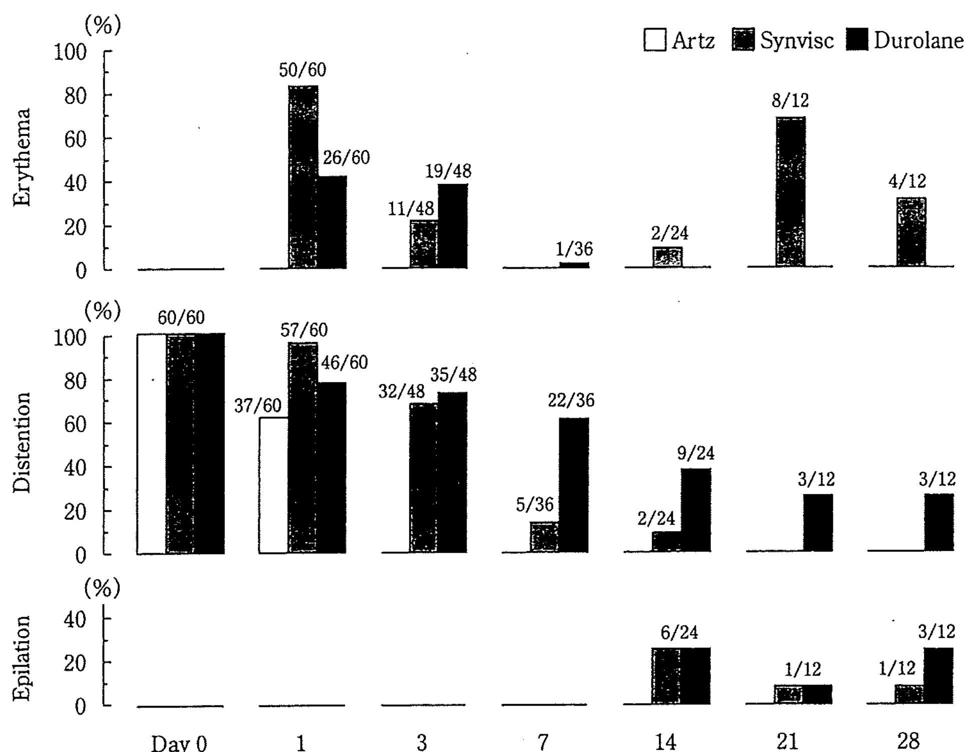

Fig. 1 Skin reaction at the sites injected with Artz®, Synvisc® or Durolane®
Incidence of skin reaction is shown.
Day 0, day of injection. 1～28, the next day of ～ the 28th day from injection.
Numerator, sites with reaction; Denominator, sites examined.

ed animals. Moreover, induction of PCA was also done using Artz® in 2 animals to examine if there was cross reaction between the 2 HA products.

Results

1 General condition, body weight and skin reaction

Throughout the study there were no death or abnormal signs that were attributable to the injection of HA products or saline. Moreover, the animals of all the groups showed normal body weight gain. Skin reactions found after the injection of HA products or saline are represented in **Fig. 1**. More than half of the sites showed distention by Artz® injection, but it disappeared at the 2nd day from the injection. Synvisc® also caused dermal distention at more than half of the sites injected for 4 days after injection. The incidence of distention went down and the reaction became weaker at the 5th day or later, and disappeared at the 15th day. On the other hand, erythema appeared at most of the sites injected with Synvisc® from the next day of the injection. This reaction attenuated gradually and disappeared at the 6th day. At the 14th day, however, erythema recurred in one/six animals (2 sites) and was found continually in one or two/three animals (4 to 8 sites) from the 15th day until the 28th day. Moreover, epilation was observed at the sites injected in a few animals from the 8th day until the 28th day. In the Durolane® group dermal distention was observed at more than half of the sites injected. The incidence tended to decrease, but the distention was continuously observed until the 28th day. Erythema was also found in the Durolane® group on the next day of the injection, but was at less than half of the sites injected, and was found weakened from the 6th day and disap-

Table 2 Skin reaction at the sites injected with hyarulonan preparations at the 1st day of observation

	Skin reaction	Grading	−	±	+	++	+++
Artz®	Epidermis						
	Focal erosion		11/12	−	1/12	−	−
	Neutrophil infiltration		11/12	1/12	−	−	−
	Dermis						
	Edema		2/12	2/12	8/12	−	−
	Alcian blue-stained fine granular material		1/12	11/12	−	−	−
	Subcutis						
	Edema		−	4/12	8/12	−	−
	Alcian blue-stained fine granular material		2/12	10/12	−	−	−
	Neutrophil infiltration		8/12	4/12	−	−	−
	Plasma cell infiltration		1/12	11/12	−	−	−
	Histiocyte infiltration		3/12	9/12	−	−	−
		Grading	−	±	+	++	+++
Synvisc®	Epidermis						
	Focal erosion		9/12	−	3/12	−	−
	Neutrophil infiltration		9/12	3/12	−	−	−
	Dermis						
	Edema		1/12	−	9/12	2/12	−
	Alcian blue-stained cluster shaped material		1/12	−	3/12	8/12	−
	Alcian blue-stained fine granular material		−	1/12	9/12	2/12	−
	Subcutis						
	Edema		1/12	1/12	10/12	−	−
	Alcian blue-stained cluster shaped material		1/12	1/12	9/12	1/12	−
	Alcian blue-stained fine granular material		−	2/12	10/12	−	−
	Neutrophil infiltration		1/12	10/12	1/12	−	−
	Plasma cell infiltration		1/12	11/12	−	−	−
	Eosinophil infiltration		3/12	9/12	−	−	−
	Histiocyte infiltration		1/12	2/12	9/12	−	−
		Grading	−	±	+	++	+++
Durolane®	Epidermis						
	None	None					
	Dermis						
	Edema		5/12	7/12	−	−	−
	Alcian blue-stained cluster shaped material		1/12	−	−	−	11/12
	Alcian blue-stained fine granular material		1/12	11/12	−	−	−
	Subcutis						
	Edema		9/12	−	3/12	−	−
	Alcian blue-stained cluster shaped material		1/12	−	11/12	−	−
	Alcian blue-stained fine granular material		1/12	11/12	−	−	−
	Neutrophil infiltration		1/12	11/12	−	−	−
	Plasma cell infiltration		1/12	11/12	−	−	−
	Eosinophil infiltration		8/12	4/12	−	−	−
	Histiocyte infiltration		1/12	11/12	−	−	−

Three guinea pigs injected a hyarulonan preparation at 4 sites on the back for each were killed on the next day of injection.
Incidences of skin reactions are shown. Numerator, sites with reaction ; Denominator, sites examined
Grading : −, negative ; ±, very slight ; +, slight ; ++, moderate ; +++, severe

peared at the 8th day. Also after the injection of saline skin distention was observed immediately, but it disappeared on the next day. Throughout the experiment no other changes were observed in the sites.

2 Histological examination

The specimens of the sites injected with Artz®, obtained from the animals killed on the next day of injection, showed edema in the dermis and the subcutis, and infiltration of neutrophils, plasma cells and histiocytes (**Table 2**). These changes were weakened time-dependently at the 3rd day after injection or later, and disappeared at the 28th day (**Table 3-6**). Besides, there were focal erosion, crust formation and infiltration of neutrophils in the epidermis, and microgranuloma in the dermis. In the specimens treated with Alcian blue, blue-stained materials were found in the dermis and subcutis around the sites injected, though the tinction was light (**Photo. 1a**). The stained materials disappeared by hyaluronidase treatment in advance. Alcian blue-stained materials were observed until the 7th day after the injection. At 28 days after injection almost no influence of injection was observed.

In the sites injected with Synvisc® Alcian blue-stained materials were found in the dermis and subcutis (**Table 2**). Edema was found in the dermis and subcutis, and the latter had infiltration of neutrophils, plasma cells, histiocytes and eosinophils. These changes became not evident at the 3rd day or later (**Table 3-6**). However, infiltration of inflammatory cells, predominantly with histiocytes and eosinophils, which were scored from very slight to moderate, reappeared at the 14th day in the dermis and subcutis (**Table 5**). Moreover, a few foreign body giant cells were also found (**Table 4-6**). Fibroconnective tissue proliferated surrounding the cluster was observed at the 14th and the 28th days after the administration (**Table 5, 6**). At the 28th day the incidence of infiltration of histiocytes and eosinophils increased. In addition, focal erosion, crust and neutrophil infiltration in the epidermis, and microgranuloma in the cutes were observed in the small number of the sites. Cluster-shaped materials and granules were found stained with Alcian blue in the dermis and subcutis around the sites injected (**Photo. 1b**). The blue tinction disappeared by pretreatment with hyarulonidase. The clustered materials in the dermis became vague and granular materials disappeared at the 28th day.

At the sites injected with Durolane® Alcian blue-stained materials were found in the dermis and subcutis on the next day after the injection (**Table 2**). Surrounding the sites injected, there were very slight or slight edema in the dermis, infiltration of neutrophils, plasma cells, histiocytes and eosinophils in the subcutis. The degrees of these changes became weaker at the 3rd day or later (**Table 3-6**). At the 7th day or later, infiltration of fibroblasts and proliferation of fibroconnective tissue were found (**Table 4-6**). At the 14th day and the 28th day after injection slight or moderate fibrous capsulation around the cluster shaped materials was found in the subcutis at a few sites (**Table 5, 6**). Besides, focal erosion, ulcer, infiltration of neutrophils and hyperplasia of squamous cells were found in the epidermis at a few sites injected. Similar to the sites injected with Synvisc®, cluster and fine granulous materials were stained with Alcian blue (**Photo. 1c**) and they could not be found by pretreatment with hyaluronidase. Alcian blue-stained materials were found until the 28th day.

In the sites injected with saline very slight or slight focal erosion, crust in the epidermis, edema and microgranulomas in the dermis, and hemorrhage, infiltration of histiocytes and eosinophils in the subcutis were observed in a few sites at the 1st and the 3rd days.

3 PCA reaction

Intravenous, simultaneous injection of Artz® or Durolane® with Evans blue caused no positive reaction at the sites injected in advance with sera from any animals given Artz® or Durolane®, respectively (**Photo. 2a, 2b**). In contrast, positive reaction was found after intravenous injection with Synvisc® and the dye at the sites injected with

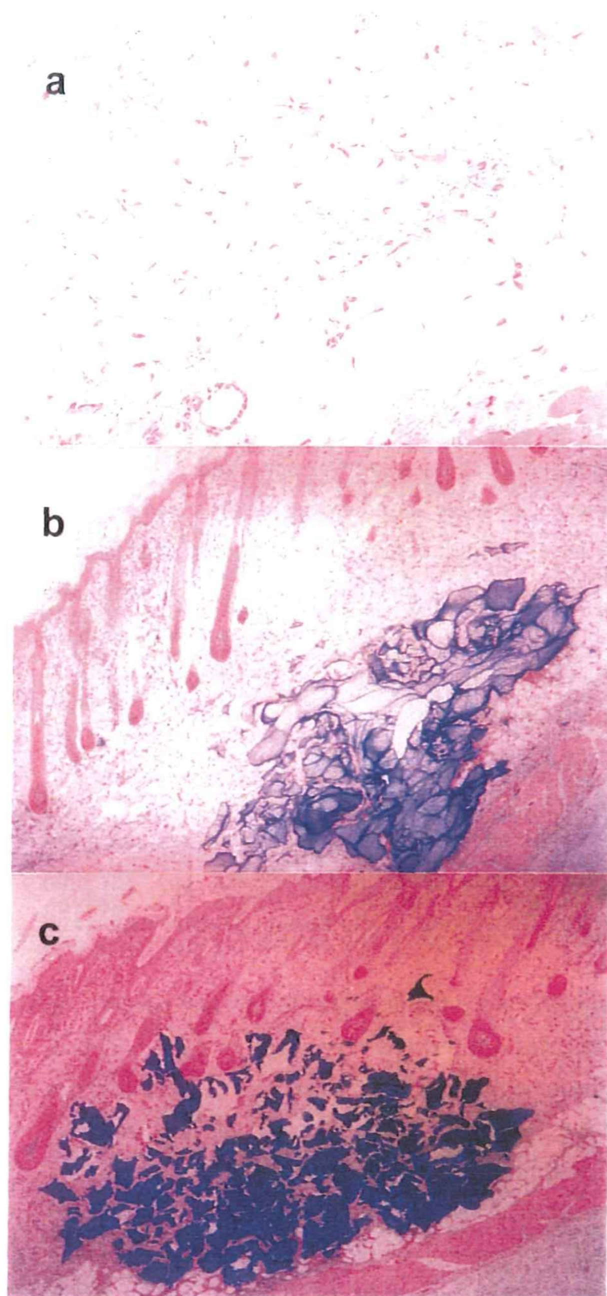

Photo. 1 Microphotographs of guinea pig skin injected with HA products
Artz® (a, × 170), Synvisc® (b, × 35) and Durolane® (c, × 35) at the 3rd day from the injection. Alcian blue-stained materials were observed.

sera from the animals of the Synvisc® group that were sacrificed at 28 days after the injection (**Photo. 2c**). Sera from the animals given Synvisc®, sacrificed at 1, 3, 7 and 14 days after the injection did not cause any reaction (data not shown). PCA positive sera showed a dilution-dependent decline of the reaction (**Photo. 3a**). Endpoint titer was 1 : 9, 1 : 9 or 1 : 27 for each serum obtained from 3 animals. In addition, intravenous injection with Artz® and Evans blue did not cause any reaction at the sites injected in advance with Synvisc®, indicating that there was no cross-over reaction (**Photo. 3b**).

Table 3 Skin reaction at the sites injected with hyarulonan preparations at the 3rd day of observation

	Skin reaction	Grading	−	±	+	++	+++
Artz®	Epidermis						
	Crust		10/12	2/12	−	−	−
	Dermis						
	Alcian blue-stained fine granular material		−	12/12	−	−	−
	Subcutis						
	Alcian blue-stained fine granular material		1/12	11/12	−	−	−
	Neutrophil infiltration		8/12	4/12	−	−	−
	Eosinophil infiltration		11/12	1/12	−	−	−
	Histiocyte infiltration		−	12/12	−	−	−
		Grading	−	±	+	++	+++
Synvisc®	Epidermis						
	Focal erosion		11/12	1/12	−	−	−
	Crust		11/12	1/12	−	−	−
	Neutrophil infiltration		10/12	2/12	−	−	−
	Dermis						
	Alcian blue-stained cluster shaped material		−	−	2/12	10/12	−
	Alcian blue-stained fine granular material		−	−	12/12	−	−
	Subcutis						
	Edema		10/12	2/12	−	−	−
	Alcian blue−stained cluster shaped material		−	−	12/12	−	−
	Alcian blue−stained fine granular material		−	−	12/12	−	−
	Neutrophil infiltration		10/12	2/12	−	−	−
	Eosinophil infiltration		11/12	1/12	−	−	−
	Histiocyte infiltration		−	1/12	11/12	−	−
	Hemorrhage		11/12	1/12	−	−	−
		Grading	−	±	+	++	+++
Durolane®	Epidermis						
	None	None					
	Dermis						
	Alcian blue-stained cluster shaped material		−	−	−	11/12	1/12
	Alcian blue-stained fine granular material		−	12/12	−	−	−
	Subcutis						
	Alcian blue-stained cluster shaped material		−	2/12	10/12	−	−
	Alcian blue-stained fine granular material		−	11/12	1/12	−	−
	Neutrophil infiltration		−	12/12	−	−	−
	Eosinophil infiltration		10/12	2/12	−	−	−
	Histiocyte infiltration		−	7/12	5/12	−	−
	Hemorrhage		10/12	2/12	−	−	−

Three guinea pigs injected a hyarulonan preparation at 4 sites on the back for each were killed at the 3rd day after injection.
Incidences of skin reactions are shown. Numerator, sites with reaction ; Denominator, sites examined
Grading : −, negative ; ±, very slight ; +, slight ; ++, moderate ; +++, severe

Table 4 Skin reaction at the sites injected with hyarulonan preparations at the 7th day of observation

	Skin reaction	Grading	−	±	+	++	+++
Artz®	Epidermis						
	None	None					
	Dermis						
	Alcian blue-stained fine granular material		−	12/12	−	−	−
	Histiocyte infiltration		11/12	1/12	−	−	−
	Subcutis						
	Alcian blue-stained fine granular material		1/12	11/12	−	−	−
	Histiocyte infiltration		10/12	2/12	−	−	−
		Grading	−	±	+	++	+++
Synvisc®	Epidermis						
	Crust		10/12	2/12	−	−	−
	Dermis						
	Alcian blue-stained cluster shaped material		−	1/12	4/12	7/12	−
	Alcian blue-stained fine granular material		2/12	7/12	3/12	−	−
	Histiocyte infiltration		−	12/12	−	−	−
	Subcutis						
	Alcian blue-stained cluster shaped material		−	2/12	10/12	−	−
	Alcian blue-stained fine granular material		2/12	8/12	2/12	−	−
	Eosinophil infiltration		10/12	2/12	−	−	−
	Histiocyte infiltration		−	5/12	7/12	−	−
	Foreign body giant cell		11/12	1/12	−	−	−
		Grading	−	±	+	++	+++
Durolane®	Epidermis						
	Focal erosion		11/12	1/12	−	−	−
	Dermis						
	Alcian blue-stained cluster shaped material		1/12	−	1/12	10/12	−
	Alcian blue-stained fine granular material		3/12	9/12	−	−	−
	Histiocyte infiltration		2/12	−	10/12	−	−
	Subcutis						
	Alcian blue-stained cluster shaped material		2/12	−	3/12	4/12	3/12
	Alcian blue-stained fine granular material		3/12	6/12	3/12	−	−
	Fibrous capsulation around cluster		4/12	−	8/12	−	−
	Eosinophil infiltration		11/12	1/12	−	−	−
	Histiocyte infiltration		1/12	9/12	2/12	−	−
	Fibroblast proliferation		4/12	3/12	3/12	2/12	−

Three guinea pigs injected a hyarulonan preparation at 4 sites on the back for each were killed at the 7th day after injection.
Incidences of skin reactions are shown. Numerator, sites with reaction ; Denominator, sites examined
Grading : −, negative ; ±, very slight ; +, slight ; ++, moderate ; +++, severe

Discussion

Since HA molecules are ubiquitously distributed in animal tissues for biological homeostasis[19, 20], purified HA products used in the present study were expected to have no serious acute adverse effects following intracutaneous injection in guinea pigs. Throughout the observation period, as expected, there were no death, abnormal changes in the general condition nor influence on body weight gain. Thus, the intracutaneous injection of the HA solutions was

Table 5　Skin reaction at the sites injected with hyarulonan preparations at the 14th day of observation

	Skin reaction	Grading −	±	+	++	+++
	Epidermis					
	Crust	11/12	1/12	–	–	–
	Dermis					
Artz®	Histiocyte infiltration	10/12	2/12	–	–	–
	Microgranuloma	11/12	1/12	–	–	–
	Subcutis					
	Nutrophil infiltration	11/12	1/12	–	–	–
	Histiocyte infiltration	11/12	1/12	–	–	–
	Grading	−	±	+	++	+++
	Epidermis					
	Focal erosion	11/12	–	1/12	–	–
	Crust	11/12	1/12	–	–	–
	Dermis					
	Alcian blue-stained cluster shaped material	1/12	–	3/12	8/12	–
	Histiocyte infiltration	4/12	5/12	–	3/12	–
	Eosinophil infiltration	7/12	2/12	1/12	2/12	–
	Foreign body giant cell	6/12	6/12	–	–	–
	Microgranuloma	11/12	–	1/12	–	–
Synvisc®	Subcutis					
	Alcian blue-stained cluster shaped material	–	–	11/12	1/12	–
	Alcian blue-stained fine granular material	–	12/12	–	–	–
	Nutrophil infiltration	10/12	2/12	–	–	–
	Eosinophil infiltration	4/12	3/12	5/12	–	–
	Histiocyte infiltration	1/12	3/12	3/12	5/12	–
	Foreign body giant cell	7/12	5/12	–	–	–
	Fibroconnective tissue proliferation	6/12	5/12	1/12	–	–
	Grading	−	±	+	++	+++
	Epidermis					
	Focal erosion	11/12	–	1/12	–	–
	Dermis					
	Alcian blue-stained cluster shaped material	1/12	–	2/12	9/12	–
	Histiocyte infiltration	5/12	7/12	–	–	–
	Foreign body giant cell	11/12	1/12	–	–	–
Durolane®	Subcutis					
	Alcian blue-stained cluster shaped material	5/12	–	–	1/12	6/12
	Alcian blue-stained fine granular material	6/12	6/12	–	–	–
	Fibrous capsulation around cluster	6/12	–	1/12	5/12	–
	Histiocyte infiltration	6/12	6/12	–	–	–
	Fibroconnective tissue proliferation	1/12	6/12	5/12	–	–

Three guinea pigs injected a hyarulonan preparation at 4 sites on the back for each were killed at the 14th day after injection.
Incidences of skin reactions are shown. Numerator, sites with reaction；Denominator, sites examined
Grading：−, negative；±, very slight；+, slight；++, moderate；+++, severe

Table 6　Skin reaction at the sites injected with hyarulonan preparations at the 28th day of observation

	Skin reaction	Grading	−	±	+	++	+++
Artz®	Epidermis						
	None	None					
	Dermis						
	None	None					
	Subcutis						
	None	None					
		Grading	−	±	+	++	+++
Synvisc®	Epidermis						
	None	None					
	Dermis						
	Alcian blue-stained cluster shaped material		−	2/12	2/12	8/12	−
	Histiocyte infiltration		1/12	4/12	2/12	5/12	−
	Eosinophil infiltration		−	5/12	3/12	4/12	−
	Foreign body giant cell		9/12	3/12	−	−	−
	Fibroconnective tissue proliferation		5/12	6/12	1/12	−	−
	Subcutis						
	Alcian blue-stained cluster shaped material		−	4/12	8/12	−	−
	Eosinophil infiltration		−	3/12	5/12	4/12	−
	Histiocyte infiltration		−	3/12	4/12	5/12	−
	Fibroconnective tissue proliferation		3/12	7/12	2/12	−	−
		Grading	−	±	+	++	+++
Durolane®	Epidermis						
	Ulcer		11/12	−	1/12	−	−
	Neutrophil infiltration		11/12	−	−	1/12	−
	Squamous cell hyperplasia		11/12	−	1/12	−	−
	Dermis						
	Alcian blue-stained cluster shaped material		1/12	2/12	1/12	7/12	1/12
	Histiocyte infiltration		2/12	10/12	−	−	−
	Fibroconnective tissue proliferation		3/12	4/12	5/12	−	−
	Subcutis						
	Alcian blue-stained cluster shaped material		7/12	2/12	−	1/12	2/12
	Fibrous capsulation around cluster		8/12	−	4/12	−	−
	Histiocyte infiltration		1/12	9/12	2/12	−	−
	Fibroconnective tissue proliferation		2/12	4/12	6/12	−	−

Three guinea pigs injected a hyarulonan preparation at 4 sites on the back for each were killed at the 28th day after injection.
Incidence of skin reaction are shown. Numerator, sites with reaction；Denominator, sites examined
Grading：−, negative；±, very slight；+, slight；++, moderate；+++, severe

confirmed to have no systemic effects in guinea pigs.

While skin distention was observed immediately after injecting any of solutions including saline, the persistence time appeared to depend on absorptivity of each solution injected. Skin distention at the site injected with saline disappeared on the next day of injection, but remained longer at the sites injected with any of HA products. Particularly, Synvisc® and Durolane® induced a longer persisted distention, which remained evident until the 14th day and the 28th day, respectively, and polysaccharide was confirmed by Alcian blue staining even at the 28th day for both products. In contrast Artz®-induced distention disappeared on the 2nd day and Alcian blue-stained materi-

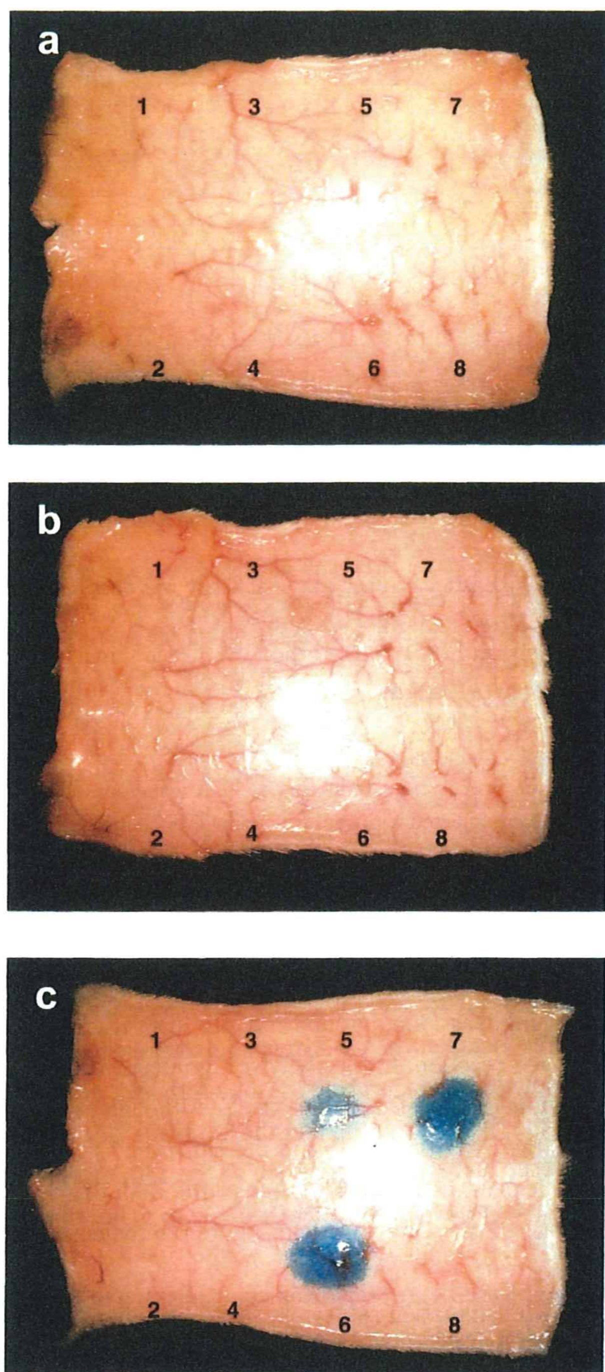

Photo. 2 PCA reactions induced by intravenous injection of Artz®, Durolane® or Synvisc®

a PCA reactions induced by intravenous injection of 1 mg Artz®
A representative preparation. 1–7 sites are injected with sera from individual animals given intracutaneous Artz® in the skin reaction study. 1, injected with serum at 7th day ; 2–4, at 14th day ; 5–7, at 28th day ; 8, saline.

b PCA reactions induced by intravenous injection of 1 mg Durolane®
A representative preparation. 1–8 sites are injected with sera from individual animals given intracutaneous Durolane® in the skin reaction study. 1, injected with sera at 7th day ; 2–4, sera at 14th day ; 5–7, sera at 28th day ; 8, saline.

c PCA reactions induced by intravenous injection of 1 mg Synvisc®
A representative preparation. 1–7 sites are injected with sera from individual animals given intra-cutaneous Synvisc® in the skin reaction study. 1, injected with sera at 7th day ; 2–4, at 14th day ; 5–7, at 28th day ; 8, saline.

als could not be found at the 14th day. The persistent skin distention by Synvisc® and Durolane® was thought to be due to increased molecular weight with the artificially given cross-linking.

Although the long residence time of HA products with the cross-linked large molecules has a certain benefit for clinical use, Synvisc®, one of such HA products has been reported to cause acute local inflammation[11,21], gout[12,22], recurrent calcium pyrophosphate dihydrate arthritis or pseudogout[13~15]. Also in the present study, erythema was found in the Synvisc® group and the Durolane® group for 7 days and for 5 days after injection, respectively, whereas such a reaction was not found in the Artz® group. The skin reaction may be due to foreign body reaction against HA products with a longer residence time. Besides, physical stimulation is also a possible cause for

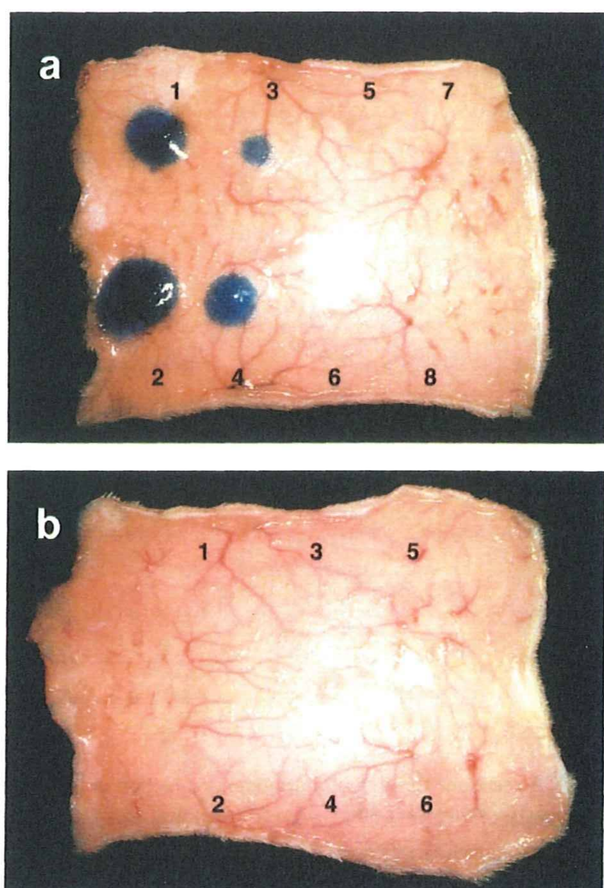

Photo. 3　Determination of titer unit and examination for cross reaction

a　Determination of titer unit by PCA reactions induced by 1 mg of intravenous injection of Synvisc®
A representative preparation. Titer unit of antibody was determined sera from the Synvisc®-treated, PCA positive animals. 1, 3, 5 and 7 sites are injected with 1/3, 1/9, 1/27 and 1/81 diluted serum from one animal given intracutaneous Synvisc® in the skin reaction study. 2, 4, 6 and 8 sites are injected diluted serum from another animal.

b　Examination for cross reaction between Artz® and Synvisc®
A representative preparation. Induction of PCA was done using Artz®. 1-6 sites are injected with sera from individual animals given intracutaneous Synvisc® in the skin reaction study. 1, 3 and 5 sites are injected with sera at 14th day and 2, 4 and 6 sites are with sera at 28th day. No cross reaction was found.

such a topical reaction, because epilation probably by repeated scraping was found around the site distended. An interesting finding in the present study was Synvisc®-induced delayed reaction. In the Synvisc® group erythema recurred on the 14th day and later, but such a recurring could not be found in the Durolane® group. Although cellular infiltration and proliferated connective tissue were found in both, the Synvisc® group and the Durolane® group in histological examination, the degree of reactions tended to be evident in the Synvisc® group at the 28th day as compared with the Durolane® group. Thus it is indicated that the delayed reaction was not attributed to molecular size or existence of cross-linking, but to a specific property to Synvisc®.

Fragments of HA molecules had been thought as a trigger for the inflammation responses after injection of HA products, since fragments of native HA were found in the synovial fluid with decreased elastoviscosity in the knee of inflammatory diseases[1,2,23,24]. However, recently it has been reported that a certain HA product and its fragments equally induced the production by human monocytic cells of pro-inflammatory cytokines, IL-2 and TNF α, and treatment with DNase attenuated the production of cytokines[25]. Thus, not HA molecules but substances contaminated in HA products such as DNA may give rise to inflammation responses. Although the purified HA of animal origin contains a small amount of protein, a previous report demonstrated no immunogenicity of purified HAs from chicken comb by passive cutaneous anaphylaxis test using rabbits[26]. Recently, in contrast, immunogenicity has been reported for Synvisc® using rabbits and guinea pigs[17,27]. That is, a product from chicken comb HA without cross-linking, Hyalgan® did not induce immunogenic responses, while Synvisc® elicited significant titer to chicken protein[27]. The authors of the study pointed out an immunogenic role of residual proteins in cross-linked molecules of Synvisc®. With regard to residual proteins, nucleic acids and bacterial endotoxins, a higher level of contamination

in Synvisc® than purified intact HA products including Artz® has been demonstrated[28]. Moreover, it has been reported that Synvisc® revealed immunogenic responses in acute cutaneous anaphylaxis and delayed type hypersensitivity assays in guinea pigs, whereas Artz® showed no immunogenicity[17].

Although Durolane® and Synvisc® are both cross-linked HA with long residence time in the tissues, not the former, a biosynthesized HA but the latter, a purified chicken comb HA showed relapsing erythema in the present study. Since Synvisc® has been reported to be potentially immunogenic, we performed PCA test using sera obtained from the Synvisc® group and the Artz® group that showed no inflammatory reactions. Sera from all the 3 animals of the Synvisc® group which killed at the 28th day caused positive cutaneous reactions with endpoint titer of 1：9～1：27, though Artz® revealed no reactions or cross reactivity with Synvisc®. Thus, it is concluded that Synvisc® is suspected to exhibit immunogenicity which might be due to a very small amount of contaminated substances such as proteins and peptides of chicken origin[17, 27], or co-existence of contaminated substance such as a protein and endotoxin[28]. By recent analysis of Toll-like receptor functions, it has been indicated that an activation of innate immunity is a prerequisite to induction of acquired immunity[29]. Furthermore the long residence time seems to provide an opportunity for eliciting immune responses.

In conclusion, Synvisc® and Durolane® both of which have molecular cross-linking resided longer in the tissue than Artz®, a HA of natural molecules without cross-linking. Both Synvisc® and Durolane® caused erythema while Artz® did not. Moreover, Synvisc® exhibited the delayed skin reaction probably due to its immunogenicity that was confirmed by the elicited titer in PCA test using sera from Synvisc®-treated animals. Although cross-linked HA products have an advantage of longer residence time, persistent inflammation-related symptoms due to immunogenicity should be considered, because artificial cross-linking appears to give difficulty in refining the products and immunogenic residues prone to remain in the molecules[28]. In contrast natural molecule HA products appear far less inflammatogenic, though its residence time is somewhat shorter.

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Validation Activities in Japan: A Report

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Summary — Japanese activities in the validation of alternative test methods are reviewed. The validation of the *Limulus* endotoxin test as an alternative method to the rabbit pyrogen test was initiated by the Japanese Pharmacopoeia Society, and was successfully conducted under the direction of the National Institute of Health Sciences (NIHS), with the participation of many pharmaceutical company laboratories. The validation of *in vitro* alternatives to the Draize eye test for cosmetics were carried out by the NIHS and cosmetic company laboratories. Research on alternatives in Japan has been promoted by the Japanese Society of Alternatives to Animal Experiments (JSAAE) since 1989. Validation studies on alternatives were also attempted by the JSAAE. Interlaboratory reproducibility studies were carried out by the JSAAE on five different cytotoxicity tests. The JSAAE is planning the validation of *in vitro* alternatives to a local irritation test for intramuscular injection. Cell transformation assays have been carried out in Japan as adjuncts to the *in vivo* carcinogenicity test.

Key words: *cell transformation, endotoxin test,* in vitro *tests, validation.*

Introduction

A previous report on the validation activities of alternative test methods in Japan was made at the First World Congress on Alternatives, held in Baltimore in 1993 (1). In the years since then, several international collaborative validation studies on a large scale have been organised, and some Japanese laboratories have participated. These studies have resulted in the acceptance of the alternative methods in regulatory procedures in a few cases. Established alternatives are limited to those for an acute oral toxicity test, a phototoxicity test and a skin corrosivity test. However, the development of alternatives to other kinds of animal tests for toxicology has not reached full-scale interlaboratory validation. Criteria for validation and regulatory acceptance were established, and intensive efforts for validation of alternatives have been taken by specialised organisations such as the European Centre for the Validation of Alternative Methods (ECVAM) and the (US) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Though we have no such comparable body for validation under governmental auspices in Japan, some alternative research activities have been carried out in Japan.

Research on alternatives to animal experiments in Japan started much later than in Western countries. The Japanese Society of Alternatives to Animal Experiments (JSAAE) was established in 1989. It has now enrolled some 300 scientist members, who are interested in alternative experimental methods to animal experiments. An annual meeting is held for scientific communication, and its official journal, *Alternatives to Animal Tests and Experiments*, is published.

Needs for Alternatives and Validation

As in many other countries, the Japanese government requires many toxicological tests for the safety management of various kinds of chemicals. Different sets of toxicological data are required for different categories of chemicals, such as pharmaceuticals, pesticides, cosmetics, novel chemicals, and materials for medical devices. Most of these toxicological tests are animal tests. Only a limited number of tests have alternatives, such as the acute oral toxicity test, skin and eye irritation tests, and the pyrogenicity test. Many others are waiting for the development of the alternative testing methods. Replacing long-term toxicity tests or reproductive/developmental toxicology studies with non-animal procedures will be very difficult; however, researchers are persevering in their efforts to investigate the possibilities.

Japanese Participation in Validation Studies

The participation of Japanese laboratories in various validation activities is depicted in Table 1. The first four studies listed in the table were international collaborative studies. Several Japanese laboratories have participated individually in these international collaborations; initially, in the validation study for the fixed dose proce-

Table 1: Participation of Japanese laboratories in international and national validation studies

Alternative to	Title of study	Study term	Sponsor/Steering body
Acute oral toxicity (LD50 test)	Fixed Dose Procedure (BTS Method)	1988–1989	EC/UK Home Office
Acute oral toxicity (LD50 test)	Toxic Class Method (BGA Method)	1991–1994	BGA (FRG)/BGA
Acute oral toxicity (LD50 test)	*In vitro* Cytotoxicity (MEIC Project)	1989–1995	SSCT/MEIC
Eye irritation test (Draize test)	*In vitro* Draize Alternatives	1992–1994	EC/UK Home Office
Eye irritation test (Draize test)	JSAAE Cytotoxicity Study	1992–1996	JSAAE/JSAAE Working Group
Eye irritation test (Draize test)	Safety Evaluation of Cosmetic Ingredients	1993–1996	MHW (JPN)/NIHS-JCIA
Pyrogenicity test (rabbit)	Endotoxin Test (*Limulus* Amebocyte Lysate)	1991–1992	Soc. Jpn Pharmacopoeia/ Osaka and Tokyo PMA
Intramuscular irritation test	Safety Evaluation Test for Intramuscular Injections	Planning in progress	JSAAE/JSAAE-JPMA
Carcinogenicity test (Cell transformation)	3T3 Cell Transformation Validation Study	1995–1997 1998–1999	JEMS/JEMS Working Group
In vivo micronucleus test	*In vitro* micronucleus test	1998–2001	JEMS/SFTG

dure of acute oral toxicity test (British Toxicology Society [BTS] method) promoted by the European Commission (EC) and the UK Home Office (2), and then in an international collaborative study for the acute toxic class method, organised by German Bundesgesundheitamt (BGA) (3). The Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) project, conducted by the Scandinavian Society for Cell Toxicology (SSCT), made a unique approach to *in vitro* alternatives to acute toxicity testing, and seven Japanese laboratories have participated in this project (4). A few Japanese laboratories participated in the EC/UK Home Office validation project of *in vitro* alternatives to the Draize eye irritation test.

High Hurdles of International Validation Studies

Through these experiences, the difficulties of international collaborative studies became evident, i.e. different national regulations defining different goals in developing alternative tests; different regulations for chemical transportation, which formed a hurdle against studies proceeding by using common chemicals in a blind manner; different standards for laboratory conditions, such as room temperature and humidity, feed for animals; geographical distances that hinder frequent meetings and sufficient communication; different Good Laboratory Practice (GLP) regulations; language barriers; and, finally, but most importantly, financial matters.

Nevertheless, international and interlaboratory validation studies have been of considerable importance in establishing the robustness of the test procedures across international and interlaboratory barriers.

Validation Studies Within the Country

Thereafter, validation activities involving Japanese laboratories were mostly conducted within the country. In the early 1990s, two major interlaboratory studies were performed in Japan. Both studies were intended to confirm the usefulness of *in vitro* tests as alternatives to Draize eye and skin irritation tests.

The project on the interlaboratory reproducibility of cytotoxicity assays was initiated by the JSAAE, gathering 47 laboratories from among the society's members to participate in the project (5, 6). Comparisons were made between five different cytotoxicity tests, each using two different cell preparations. Data for six surfactants with each test method and each cell line were collected and analysed for reproducibility among the laboratories. Extensive data analysis revealed the least interlaboratory variation with the crystal violet staining method, and the results also showed no superiority of any cells over other kinds of cells in testing for cytotoxicity of surfactant chemicals.

The EC directive to prohibit animal testing for cosmetic ingredients had a great impact internationally, and the Japanese Cosmetic Industry Association (JCIA) entered into a collaboration to evaluate *in vitro* test methods as possible alternatives to the Draize rabbit eye test. The project was supported with a grant from the Ministry of Health and Welfare (MHW) and was conducted by the National Institute of Health Sciences (NIHS) (7). In this study, nine different *in vitro* test methods, i.e. tests using the chorio-allantoic membrane of the hen's egg, sheep erythrocytes, artificial skin models (SKIN2™ and MATREX™), cytotoxicity tests using several different cells, and EYTEX™, were evaluated for reproducibility among the different

laboratories. In total, 17 laboratories participated in this 3-year project, producing data for 45 test chemicals. The results were subsequently published (8), and a scheme for the safety evaluation of cosmetic ingredients has been proposed for regulatory acceptance.

Prior to these two projects concerned with the rabbit eye test, a validation study for an endotoxin test using the *Limulus* amebocyte lysate as the replacement alternative to the pyrogenicity test using rabbits was carried out. The rabbit pyrogen test had been frequently performed, as the test was described in monographs of the *Japanese Pharmacopoeia* (*JP*) for drugs for injection, antibiotics and biological preparations. The project was planned on the initiative of the Society of Japanese Pharmacopoeia and was conducted by the NIHS, in collaboration with 27 laboratories of the Osaka and Tokyo Associations of Pharmaceutical Manufacturers (PMA). They tested all the 67 preparations for the pyrogen test, required in the monograph in *JP XI* (1986). The result was satisfactory, and for most of these preparations, the pyrogen test was replaced by an endotoxin test in the next version of the *JP*, *JP XII* (1991).

In this validation study, the *Limulus* test actually validated was the "gel-formation method", and *JP XII* described only this method for the endotoxin test. Later, when more-sophisticated photometric methods based on the same principle were developed, these improved procedures were described in the next revision, *JPXIII* (1996), without any large-scale collaborative validation study.

The Intramuscular Irritation Test

The validation of an *in vitro* cytotoxicity test as an alternative to the intramuscular local irritation test is planned by the JSAAE. A local irritation test using intramuscular injections in rabbits is required in Japan for every drug used by intramuscular injection. This requirement was made mandatory after outbreaks of thigh muscle contracture and gait disturbance in children who had received intramuscular injections for the treatment of febrile diseases. The drug preparations were injections of antipyretic pyrazolone and antibiotics. It was demonstrated that the potential tissue-damaging property could be detected by an intramuscular injection test of these drugs using rabbits. This test has since been required for every drug to be used by the intramuscular route. The use of cytotoxicity tests as an alternative to this irritating property test is promising, and the JSAAE is preparing the validation in collaboration with the Japanese Pharmaceutical Manufacturers Association (JPMA). This project has been postponed for some years, because there was a reluctance among pharmaceutical industries intending to drop rather than sustain the intramuscular injection method. Thus, this project has not been launched, although the need for alternatives still exists. A survey by the JSAAE in 2000 revealed that 67 preparations for intramuscular injection have been developed during the past five years.

Alternatives to *In Vivo* Carcinogenicity Studies

Cell transformation assays hold promise for serving as predictive indicators of the carcinogenic potential of chemicals (9), and the transformation assay with Syrian hamster embryonic (SHE) cells has now been proposed for an OECD test guideline. On the other hand, the transformation assay using an established cell line, Balb/c 3T3, has also been developed and recognised as useful. A multi-laboratory validation study for an improved cell transformation assay using Balb/c 3T3 cells was conducted by the Japan Environmental Mutagen Society (JEMS) in 1995–1997 (the first phase), and in 1998–1999 the second phase study took place. The results of the study were published in *ATLA* (10), and are being presented at this Congress by Makoto Umeda.

Instead of a cytogenetic test on whole animals, the usefulness of an *in vitro* micronucleus test using CHL cells and L5178Y cells has been studied by international collaboration with JEMS and the Société Française de Toxicologie Génétique (SFTG), including 38 laboratories, in 1998–2001.

Discussion

The validation of a test method is not only a process for showing its scientific usefulness, but also the way forward to public and regulatory acceptance. The most remarkable progress of the last decade in the area of alternative research has been in developing and agreeing on the criteria for validation and regulatory acceptance.

Japanese scientists have carried out several validation studies within the nation or have participated in international collaborative studies. Through these studies, we have recognised considerable difficulties in the huge workload of validation, as well as its cost and time-consuming nature. In addition to inadequate financial support, we have difficulties in finding sufficient human resources that are capable of conducting a complicated collaboration, dealing with a large volume of data and completing the independent review of the results.

A new trend in genomic sciences is emerging in the world of toxicology. Research in toxicogenomics might produce great changes in toxicology and also in animal use in safety assessment. However, we have not yet focused much upon the validation of this new approach to predicting toxicity. We must make a fresh assessment of validation procedures

for these new methods in toxicology. It is not too early to consider this, since the toxicogenomics investigation is a large-scale enterprise with a very promising future.

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総説

急性毒性試験法ガイドラインの改定とその意義

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要約—OECD 急性経口毒性試験ガイドライン（TG 401）の廃止の措置と 3 種の代替法（TG420，TG423，TG425）の提示に応じて，毒性学者の対応しなければならない諸問題について論じた。3 代替法の試験操作について略述した。これらの試験法は化学物質の分類，表示を行う目的に適うように設計されており，万国共通化学物質分類方式（GHS）に合わせて用量設定がなされている。これら代替試験法の共通の問題として，毒性の雌雄差の検査を無視していること，いずれも逐次試験法であるため，試験の始めと終わりでは相当に時日の経過があること，毒性の症状観察に熟達することが要求される問題を論じた。

はじめに

急性毒性試験における半数致死量 LD_{50} は，これまで化学物質の毒性の程度を表現する基本的数値として，原則としてすべての物質について動物実験によって求められてきており，わが国では概略の致死量でよいとされている医薬品は別格として，農薬等の安全性評価資料として用いられてきたばかりでなく，毒物劇物取締法の評価ではほとんど必須の資料といってよいものであった。毒性学の理論においても，LD_{50} は毒性学の基本概念の一つとして大きな位置を占め，たとえば，日本トキシコロジー学会の認定トキシコロジスト資格試験問題には毎回少なくとも候補問題として取り上げられる。この LD_{50} が，動物福祉の観点から不適切なものであり，科学的に価値が低く，必要性がないとして，それを求める従来の急性毒性試験法（「従来法 traditional method」と称するようになった）が，OECD 試験法ガイドラインから削除されることになった。この決定は，OECD 理事会において 2001 年 12 月 17 日に行われ，1 年間の猶予を置いて，2002 年 12 月 17 日以降は従来法の急性毒性試験は行わないよう、またその時以降に実施された従来法急性毒性試験の結果の行政的受け入れをしないように各国に勧告されたのである。この総説では，この措置に伴う毒性試験実施の諸問題について要点を述べる。

OECD 試験法ガイドラインにおける急性毒性試験

経済開発協力機構（OECD）の化学物質試験法ガイドラインは，化学物質の物理化学的性質の試験法から，生態系への影響，分解性，蓄積性，および健康への影響の試験（毒性試験）の各種試験法を含んでおり，加盟各国の合意によって成立した harmonized guidelines である。これは，OECD 加盟国の科学者の協力によって 1981 に作られてから，化学物質の標準的試験法として広く用いられてきており，また，ここに記載された試験法に従って行われた試験のデータについては，加盟国間で相互に受入れを承認する「Mutual Acceptance of Data（MAD）」の原則が合意されていて，試験の重複実施を防ぐことができる利点がある。このガイドラインは，科学技術の進歩に即応して更新すべく定期的に見直しが行われてきたが，1983 年の High Level Meeting の指令に基づいて，見直しの要素として「動物福祉の観点」が加えられ，動物を用いる試験法について諸種の応急的な改定が加えられた。急性毒性試験の動物数の削減（雌雄いずれか一方のみを用いる：1987 年改定）や刺激性試験における試験除外の規定（先立って行った *in vitro* 試験によって刺激性が予想された場合は実施しない：1992 年改定）等

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の変更である。

その後「従来法」急性経口毒性試験（TG401）に対して，各種の代替法が考案され，提案され，OECD試験法として，1992年に固定用量法(TG420)が，1996年に急性毒性等級法（TG423）が採択された。また，1998年に上げ下げ法（TG425）が提案された。これらはすべて単回経口投与試験法であるが，その他の経路による急性毒性試験の代替法も現在検討されている。また，皮膚と眼の刺激性／腐食性試験についても代替法の開発は盛んに試みられてきたがまだ決定的な方法はなく，一部の代替にとどまっている。

元来，代替法とは，基準になる試験法に対して，それと同等またはそれ以上の能力を持つことが証明された上で，基準法によらずその方法によって試験をすることも正当化された試験法を言うものであり，それまで基準法となっていた試験法を廃止してそれと置換するものではないはずであった。OECDにおける急性経口毒性試験の3種の代替法（TG420，423，425）も当初は「従来法」TG401と並存していたが，その後の実態を見ると，実際に使用されることは少なかった。TG420を開発し，その使用を推進してきた英国でさえ，1998年の時点で化学物質の届出資料を見ると，急性毒性の資料のうちTG420によった物質は15％に過ぎなかった。ドイツでもTG423を用いて試験した物質は20％に過ぎなかった（OECD　専門家会議席上報告）。届出物質の約半数は，限度試験（2000 mg kgの1用量のみの試験）によっていたが，残りの大部分の物質はTG401に，しかも改定前の1981年版によって試験がなされていた。これは，代替試験法が信頼されていないこと，あるいは実施する利点が乏しいことを示すものであろうが，代替法推進派は，TG401を廃止すれば，否応なしに代替法が用いられるようになると考え，そのように提案した。

OECD理事会は，2001年12月17日，急性経口毒性試験の従来の試験法を定めたTG401を削除することを決議し，1年の猶予ののち，2002年12月17日以降にこの方法を用いて試験した物質については，加盟各国はその受入れを承認することを要しない，とする決定を行った。実際にTG401の廃止が話題に上ったのは1995年からのことであり，この間，従来法の普及していることやLD_{50}値が各種の規制に組み込まれている利用度の高さを考えると捨てがたいものであり，結局廃止は出来ないだろうと見る向きが多かった。それだけにこの決定は思い切った措置であったといえる。いずれにせよ，LD_{50}の算定を含む急性毒性試験が毒性学と安全性評価における基本的な過程であっただけに，この決定が各方面に大きな影響を及ぼすことは明らかである。これにどの様に対応するのか，規制の変更も必要であるが，それとともに試験の現場でも十分な対応を行わなくてはならない。

急性経口毒性試験代替法の要点

OECD TG401の代替法として採択されている3試験法について要点を記述する。最初の代替法(TG420)が採択されたのは1992年にさかのぼるが，その試験法が提案されたのは1984年のことであり（British Toxicology Society，1984），成立するまでにはその妥当性を証明するvalidationを行う時間が必要であった。（そうした動物実験代替法を検証するためのvalidationの手順を確立するワークショップを何度か開催する必要もあった。）さらに，これら代替法の主目的である化学物質有害性分類の基準が，国によって，国際機関によって一様でなかったので，それを統一する作業も必要であった。

1. TG420：固定用量法（Fixed Dose Procedure）

TG401の代替法として英国から提案されたTG420固定用量法（British Toxicology Society，1984；Van den Heuvel *et al.*，1987，1990）は，1992年に採択された。これは，まず動物の死亡を毒性の指標とすることを止め，さらに使用動物数を制限するために被験物質の用量を固定したものである。すなわち，嚙歯類（ラット）を用いて4段階の固定した用量に限って逐次単回経口投与して試験し，動物の致死量の観察は避け，「明らかな毒性 evident toxicity」を現す用量を以て化学物質の毒性の強さを分類する。「明らかな毒性」とは，それより上の用量では死亡が起こると予想される用量による毒性，と定義されている。固定用量は5，50，300，2000 mg kgの4用量（および例外的に5000 mg kg）である。

試験の手順は，まず少数の動物(各用量につき1匹)を用いて見当づけ試験（sighting study）を行い，「明

らかな毒性」を観察できそうな（そして動物を死亡させないような）用量を選定する。次に，選定した1つの用量で主試験（main study）を開始し，その結果によって次の用量（毒性が見られなければ高い方の用量，死亡が起これば低い方）を試験し，「明らかな毒性」を示す用量を求める。見当づけ試験の手順の概念をFig. 1に，主試験の手順をFig. 2に示す。この逐次試験の進め方は試験の開始用量とその結果によって変わる。

この方法では使用動物数を従来法より削減し，1用

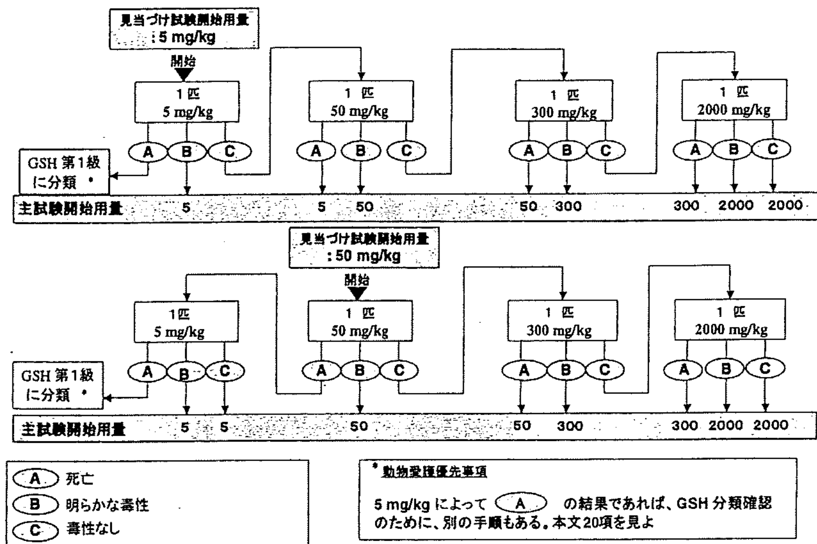

Fig. 1-1. Flowchart of Fixed Dose Procedure (TG420): Sighting Study starting at 5 and 50 mg/kg.

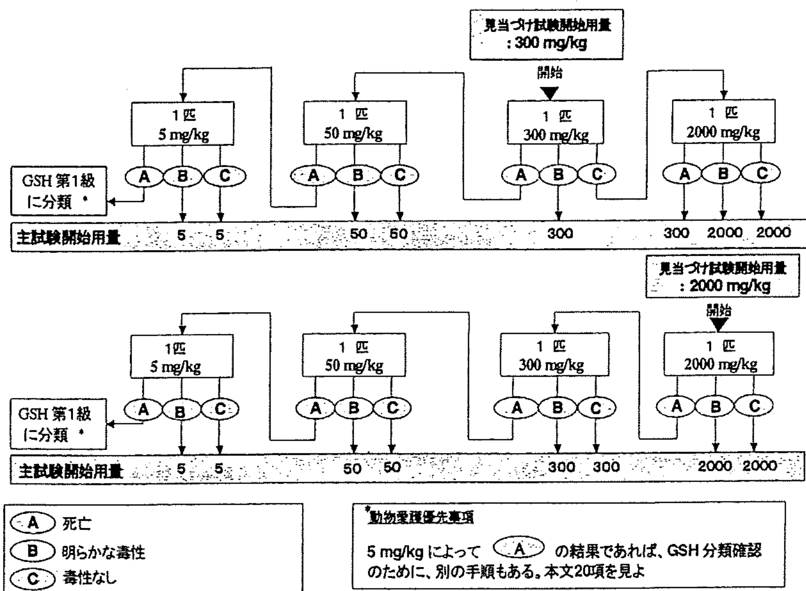

Fig. 1-2. Flowchart of Fixed Dose Procedure (TG420): Sighting Study starting at 300 and 2000 mg/kg.

量につき5匹とした。この数には見当づけ試験の動物を含むとしている。従って，動物数は見当づけ試験に各用量1匹ずつ，主試験では逐次1用量ずつ各4匹を用いる。判定は合わせて5匹として行われる。動物の性は，雌雄いずれか一方，通常は雌を用いることとされた。結果に基づいて化学物質有害性分類を行う。動物が強い毒性症状を示して苦しむときは観察を中止して動物を人道的に屠殺し，毒性による死亡と同一にみなす。観察期間は従来法と同じく原則として14日間であるが，投与の結果の判断（次の用量に進むかどう

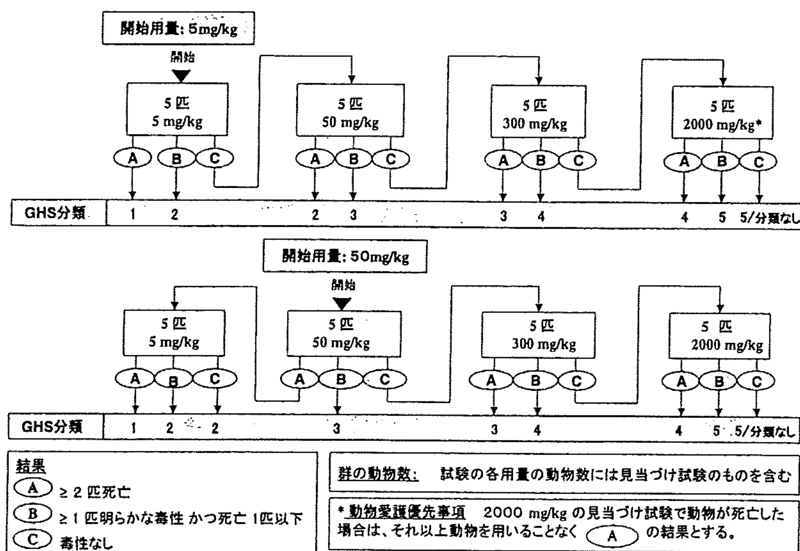

Fig. 2-1. Flowchart of Fixed Dose Procedure (TG420): Main Study starting at 5 and 50 mg/kg.

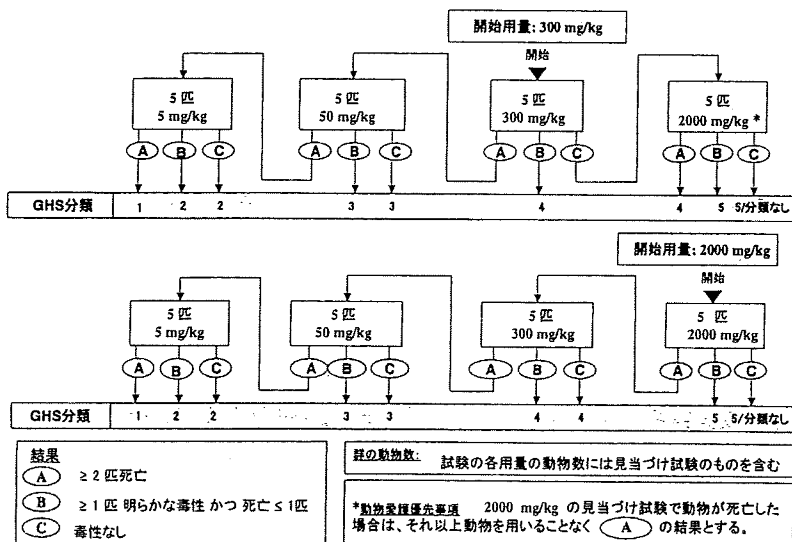

Fig. 2-2. Flowchart of Fixed Dose Procedure (TG420): Main Study starting at 300 and 2000 mg/kg.