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## TISSUE INTERACTIONS WITH DERMAL SHEEP COLLAGEN IMPLANTS: A TRANSMISSION ELECTRON MICROSCOPICAL EVALUATION

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### Abstract

Tissue interactions with discs of dermal sheep collagen (DSC), subcutaneously implanted in rats, were evaluated using transmission electron microscopy. DSC cross-linked with hexamethylenediisocyanate (HDSC) had already been tested previously. In the present study, we compared tissue interactions of non-cross-linked DSC (NDSC) and glutaraldehyde-cross-linked DSC (GDSC) with those of HDSC. To gain more insight in the relation between basophil-like cells, foreign body multinucleate giant (FBM-G) cells derived from them, and aluminum/silicate (Al/Si)-crystals, we also implanted agarose-embedded Al/Si-crystals. GDSC induced early after implantation extensive lipid formation, cell degeneration and death, but NDSC did not have any cytotoxic effects. Basophil-like (FBM-G) cells specifically dealt with Al/Si-crystals, because they were attracted by the agarose-embedded Al/Si-crystals. These cells were also attracted by GDSC and NDSC, where Al/Si-crystals are present. Accumulation of Al/Si-crystals was especially prominent with GDSC. Degradation by phagocytosis was in part carried out by FBM-G cells, derived from macrophages or fibroblasts, and observed only with GDSC, which indicates that the presence of cross-links is a prerequisite for their formation.

**Key Words:** collagen, cross-linking, tissue interactions, transmission electron microscopy, giant cells, neutrophils, basophils, aluminum silicate, calcification.

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### Introduction

Various collagen-based biomaterials have found applications in the biomedical field (7, 18, 31). Tissue interactions with implanted collagen-based biomaterials have been extensively described (e.g. 8, 15, 26, 37). However, these reports mainly concern light microscopical evaluations. In some instances, more detailed evaluations using transmission electron microscopy (TEM), have been reported as well (1, 44, 45).

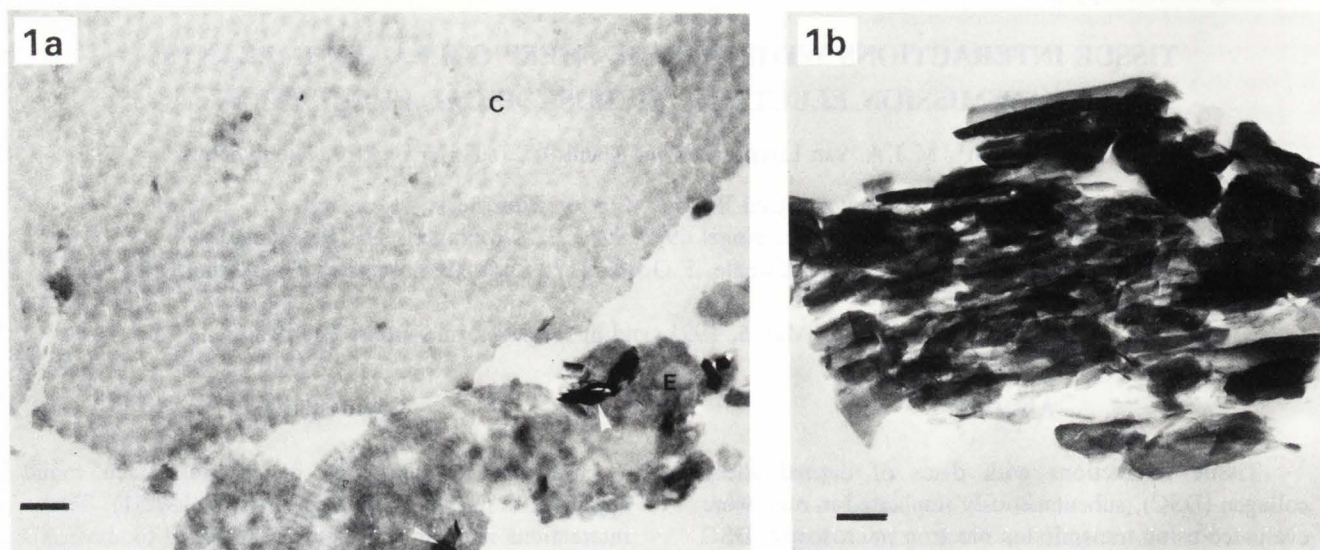
Previously we reported on possible *in vitro* (43) and *in vivo* (44) cytotoxic effects of a processed dermal sheep collagen (DSC), which had been cross-linked with hexamethylenediisocyanate. This type of DSC will subsequently be referred to as HDSC. Using our specially developed *in vitro* cytotoxicity test system (43), HDSC was shown to have cytotoxic influences on fibroblasts in the sense that it induced inhibition of cell growth and lipid degeneration.

After subcutaneous implantation of HDSC in rats, the following remarkable features were observed (44): The neutrophils, infiltrating during the first 10 days after implantation, locally showed a deviant morphology, i.e., the cytoplasm and organelles appeared to have disintegrated. Furthermore, a rather high incidence of intracellular lipid accumulation was observed. Both phenomena were possibly related to cytotoxic influences.

Another striking feature was the infiltration of a cell type which was qualified as basophil-like cell, as judged by the granules. Basophil-like cells could form foreign body multinucleate giant (FBM-G) cells. Both basophil-like cells and FBM-G cells derived from them appeared to specifically deal with crystals present in HDSC and with X-ray microanalysis shown to contain aluminum and silicon (Al/Si). Al/Si-crystals accumulated in the basophil-like granules. After 15 weeks of implantation, i.e., the final stage in degradation of HDSC, only basophil-like (FBM-G) cells with (sometimes huge) accumulations of Al/Si were observed.

At an earlier stage during degradation, two other remarkable phenomena were observed. First, HDSC was degraded by phagocytosis of the collagenous parts by





**Figure 1a:** Non-implanted HDSC with transversely cut HDSC-bundles (C), and elastin (E)-like substances with sheet-like Al/Si-crystals (arrow). Bar = 0.34  $\mu\text{m}$ .

**Figure 1b:** Non-implanted Al/Si-agarose with a typical small-sized Al/Si-crystalline particle. Bar = 0.15  $\mu\text{m}$ .

FBM-G cells from macrophage or fibroblast origin, and, second, calcium phosphate (Ca/P)-depositions were found in, mainly the larger, not yet internalized HDSC-bundles (44).

In the present study, we evaluated possible influences of DSC related to the use of tanning agents. Therefore we implanted a non-cross-linked DSC (NDSC) and a glutaraldehyde-cross-linked DSC (GDSC), and compared the tissue interactions with those of HDSC. To gain more insight in the relation between infiltration of basophil-like cells, FBM-G cells derived from them, and Al/Si-crystals, we also implanted agarose-embedded Al/Si-crystals as reference material. The tissue interactions were studied using TEM, thereby focussing on the remarkable features mentioned above.

#### Materials and Methods

##### Materials

*Dermal sheep collagen* (DSC), processed from sheep skin (41, 42), was obtained from the Zuid Nederlandse Zeemlederfabriek, Oosterhout, The Netherlands. Processing up to skin splitting means depilation, immersion in a lime-sodium sulphide solution for removal of epidermis and purification with pancreatic enzymes, i.e., mainly trypsin (41). Three DSC-versions were used, a non-cross-linked version (further referred to as NDSC, batch no. 409) and two cross-linked versions. The hexamethylenediisocyanate (HMDIC)-cross-linked version (batch no. 392), and the glutaraldehyde (GA)-cross-linked version (batch no. 397) will subsequently be referred to as HDSC and GDSC. In short, HDSC was

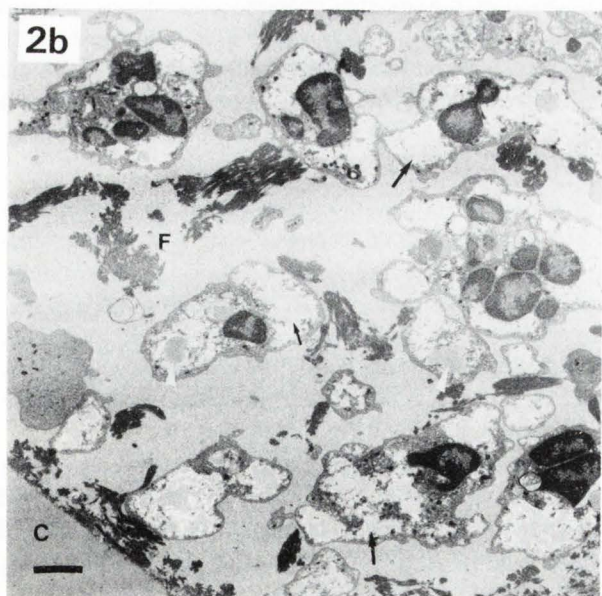
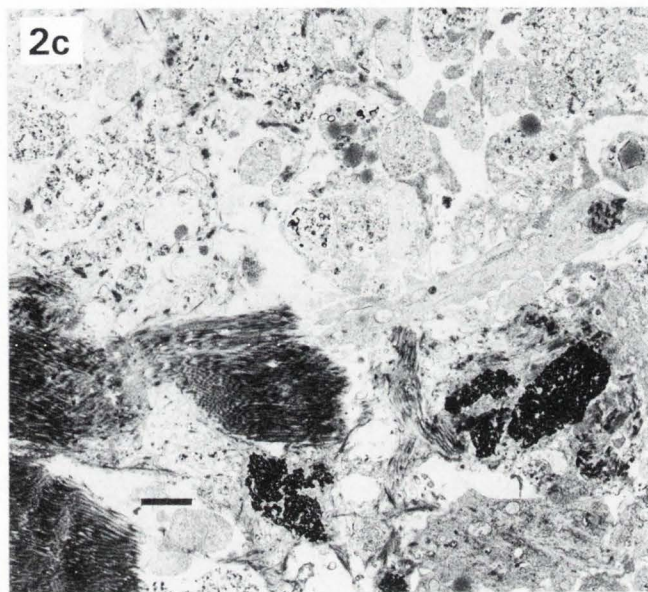
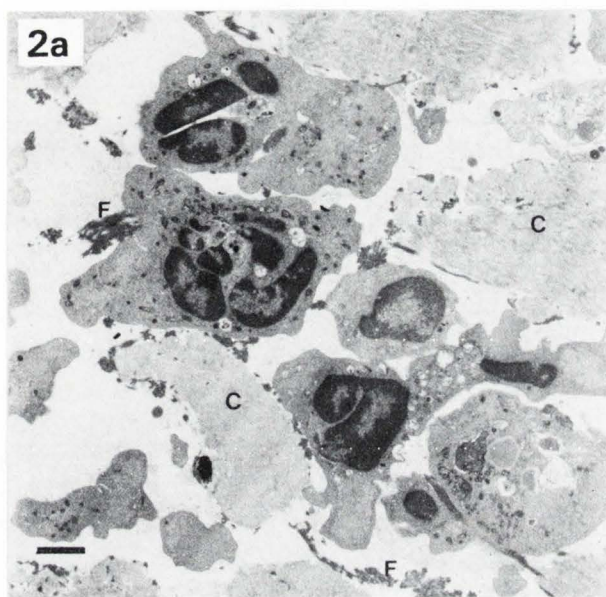
cross-linked during 15 hours with a HMDIC-solution of 1.25% (Desmodur<sup>®</sup>) containing 0.3% of surfactant (Emulvin W<sup>®</sup>), both obtained from Bayer, Wuppertal, Germany. GDSC was cross-linked during 17.5 hours with a 0.5% GA-solution (Relugan GT 50<sup>®</sup> obtained from BASF, Basel, Switzerland). Both HDSC and GDSC have shrinkage temperatures of approximately 70°C. Discs with a diameter of 8 mm were punched from each material. The weight of the discs was approximately 30 mg. Discs were sterilized by gamma-irradiation (2.5 Mrad, Gammaster, Ede, The Netherlands).

*Aluminum silicate* ( $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$ ) in powdered form was obtained from Fluka Chemie AG, Buchs, Switzerland. Five mg of Al/Si-crystals were evenly suspended in 1 ml of a 2% agarose/phosphate buffered saline (PBS, pH = 7.4) solution at 40°C. The agarose had been obtained from Sigma Chemical Co., St. Louis, U.S.A.. Thereafter, the suspension was quickly coagulated at 4°C during one hour in tubes with a diameter of 8 mm from which the Al/Si-agarose gel was harvested and cut into discs with a thickness of 2 mm.

##### Implantations

Male AO/G-rats ( $n = 2$  per implantation period) of approximately three months of age were ether-anaesthetized and subcutaneous pockets were made to the right and left of two midline incisions. DSC-discs were implanted in the pockets at a distance of about 1 cm from the incisions. Al/Si-agarose-discs were implanted in the same way. Implants with surrounding tissue were harvested at 2, 5 or 10 days and at 3, 6, and, if possible at 10 and 15 weeks.





**Figure 2a:** Invading neutrophils in between fibrin (F) and collagen bundles (C) of NDSC, 2 days after implantation. Bar = 1.4  $\mu\text{m}$ .

**Figure 2b:** Deviant morphology of neutrophils within HDSC at 2 days after implantation. F: fibrin network; C: collagen. The neutrophils show a disintegration of the glycogen in the cytoplasm (black arrows), swollen mitochondria and lipid formations (white arrows). Bar = 2.1  $\mu\text{m}$ .

**Figure 2c:** Extensive cell death within GDSC, 10 days after implantation. Bar = 2.1  $\mu\text{m}$ .

### Microscopy

Implants were fixed in 2% (v/v) GA in 0.1 M PBS. Specimens were cut into small blocks (2 x 2 x 2 mm) after at least 24 hours of fixation at 4°C. Representative blocks were postfixed in 1% OsO<sub>4</sub>, 1.5% K<sub>4</sub>Fe(CN)<sub>6</sub> in PBS (19), dehydrated in graded alcohols and embedded in Epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined with a Philips EM 201 transmission electron microscope, operated at 40 kV.

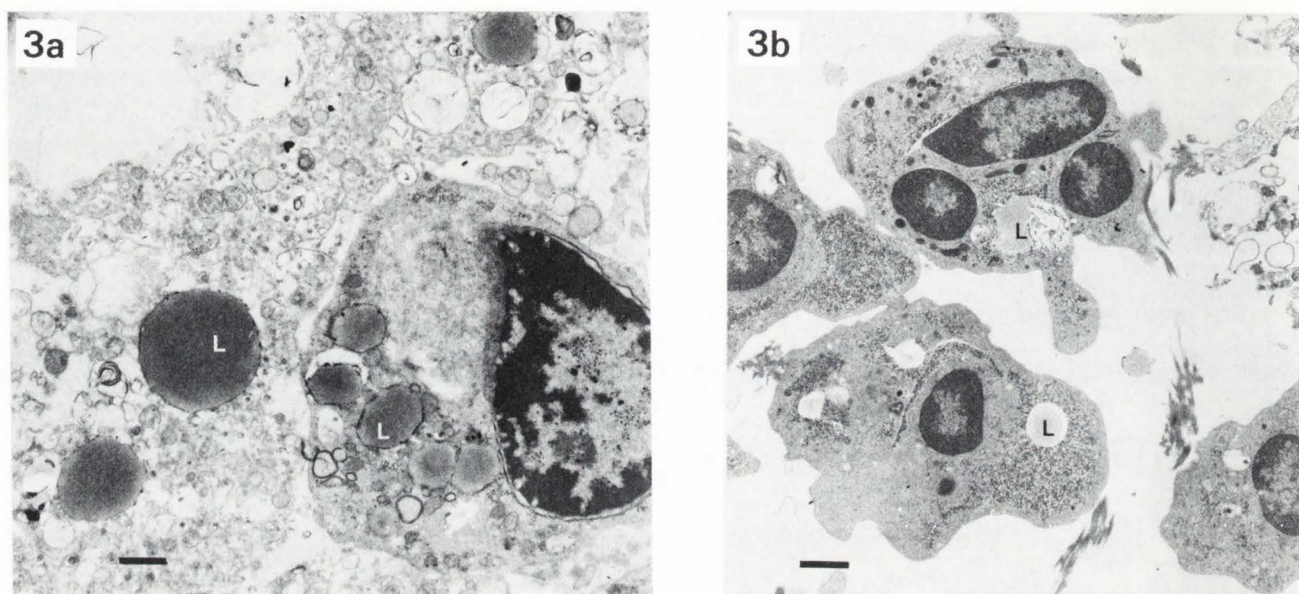
### Results

#### Characterization and harvesting of implant materials

Macroscopically, the three types of DSC were similar, i.e., they had a fibrous and supple structure. Microscopically, the three types of DSC also had similar structures. Observed with TEM, they all consisted mainly of a matrix of collagen bundles, as shown for HDSC in Figure 1a. Gelatin-like substances, i.e., collagen, which had lost the original fibril structure, were found in each type of DSC, but mostly in NDSC. Elastin-like substances, recognized because they had the same morphology and electron-density as remnants of (elastic laminae of) former blood vessels, sometimes lined collagen bundles. The latter often contained crystal(like) structures which we previously had identified as aluminum silicate (Al/Si)-crystals (Fig. 1a). Macroscopically, Al/Si-agarose had a whitish gel-like appearance. With TEM, an even distribution of small-sized Al/Si-crystalline aggregations was seen within the agarose (Fig. 1b).

Harvesting of implanted materials was easy during





**Figure 3a:** Intracellular lipid (L) formation combined with cell death in GDSC at 5 days after implantation. Bar = 0.65  $\mu\text{m}$ . **Figure 3b:** Intracellular lipid droplets (L) in neutrophils within NDSC, 10 days after implantation. Bar = 0.93  $\mu\text{m}$ .

the first three weeks after implantation. Both HDSC and GDSC were macroscopically hard to localize from 6 weeks onwards. At 15 weeks, the HDSC-implants (remnants) were not recognized macroscopically; harvested tissue was only at the microscopical level found to contain indications of the former presence of HDSC-implants. The remnants of GDSC were at that time macroscopically recognized as a small metallic-like chip. NDSC-implants could only be harvested until 6 weeks after implantation. Al/Si-agarose implants might break at implantation, but localization and harvest of (parts of) these implants was easy after each time of harvesting. By 15 weeks, implants appeared slightly smaller than originally.

#### Tissue interactions with implant materials

**Neutrophils.** Numbers of infiltrating neutrophils differed per material. High numbers of neutrophils were observed within GDSC as compared to the medium numbers found with HDSC. Neutrophils completely infiltrated both materials. Only few neutrophils were present with NDSC and Al/Si-agarose and mostly observed at/in the edges.

Except when HDSC had been implanted, infiltrating neutrophils at 2 and 5 days always had the normal morphology of wound healing-associated neutrophils, i.e., activated or degenerated (Fig. 2a). With HDSC, locally a *deviant* neutrophil morphology (Fig. 2b), with disintegration of glycogen and organelles in the cytoplasm but with an intact nucleus, was observed. This was observed at 2 days after implantation in both the implant and

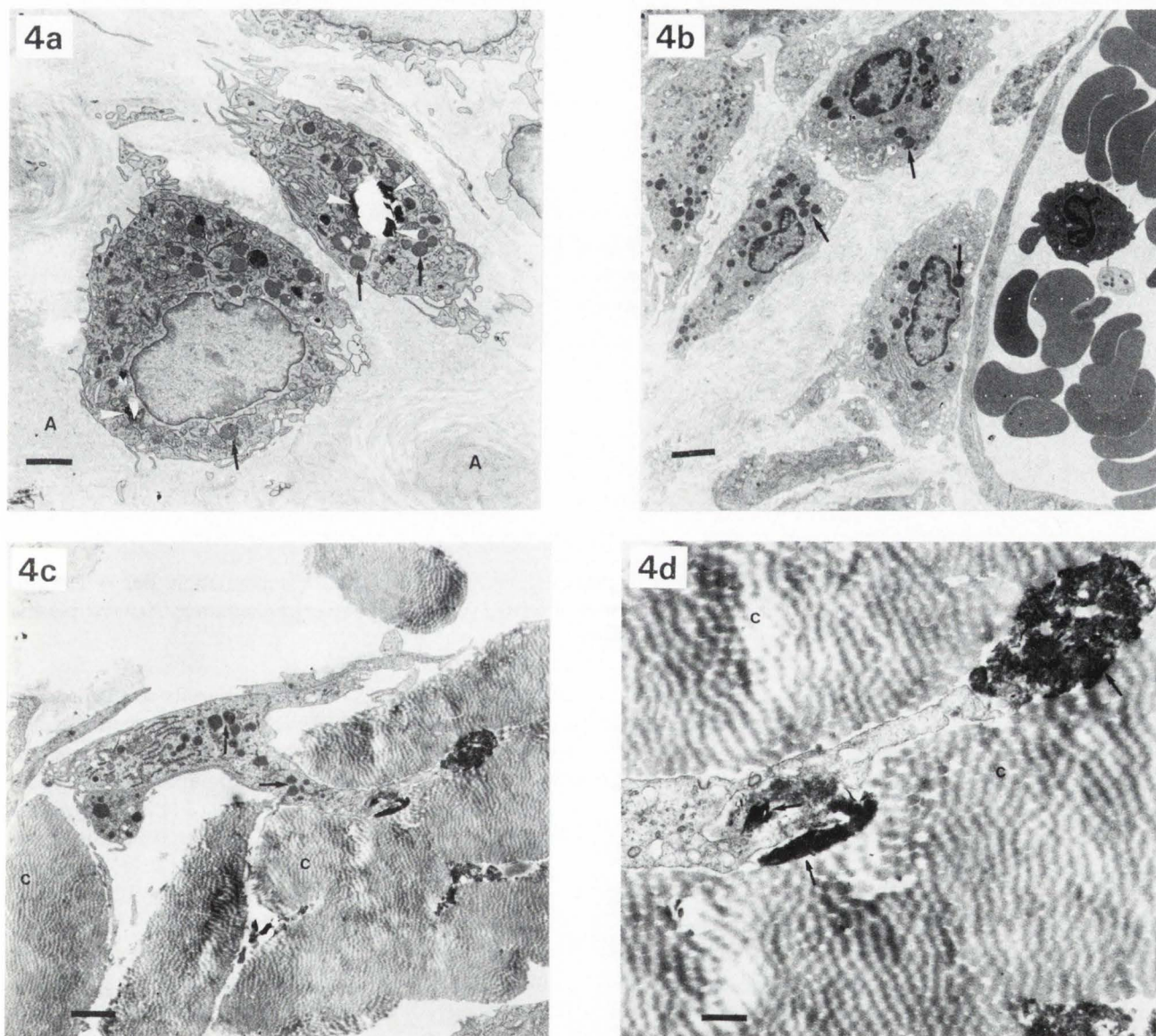
sometimes in the surrounding connective tissue, and still observed within the implant, although less intense, at 10 days. Deviant morphology of neutrophils was not found with GDSC. This material induced extensive (normal) cell death of mainly neutrophils from day 2 till 10 (Fig. 2c).

**Lipid accumulation.** Intracellular lipid accumulation, observed as lipid droplets, occurred after implantation of each material, but most intensively with GDSC and to a lesser degree with HDSC. Neutrophils and macrophages contained lipid droplets at day 2. Especially with GDSC, many cells with lipid accumulations had degenerated at days 5 and 10, leaving pronounced accumulations of extracellular lipid (Fig. 3a).

In the case of NDSC, neutrophils, but also other cell types, sometimes contained a different type of lipid accumulation, observed as many medium-sized lipid droplets at 10 days (Fig. 3b). Cell degeneration only sporadically occurred in NDSC.

**Infiltration of other cell types.** Apart from neutrophils, macrophages migrated to or into the materials at day 2. Compared to the other materials, low numbers of macrophages migrated to NDSC. *Basophil-like cells* (Fig. 4), containing basophil-like granules (5, 14, 48) were found from day 2 on at the edges of Al/Si-agarose and from day 5 on at the edges or within each type of DSC (see below). From 10 days on, basophil-like cells were the most common cell type in the hardly infiltrated NDSC. High numbers of basophil-like cells were especially seen when Al/Si-agarose (Fig. 4a) and GDSC





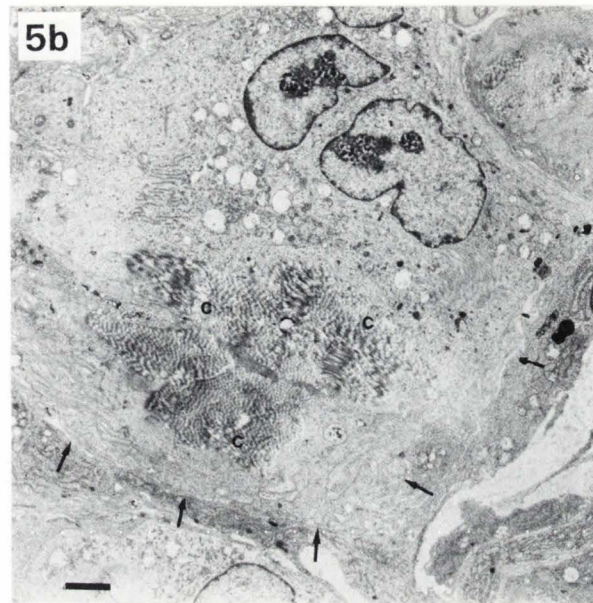
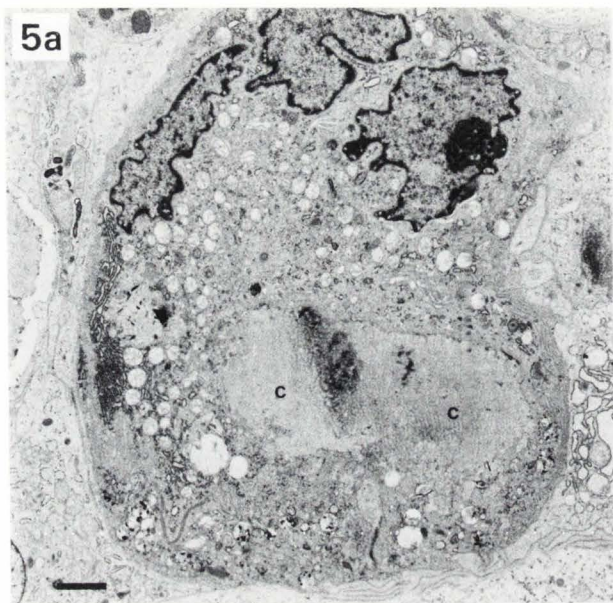
**Figure 4a:** Basophil-like cells with basophil-like granules (arrows) and Al/Si-crystals (white arrow) within Al/Si-agarose (A) and collagen of the subcutaneous tissue, at 10 days after implantation. Bar = 1.4  $\mu\text{m}$ . **Figure 4b:** Basophil-like cells with basophil-like granules (arrows) infiltrating GDSC at 10 days after implantation. Bar = 2.1  $\mu\text{m}$ . **Figure 4c:** Basophil-like cell with basophil-like granules (arrows) infiltrating GDSC (C), at 3 weeks of implantation. One pseudopod is reaching out to and surrounding elastin-like substances with Al/Si-crystals. Bar = 1.4  $\mu\text{m}$ . **Figure 4d:** Detail; arrows: Al/Si-crystals within elastin-like substance of GDSC. Bar = 0.48  $\mu\text{m}$ .

(Fig. 4b) had been implanted. Basophil-like cells had cytoplasmic extensions closely associated with Al/Si-crystals present in elastin-like substances (Figs. 4c, d).

From day 5 on, fibroblast activity resulted in the formation of a fibrous capsule around each type of implant, and in fibroblast infiltration into both HDSC and GDSC. At days 5 and 10, increased numbers of eosinophils, as compared with the other materials, were observed in close proximity of NDSC.

**Foreign body multinucleate giant (FBM-G) cells.** From day 5 on, three types of FBM-G cells were observed, though not with each type of implant. *Macrophage-derived FBM-G cells* were found only when HDSC (Fig. 5a) and GDSC (Fig. 5b) had been implanted. These cells were specifically involved in phagocytosis, i.e., in surrounding, internalization and subsequent degradation of DSC-fragments. Macrophage-derived FBM-G cells also phagocytosed rat collagen,





**Figure 5a:** Macrophage-like FBM-G cell internalizing a part of HDSC (C), 10 days after implantation. Bar = 1.4  $\mu\text{m}$ .  
**Figure 5b:** Macrophage-like FBM-G cell internalizing a part of GDSC (C), 10 days after implantation. Arrows: plasma membrane. Bar = 2.1  $\mu\text{m}$ .

which had been formed between weeks 6 and 10 during degeneration of both HDSC- and GDSC-implants. Rat collagen could be discriminated from DSC, because it consisted of smaller caliber fibers and contained entrapped remnants of degenerated cells (44).

*Fibroblast-derived FBM-G cells*, formed from fibroblasts, were present in lower numbers, compared to the macrophage-derived FBM-G cells, and also only observed when HDSC and GDSC had been implanted. These cells were discriminated from macrophage-like FBM-G cells by the abundance of rough endoplasmic reticulum and the absence of azurophilic granules. Fibroblast-derived FBM-G cells phagocytosed collagen in a similar way, i.e., by internalization, as the macrophage-derived FBM-G cells did (44).

*Basophil-derived FBM-G cells*, derived from basophil-like cells, were observed with each type of implant. Their specific granules were of various sizes and (semi-)round in form, often containing densely-packed particles (Figs. 6a, b). Fine parallel-membranous structures were observed in some larger granules of multiglobular form (Fig. 6b). Multiglobular granules appeared to originate from fusion of many smaller granules (44). The granules often contained accumulations of Al/Si-crystals (Figs. 6c, d). Basophil-derived FBM-G cells were especially prominent when GDSC or Al/Si-agarose had been implanted.

Al/Si-crystals accumulated in smaller sized basophil-like (FBM-G) cells (Fig. 6d) from 3 weeks on in NDSC and Al/Si-agarose and from 10 weeks on in HDSC and

GDSC. At 15 weeks, the Al/Si-crystals accumulated in smaller sized basophil-like (FBM-G) cells were the only remnants of the original HDSC or GDSC implants.

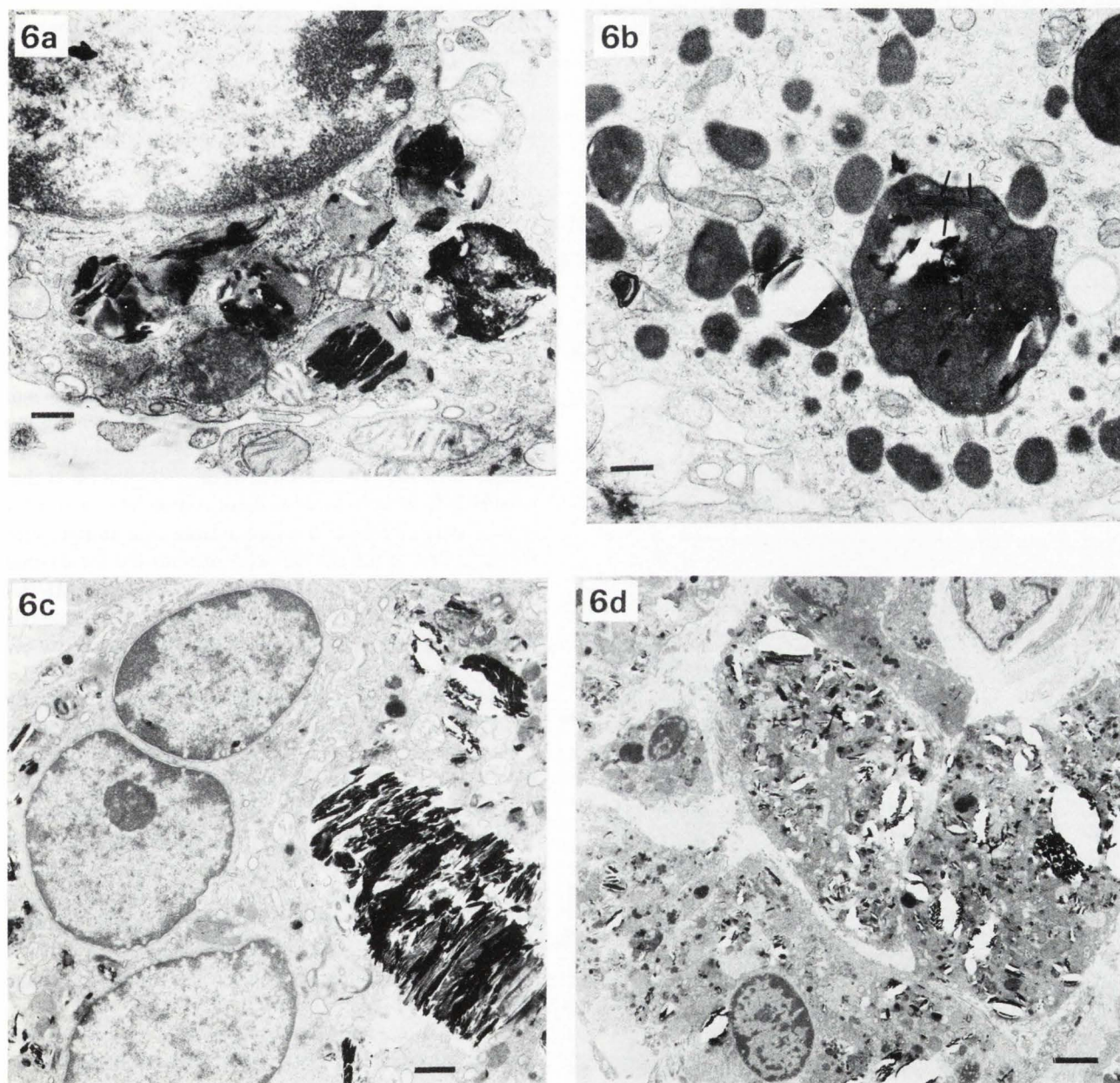
**Calcification.** Deposits of calcium (Ca)- and phosphorus (P)- containing crystals, possibly hydroxyapatite (44), were present only in HDSC implants. Small deposits were sometimes found intracellularly, in macrophages, fibroblasts or macrophage- or fibroblast-derived FBM-G cells from day 10 (Fig. 7a) till 3 weeks. Large Ca/P-deposits were present in some of the larger, not-internalized, collagen bundles of the 10 day-implant. The morphology of the latter Ca/P-deposits appeared to have changed in the 2 and 3 week-implant (Fig. 7b), since in those implants, increasing numbers of deposits with cleared central parts, leaving only contours of collagen fibrils, were observed.

## Discussion

The tissue interactions with discs of HMDIC- or GA-cross-linked and non-cross-linked DSC (respectively HDSC, GDSC and NDSC), subcutaneously implanted in rats, were evaluated using a TEM to advantage. Cytotoxic influences due to tanning were observed. Macrophage-derived FBM-G cells were specifically formed to phagocytose HDSC and GDSC. Basophil-like cells and FBM-G cells derived from them specifically phagocytosed Al/Si-crystals in each type of DSC. Al/Si-crystals were found to accumulate in these cell types. Calcification only occurred in HDSC. The different observations are discussed below.



## Dermal Sheep Collagen Implants



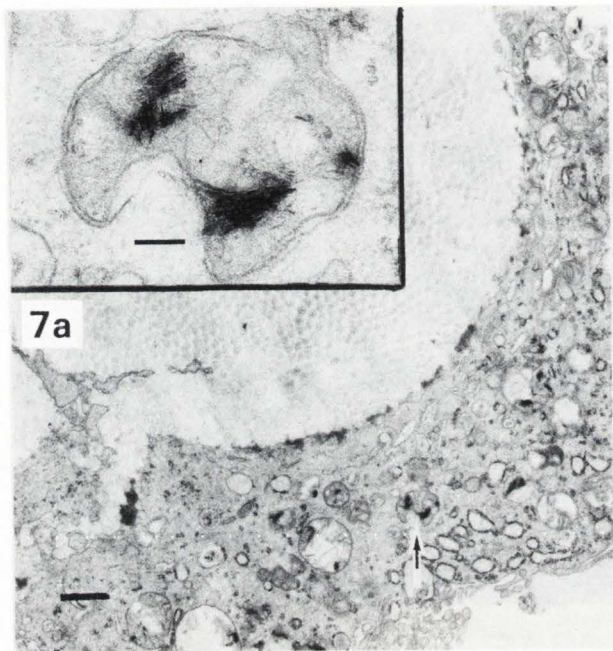
**Figure 6a:** Basophil-like FBM-G cell containing round or semi-round granules with densely-packed particles and Al/Si-crystals in GDSC at day 10. Bar =  $0.34 \mu\text{m}$ . **Figure 6b:** Basophil-like granules with densely-packed granules, Al/Si-crystals and parallel membranous structures (arrows) in GDSC at day 10. Bar =  $0.48 \mu\text{m}$ . **Figure 6c:** Basophil-like FBM-G cell with huge Al/Si-accumulation in GDSC implanted for 15 weeks. Bar =  $0.93 \mu\text{m}$ . **Figure 6d:** Smaller sized basophil-like (FBM-G) cells with basophil-like granules and Al/Si-accumulations in GDSC at 15 weeks (arrows). Bar =  $2.1 \mu\text{m}$ .

### Cytotoxicity, neutrophils and lipid accumulation.

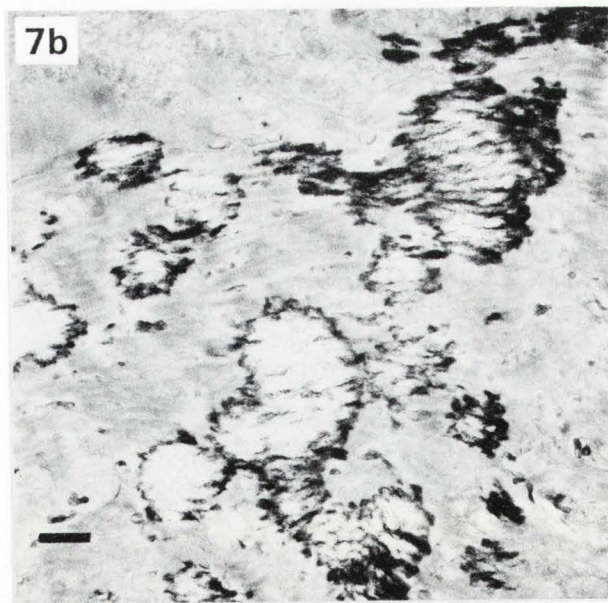
Deviant morphology of neutrophils was observed only with HDSC. Deviant neutrophil morphology, lipid formation and more than normal cell degeneration were the combined result of cytotoxic influences of HDSC. GA-cross-linking of DSC resulted in a cytotoxicity ob-

served as both extensive lipid formation and extensive cell degeneration. NDSC did not induce cytotoxic effects. In fact only few neutrophils were present in case of NDSC, which may be due either to the fact that NDSC was not experienced as 'very foreign' or to the fact that the collagen bundles turned into a impermeable gelatinous mass.





7a



7b

**Figure 7a:** Ca/P-crystals within mitochondria of a macrophage-like FBM-G cell. HDSC, implanted for 10 days. Bar = 0.65  $\mu\text{m}$ . Insert: detail. Bar = 0.10  $\mu\text{m}$ .  
**Figure 7b:** Deposits of Ca/P-crystals present in HDSC-bundles after 3 weeks of implantation. Most deposits have cleared central parts. Bar = 0.65  $\mu\text{m}$ .

The results more or less agree with the results presented in our *in vitro* studies (43, van Luyn *et al.*, unpublished results). When cytotoxicity was expressed as inhibition of fibroblast growth, GDSC induced a high in-

hibition of cell growth, whereas HDSC was moderately and NDSC only very slightly cytotoxic. Cytotoxicity of HMDIC-cross-linked collagen was reported previously (39, 43), notwithstanding the fact that Chvapil *et al.* (8, 9) evaluated HMDIC-cross-linked burn-dressings and collagen sponges in rats as toxicologically quite acceptable. We hypothesized before (44), that deviant neutrophil morphology might be related to HMDIC-tanning. This was confirmed by the observation that deviant neutrophil morphology was not found when GDSC or NDSC had been implanted. Deviant neutrophil morphology appears to be the result of *primary* release of cytotoxic substances, i.e., substances which were released without the direct interference of cells (43). The deviant neutrophil morphology at 2 days in both the surrounding tissue and the implant support this hypothesis. Deviant morphology can still be found at day 10 because at that time HDSC in those areas had not completely been infiltrated, which resulted in a slow release of cytotoxic substances. One of the substance(s) responsible for deviant morphology may be the hydrolysis product of HMDIC, i.e., diaminoethane (DAH), since diamines in general, and DAH specifically, were reported to be cytotoxic (4, 20, 47).

GA-cross-linking of DSC results in a cytotoxicity observed as both extensive lipid formation and extensive cell degeneration. Cytotoxicity of GA-tanned collagens has been reported previously (8, 10, 11, 17, 25, 36, 43). Extracellular lipid may result from degeneration of cells with high intracellular lipid content occurring after intracellular lipid formation, i.e., lipid-degenerative effects (46). Lipid-formation agrees with the results presented in our *in vitro* study (43), in which cultured fibroblasts, with HDSC, but especially with GDSC, showed, apart from high cell growth inhibition, huge lipid-accumulations.

It is hypothesized that, in general, cytotoxic effects from implanted cross-linked collagens are bound to occur, even when *primary* release of cytotoxic substances, i.e., substances, which can be removed by extraction independent of cellular interaction, would have been eliminated by extensive washing of the collagens. The reason is that *secondary* cytotoxicity, which is a cell interaction dependent cytotoxicity and involves the release of enzymes which degrade the collagen, will result in the release of various degradation products. Part of these degradation products will be cytotoxic, depending on the tanning agent used (43, Van Luyn *et al.*, unpublished results). Intracellular lipid formation is not necessarily the result of cytotoxic influences, since lipid droplets-filled neutrophils, but hardly any cell degenerations, were observed with NDSC. The presence of lipid droplet-filled neutrophils in NDSC is possibly explained by phagocytosis of lipid (48).



### Degradation of DSC, and migration and formation of other cell types

NDSC seems only to be degraded by an extracellular degradation pathway, since hardly any signs of phagocytosis were found. At three weeks, an increase in gelatinous (denatured collagen) substances as well as the original fibril structure were easily recognized; from this it appears that the degradation appears to occur at a slow rate. Both HDSC and GDSC are intracellularly degraded by phagocytosis of macrophage(-derived FBM-G cell)s and/or fibroblast(-derived FBM-G cell)s. This finding strongly suggests that the reason for formation of macrophage- and fibroblast-derived FBM-G cells is (in this case) the presence of cross-linked DSC, eligible for phagocytosis. Obviously, the non-cross-linked NDSC does not elicit a foreign body response in this sense. But in another sense it does. Basophil-derived (FBM-G cell)s were attracted to infiltrate NDSC, notwithstanding that NDSC appeared unattractive for other cell types. This indicates that the presence of basophil-derived (FBM-G) cells is related to the presence of Al/Si-crystals. Proof is also found in the fact that basophil-like (FBM-G) cells were also found with the Al/Si-agarose implants. Previously (44), as found during HDSC-degradation, a point of criticism was the possibility of basophil-like granules consisting (in part) of phagocytosed collagenous material, which was with time degraded, thereby accumulating the Al/Si-crystals, indicating that basophil-like cells were just other macrophages. Since in the case of Al/Si-agarose, the basophilic granules can not contain phagocytosed collagenous material, cells, with the specific granules, found with Al/Si-agarose further indicate the existence of basophil-like cells and basophil-derived FBM-G cells. The current theories on FBM-G cells (6, 22, 24, 27-30, 33) describe fibroblast-derived (6) and macrophage-derived (22, 24, 27-30, 33), but not basophil-derived FBM-G cells. Our results may therefore extend current theories.

Basophils, mast cells and eosinophils are cell types usually involved in allergic reactions (5, 14, 48). The attraction of basophil-like cells by DSCs and Al/Si-agarose may therefore be qualified as a kind of 'allergic' reaction, the 'allergens' being the Al/Si-crystals. Basophils and mast cells may originate from the same precursor cell (5, 14, 48), which may explain why in case of some types of basophilic granules, i.e., the multiglobular formed granules with fine parallel membranous structures, one could even qualify them as 'mast cell-like' granules. In case of NDSC, many eosinophils were observed at days 5 and 10. This may indicate that another 'allergic' reaction with this material occurred, the 'allergen' now being the collagen. An immunogenic reaction, in the sense that lymphocytes were attracted was not found with any of the materials. In a way, the presence

of lymphocytes was expected (23), especially in case of NDSC, since one reason for the tanning of collagen-based prostheses is to render this non-autologous tissue non-immunogenic (2, 32, 36). However, our results agree with other observations (1), and can be explained by this implantation not having enough presentation of antigens or being the first presentation; a second and third time may indeed result in a high incidence of lymphocytes (23). The other reason for cross-linking of collagens is the reduction in degradation rates, which is confirmed by the higher degradation rate of NDSC versus the reduced rates of HDSC and GDSC.

### Al/Si-crystals and calcification

Al/Si-crystals and their accumulations are undesirable. Before implantation, the three types of DSC appear to contain equal amounts of small-sized Al/Si-crystals. During degradation, these crystals are accumulated by cellular interactions, which were also observed with the Al/Si-agarose implants. However, a net increase in the amount of Al/Si-crystals also appears to occur, possibly not with NDSC, but to some extent with HDSC and even more so with GDSC, thereby leaving a 'metallic-like chip' at the implantation site after 15 weeks. A relation between cross-linking procedure and new formation of Al/Si, followed by Al/Si-accumulation, may therefore be possible, but is not understood. Calcification of collagen-based bioprotheses has previously been reported (3, 12, 13, 16, 35, 36, 38, 40). In general, the process of calcification is associated with well cross-linked collagen fibers, the cross-linker being glutaraldehyde or formaldehyde (21, 34, 36). Calcification is the major cause of failure for, e.g., implanted fixed heart valves. In contrast with these reports, we did not observe calcification during GDSC-degradation, but only during HDSC-degradation. The observed sequence of morphologies, i.e., the change from completely electron-dense to electron-dense edges with cleared central parts, where no more collagen could be observed, suggests another pathway of collagen degradation (21, 34, 44). As to the small deposits in mitochondria of macrophage and fibroblast (-derived FBM-G cell)s, we are unsure whether the mitochondria function as nucleation sites themselves or whether Ca/P-crystals ended up within these structures in the second instance.

### Conclusion

With this study, details, occurring during tissue interactions with DSC, were obtained. These details give more insight in cytotoxic influences from tanning of DSC and in processes such as the formation of foreign body multinucleate giant (FBM-G) cells, accumulation of Al/Si-crystals and calcification. These results may be



of use for a better understanding and further improvement of the function of collagen-based biomaterials in general. Cross-linking agents will always in some way be released, which remains a point of concern. Although it is claimed that, e.g., GA-tanning of porcine heart valves, which leak toxic substances, improves their function by prevention of cell ingrowth and therefore degradation (11, Van Luyn *et al.*, unpublished results), our group prefers the way of trying to modify collagen, in this case DSC, by using non-toxic cross-linking agents. Another point of concern is the presence of Al/Si-crystals. In order to get rid of them and the possibility of granuloma formation, we need to know more about their origin and the background of their possible formation during HDSC and GDSC-degradation.

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### Discussion with Reviewers

**F.H. Silver:** How are the results an extension of the previous paper (44) except to indicate that GDSC elicits a toxic response and NDSC does not?

**Authors:** The results of this paper are an extension of



the previous paper, because it proves that both HDSC and GDSC have cytotoxic influences, because NDSC does not induce the observed effects. The cytotoxic influences of HDSC differ from those of GDSC. This paper also shows that basophil-like (FBM-G) cells are attracted by and specifically deal with Al/Si-crystals (unrelated to collagen), because they were also found with Al/Si-agarose. Furthermore, the paper indicates that the presence of cross-links is a prerequisite for the formation of macrophage and fibroblast derived FBM-G cells, since these cells were not observed with NDSC and Al/Si-agarose.

**D.F. Williams:** The conditions of cross-linking are hardly mentioned. Were any studies undertaken to characterize the nature or degree of cross-linking with the two methods? Does gamma irradiation have any effect on the resulting structure?

**Authors:** The three materials were obtained from the Zuid Nederlandse Zeemlederfabriek; the cross-linking was not done by us. However, we measured shrinkage temperature, degradation by collagenase, and stress strain. These parameters as well as the *in vitro* (43) and *in vivo* characteristics function as starting points for modifications of DSC.

Yes, gamma irradiation results to some degree in chain scission. Therefore, gamma irradiated DSC will degrade more easily than, e.g., ethyleneoxide-sterilized DSC.

**F.H. Silver:** Is there any evidence that the purification enzymes were totally removed prior to cross-linking? What evidence exists the enzymes were not cross-linked to DSC? What concentration of cross-linking agents were used and for how long? How is the product characterized as collagen. How do we know it is triple-helical collagen? What types of collagen are present?

**Authors:** Please see the previous answer also. We did not measure biochemical parameters. Therefore, we do not know whether purification enzymes have been cross-linked to DSC. However, it seems unlikely since, after trypsin treatment, skins were pickled at pH 1-2 during one hour and thereafter stored during a (maximum) period of one year. The types of (denatured) collagen present are probably mainly types I and III, since these are the main collagen components of dermis, and the processing of DSC is mainly focussed on the retaining of the collagenous parts of the dermis.

The 68-nm periodicity of collagen fibrils (quaternary structure) is based on the tertiary structure (triple helix). The periodicity can be recognized in Figs. 1a and 7b.

**P.J. Klopper:** In view of the influence of tanning agents, please comment on the lack of controls with

diluted tanning agents.

**Authors:** Please see answer to first question of Dr. D.F. Williams above. We did not test controls with diluted tanning agents, because we did not do the cross-linking ourselves and just examined DSCs obtained from Zuid Nederlandse Zeemlederfabriek.

**F.H. Silver:** Is it really possible to distinguish between collagen and gelatin by electron microscopy? Elastin is amorphous when observed by TEM. How can one identify elastin-like substances and differentiate them from gelatin-like substances?

**Authors:** This is easily recognized in the non-implanted DSCs: collagen in cross-section is constructed of fibers, which, in transverse section at higher magnifications, show the 68 nm periodicity. Gelatin-like substances are less electron-dense than collagen, because it has lost the original fibril structure. Elastin-like substances are much more electron-dense and were defined as such because these substances had the same structure as the elastic laminae of (former) larger arteries, which were easily recognized in DSC.

**P.J. Klopper:** It is not clearly stated what the origin of the crystals in the non-implanted collagen is. Are they also present in normal sheep skin or are they the results of the processing of the material like polishing with Al/Si grindstones?

**Authors:** Apart from Al/Si-crystals being present in HDSC, they are now also found in (non-implanted) GDSC and NDSC. This proves, that these crystals have been present before cross-linking. Furthermore, there seems to be a clear relationship between these crystals and elastin-like substances. This makes formation during degeneration (upon flaying) likely and formation during some processing step, such as polishing with grindstones, unlikely. We have the impression that, especially in case of GDSC and to a lesser degree in HDSC, during implantation in the rat, new formation of Al/Si-crystals occurs. We are trying to quantitatively examine this, and first measurements confirm this hypothesis. If correct, then there is a relationship between new formation, cross-linking and the by the cross-linking induced environment.

**F.H. Silver:** The authors do not report deviant neutrophil morphology in G-DSC implant, yet conclude that it (G-DSC) is cytotoxic. What is the significance of deviant neutrophil morphology, if it is not sufficient to predict cytotoxicity?

**Authors:** The significance is that the cytotoxic influences of HDSC at the biochemical level differ from those of GDSC.



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**D.F. Williams:** A "kind of allergic reaction" seems rather imprecise and ambiguous. The conclusions appear to be suggesting some dose-response relationship. Is this consistent with existing knowledge? Is it feasible for DSCs to initiate different characteristics of an "allergic reaction" depending on the dose of "antigen"?

**Authors:** We do not know, because we did not examine it. However, it seems predictable that if higher numbers of Al/Si-crystals are present, also higher numbers of basophil-like(FBM-G) cells will interfere with them.

**D.F. Williams:** The discrimination of fibroblast-derived FBM-G cells from macrophage-derived cells is based on an abundance of endoplasmic reticulum and absence of azurophil granules. How clear-cut is this discrimination?

**Authors:** We checked many FBM-G cells, which phagocytosed collagen parts of HDSC or GDSC, and always found two morphologies, i.e., either with many azurophil granules (macrophage) or without azurophil granules, but then with an abundance of rough endoplasmic reticulum (fibroblast). However, we are aware of the possibility of intermediate forms, similar to what is discussed in literature about macrophages, fibroblasts and intermediate morphologies.

**P.J. Klopper:** In all specimens, cell death of mainly neutrophils was seen as is normal after implantation of foreign bodies, or was the death of cells more than expected? A comparison with agarose-discs and a short remark on the eventual cytotoxicity of aluminum is needed.

**Authors:** High numbers of infiltrating and dying neutrophils were especially observed when GDSC had been implanted. This is in contrast to low numbers of neutrophils with normal morphology when NDSC or Al/Si-Agarose had been implanted. Aluminum silicate is meant instead of aluminum; it is not toxic in the sense that rr are released; however, Al/Si-crystals might be considered toxic in the sense that they represent foreign bodies and evoke an 'allergic' reaction.

**P.J. Klopper:** The difference between normal and extensive cell degeneration between both types of tanned material is unclear. Was it measured by counting? The difference between both tanned materials appear to be lipid formation and extensive lipid formation, cell degeneration, and extensive cell degeneration. A score between agarose and collagens seems advisable to obtain an objective comparison.

**Authors:** We did not count cells and did not need to because differences were very obvious.

**P.J. Klopper:** In view of primary and secondary release of cytotoxic substances: it seems possible that both

tanned materials contain some remains of the tanning agents (not-washable). Also, degradation of collagen can be accompanied by the release of tanning agents. It means that there must be a correlation between the first days after implantation and the reaction seen at the time of degradation of collagen, with the restriction that transport of agents will be facilitated in latter phases by the restored circulation.

The authors introduce a third factor, degradation products of collagen following enzymatic degradation of collagen. Is this a new concept or was this also seen after degradation of catgut or related collagens.

**Authors:** Defining primary and secondary cytotoxicity is indeed a new concept; we could discriminate the two in our *in vitro* study (43) with HDSC.