48	0
			72	0
		1%	48	0
			72	0
		10%	48	0
			72	0
Negative Control Groups	9	10%	48	0
			72	0
		1%	48	0
			72	0
		0.1%	48	0
			72	0
		0%	48	0
			72	0
Positive Control Groups	6	0.1w/v %	24	100
			48	100
			72	100

5. Oral Mucosa Irritation Test

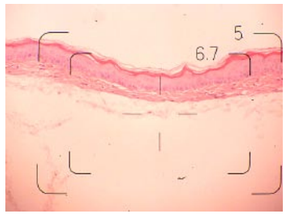
None of 1) Thickening of horny layers and epithelium2) Carcinogenesis 3) Infiltration of inflammatory cells and 4) Vasodilatation were confirmed in any of the samples. (Pic.1-3)



Pic.1: Fluorescent Pigment



Pic.2: Color Pigment



Pic.3: Primer

6. Cell Growth Inhibition Test

No cytostatic effect was confirmed in fluorescent pigment at the time of extraction. A slight reduction in viability was confirmed for resin (test model) 168 hours after extraction. A reduction in viability was confirmed for color pigment as time after extraction elapsed, and viability was reduced by 30% 168 hours after extraction. The time period after extraction during which viability reduced by 50% was ascertained at 95 hours on this occasion. No cytostatic effect was confirmed in primer at any sample solution density. In TEGDMA and UDMA, viability was reduced by 30% and 15% respectively.

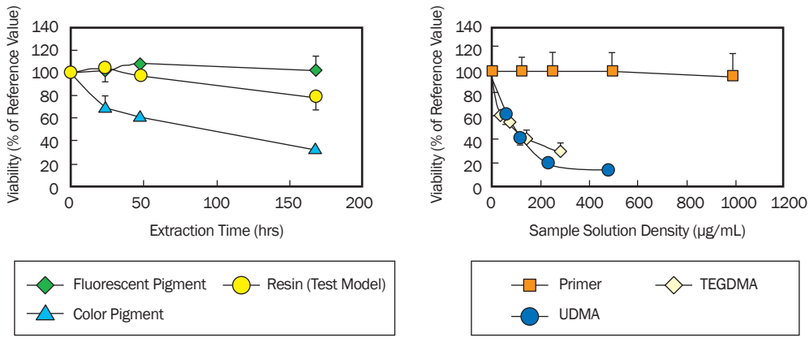


Fig. 4: Result of the *in vitro* Cytotoxicity Test

7. DNA Synthesis Test

Radiation activity volume of resin (test model) was the same as the reference value, but it increased by 78% in fluorescent pigment, reduced by 38% in color pigment, and increased by 23% and 98% in primer and UDMA and TEGDMA respectively.

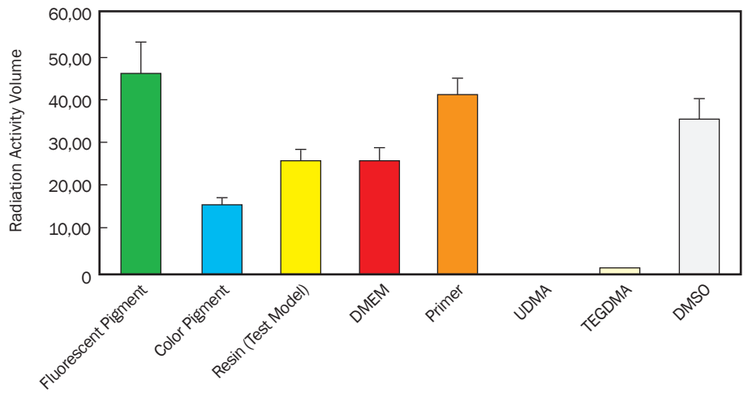


Fig. 5: Result of DNA Synthesis Test

8. Cytotoxicity Test Using Cultured Cells

No exceptional occurrence of cell death was confirmed in any of the samples.

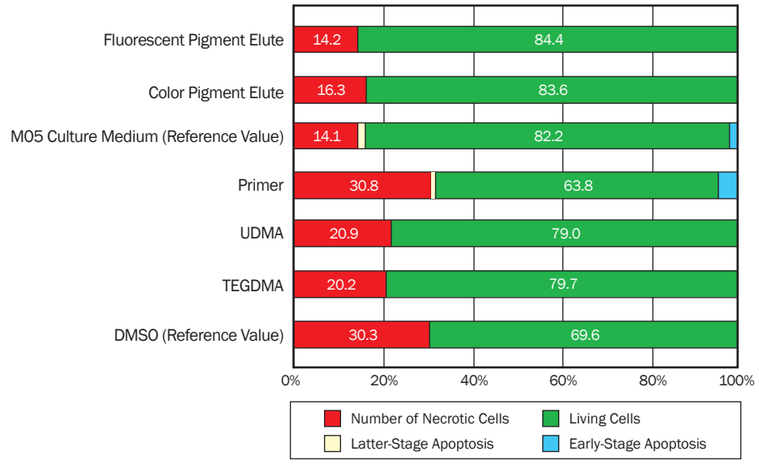
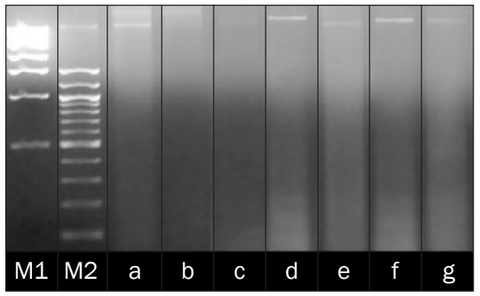


Fig. 6: Result of Cytotoxicity Test

9. DNA Fragmentation Test

DNA degradation was confirmed in all of the samples; however, the difference between reference values and specific DNA fragmentations in apoptosis were not measured (Pic. 4).



M1: Marker (1 kb DNA Ladder)
M2: Marker (100kb DNA Ladder)
a: Fluorescent Pigment
b: Color Pigment
c: Resin Test Model
d: Reference Value
e: Primer
f : UDMA
g: Reference Value of Test Primer Material

Pic. 4: DNA Fragmentation Test

10. Protein Synthesis Test

No reduction of the quantity of protein synthesis in fluorescent pigment and resin (test model) was confirmed compared to the reference value (Fig.7). However, it reduced by 23% in color pigment compared to the reference value. Also, it reduced by 6% in primer compared to the reference value. It reduced by 52% and 25% in UDMA and TEGDMA respectively.

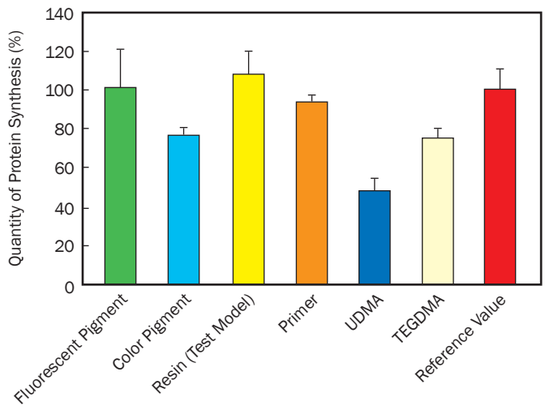


Fig. 7: Result of Protein Synthesis Test

《 Discussion 》

1. Acute Toxicity

Since no weight change in mice and no shape changes in organs were confirmed in any sample, it becomes clear that there is no acute toxicity in the substance.

2. Cytotoxicity

There are some reports of cytotoxicity for UDMA and TEGDMA in biological evaluation reports of resin materials. According to the reports, the value of IC50 is 0.12-0.26mM and 0.06-0.47mM respectively. In the fluorescent pigment, color pigment, resin (test model), prime, UDMA, and TEGDMA used in the test, color pigment in particular showed cytostatic effect. It is considered that such cytostatic effect is caused either by cell death or delay in progress of the cell cycle. To clarify these possibilities, a cytotoxicity test to observe whether or not there is apoptosis and necrosis was performed; the test results confirmed neither. From this fact, it is considered that colony-forming inhibitory action is caused not because cell death is caused by color pigment but because the cell cycle influences and delays it in some manner. In addition, it is reported that while cytostatic effect was shown particularly on uncured materials, almost no cytostatic effect was shown on cured materials, according to the result of the in vitro cytotoxicity test on resin materials.13) The resin (Test Model) used this time was cured and hardened as a test material. As a result, it is considered to have no impact on safety for color pigment on which cytostatic effect was confirmed in the test from the fact that no abnormality was confirmed in any in vitro cytotoxicity test; the same result as in the biological evaluation reports of resin materials.

3. Genetic Toxicity

In the reverse mutation test using bacteria, no increase or decrease in the number of reverse mutation colonies was confirmed compared to the negative control group for any of the samples. Therefore, mutation of the sample is considered to be negative and there is no special risk of cancer being caused by DNA mutation under the test conditions.

4. Skin Sensitization

In the result of the skin sensitization test using guinea pigs, no skin reaction was confirmed on any application site either 48 or 72 hours after application in test groups, and sensitivity was 0%. Comparing these testing results with the result of negative control groups and positive control groups, no skin reaction was confirmed on any application site in negative control groups, and sensitivity was 0% at both 48 hours and 72 hours; therefore, the substances used in this test have no risk of causing skin sensitization.

5. Oral Mucosa Irritation

No irritation response in any of the samples and solvent control cheek pouches was confirmed in observation with the naked eye. In histopathological observation, no irritation response was confirmed in any of the samples of fluorescent pigment, primer, and solvent control cheek pouches. In the color pigment, thickening of horny layers was confirmed in 8 out of 15 of the cheek pouches for the test sample. However, the affected areas were topical and very weak. Therefore, the substances used in this test are considered to have no irritation risk for oral mucosa of hamsters.

《 Conclusion 》

In the result of the in vitro cytotoxicity test, color pigment mainly showed cytostatic effect. However, neither apoptosis nor necrosis appeared in the cytotoxicity test. From these facts, the cytostatic effects observed in the colony-forming assay and the cell growth inhibition test are considered to be caused by delay in progress of the cell cycle, not by induction of cell death in color pigment.
In the reverse mutation test using bacteria, no colony formation was confirmed; it is clear that DNA mutation of bacteria is not induced. Therefore, there is considered to be no risk of incidence of disease related to DNA mutation, such as cancer.
In the skin sensitization test using guinea pigs, no sensitization was confirmed. From this fact, the substances used in the test are considered to have risk which is lower than the standard level for sensitization.

These tests were conducted in collaboration with the Oral Tumor Control Science, Tumor Pathology Course of Kochi Medical School, Kochi University.

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