Autologous Skin Grafts in the Rat. Biochemical Analysis of Mucopolysaccharides and Hydroxyproline.* (30303)

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By histological methods Medawar(1) demonstrated that the evolution of autografts passes through 3 periods, 1) a period of primary union and vascularization, 2) one of generalized hyperplasia, in which all cellular elements of the graft participate, and 3) one of partially retrograde differentiation, during which the grafts return toward the condition of normal skin. Information on chemical changes in the grafts is scarce. The concentrations and possible significance of histamine in full-thickness skin grafts of rats were reported by Marckmann and Zachariae (2). The aim of the present work was to study the levels of mucopolysaccharides and collagen in full-thickness skin autografts of rats. Biochemical determinations of hexosamine, representing both neutral and acid mucopolysaccharides, as well as determinations of hexuronic acid, representing acid mucopolysaccharides, were performed. Furthermore, the water content and content of hydroxyproline, an amino acid characteristic of collagen(3), were determined.

Material and methods. Seventy-four young male rats of an inbred Wistar strain (State Serum Institute, Copenhagen), weighing 178 $\pm$ 3.0 g were maintained on an adequate diet with free access to water. The rats were kept in the animal quarters for at least 3 days before operation. Seven rats served as controls, while 67 had full-thickness skin autografts. A circular piece of skin was excised from the right side of the back and freed of subcutaneous tissue, including the striated muscle *panniculus carnosus*, leaving the dermis proper for grafting. After turning the graft 180 degrees, it was repositioned by suture. Surgical procedures have previously been described(2). The animals were killed with ether at intervals from one to 103 days after surgery. The grafts were removed, and the utmost care was taken to include only grafted tissue in the samples. To exclude the marginal wound and the suture line a 2 mm broad brim was cut from the edge of the graft. On the left side, symmetrical skin was removed and freed of fat and *panniculus carnosus*. Representative specimens were obtained from grafts and symmetrical skin sites for histologic examination. After fixation in a 4% aqueous solution of lead subacetate for 24 hours, they were embedded in paraffin, sectioned and stained with a 0.5% aqueous solution of toluidine blue, which rather selectively stains acid mucopolysaccharides metachromatically.

Biochemical analyses were performed simultaneously on grafts and symmetrical skin samples. Symmetrical skin samples from non-wounded animals served to check the reliability of the technic and analytical procedures employed.

Determination of water content. Skin samples were weighed immediately after removal. The minced samples were lyophilized for 48 hours over phosphopentoxide at room temperature under a pressure of 2 mm Hg. After weighing, the samples were defatted by shaking one hour in acetone, one hour in ether, and, once more, one hour in acetone. Finally, the specimens were lyophilized for 48 hours. The weight now recorded is referred to as dry defatted weight. Values of the water content are given in per cent of dry defatted weight.

Biochemical studies. Thirty to fifty mg of dried, defatted tissue was homogenized in 25 ml of 0.5 N sodium hydroxide in an Ultra Turrax homogenizer for 14 minutes. The procedure was stopped midway to allow cooling in an ice bath.

Acid mucopolysaccharides were extracted in a cool room for 24 hours and isolated as described by Bollet *et al*(4) with minor modi-

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fications. Protein in 10 ml aliquots of the extract was precipitated with 0.6 ml of 70% perchloric acid. The mucopolysaccharide precipitate was redissolved in 1.5 ml of 2 M acetate buffer of pH 5. Duplicate 0.3 ml aliquots were taken for determination of uronic acid by the carbazole method of Dische(5), converted to a microscale. One and eight-tenth ml of concentrated sulfuric acid and 60 μl of a carbazole solution were added. For determination of uronic acid by Brown's orcinol method(6) converted to a microscale, other duplicate 0.4 ml aliquots were taken. One and two-tenths ml of orcinol reagent were added. The values were read in microcuvettes. Glucuronic acid was used as reference standard.

The content of hexosamine was determined on aliquots of the original tissue homogenates. The analysis was carried out as described by Kirk and Dyrbye(7) and Dyrbye(8), using a modified Elson and Morgan procedure(9) according to Boas(10). The samples were hydrolyzed for 2 hours at 118°C in 4 N hydrochloric acid in sealed glass ampoules. Previous investigation had shown that a maximum yield of hexosamine was obtained from the skin proper of rats by this procedure. Optical density was read on duplicate samples.

The content of hydroxyproline was measured on other aliquots of the original homogenates by Martin and Axelrod's modification(11) of Neuman and Logan's method (12). Optical density was read on duplicate samples.

Results. One graft removed 8 days after surgery showed gross and microscopic evidence of failure and was consequently excluded. All the rest of the grafts were considered fully viable. Hair growth in the graft was grossly observed as early as 8 days after surgery, and usually after 21 days there was a good pelt, the hairs growing in reverse direction.

In the dermis of the rat, the connective tissue ground substance shows only faint metachromatic staining with toluidine blue. The mast cells, however, are clearly demonstrated. They are most numerous towards the subcutis and regularly located around the vessels. Heavily granulated mast cells are predominant. Although no detailed countings were performed, comparison between graft and symmetrical skin revealed an obvious decrease in number of identifiable mast cells in the graft early after surgery. Eight to twelve days after surgery they reappeared in the grafts, and 16, 21, and 28 days after surgery the mast cells appeared more numerous in the graft than in symmetrical skin.

The results of the biochemical determinations are given in Tables I and II. In each group, the values of graft tissue were compared statistically to the values of symmetrical skin according to students "t" test, using the formula t = mean of differences divided by standard error of mean of differences. The data obtained from non-wounded animals confirmed that the technic was reliable.

Determination of the *water content* revealed an increase in the grafts immediately after surgery. Five days after the operation a secondary increase was noted. This increase was still pronounced 28 days after grafting. However, 54 and 103 days after surgery the water content of the grafts approached that of control skin.

The *hexosamaine* level was significantly elevated in the grafts even from the first day and remained so for about 8 weeks after surgery. One hundred and three days after grafting the content of hexosamine in the grafts did not differ significantly from control skin.

In 5-day-old grafts the content of *uronic acid* was significantly lower than in control skin. Thereafter, the concentration increased as compared to control tissue. Fifty-four and 103 days after surgery the content of uronic acid in the grafts was still slightly higher than in control skin, but the elevation was not significant.

The content of *hydroxyproline* showed a general tendency to decrease in the grafts.

Discussion. Changes in the chemistry of skin autografts cannot be discussed without reference to the alterations in the blood supply occurring simultaneously. In rats, Marckmann(13) examined the microcirculation in skin autografts, which in every respect were comparable to the grafts studied in this report. Forty-eight hours after surgery a slow

flow was noted in certain areas. The circulation was found to be normal 7 days after grafting, while, 11 to 20 days after surgery, an increased vascularity in the grafts was demonstrated.

Until the blood supply has been fully restored, the graft, or part of it, is suffering. Although an early influx into the graft by a fluid that is probably rich in hexosamine takes place, the maintenance of metabolic

No. of Animals		7	7	7	7	6	7	6	6	6	7	7
Time after Surgery		0	1d.	3d.	5d.	8d.	12d.	16d.	21d.	28d.	54d.	103d.
Water %	Graft	193°	213**	209°	238**	238*	239**	269**	273***	258**	203**	183*
		2.8	4.0	6.6	3.6	8.0	5.0	6.2	4.8	6.3	4.1	2.8
	Skin	186	193	192	209	197	196	226	205	212	178	171
		1.8	2.6	5.2	6.9	8.8	7.5	1.1	1.7	3.3	2.1	2.4
Hexosamine	Graft	3.54°	4.39***	4.54***	3.57***	4.17**	4.42**	4.60**	4.06**	4.77**	3.73*	3.29°
		.06	.06	.15	.10	.13	.21	.16	.13	.12	.15	.11
	Skin	3.60	3.58	3.50	2.79	3.29	3.43	3.75	3.32	3.52	3.24	2.94
		.07	.07	.05	.07	.16	.07	.04	.01	.19	.12	.16
Hydroxy-proline	Graft	88.1°	78.5°	77.6**	76.0*	75.2°	83.3°	88.9°	70.0*	93.4°	96.6°	89.0°
		2.6	1.3	3.2	1.8	4.1	4.7	3.6	2.8	3.4	3.1	2.0
	Skin	90.9	81.6	88.5	83.9	80.8	84.6	94.2	79.4	93.5	101.3	92.7
		2.8	1.1	1.3	3.1	4.2	2.9	1.5	1.3	1.9	1.6	2.7

TABLE I. Levels of Water, Hexosamine and Hydroxyproline in Skin and Autologous Skin Grafts of Rats.

Figures represent mean and s.e.m. values.
Water content is given in per cent of dry, fat-free weight.
Hexosamine and hydroxyproline content is given in μg/mg dry, fat-free tissue.
Values of grafts are significantly different from values of control skin at the 5% (*), 1% (**) and 0.1% (***) level of probability.
(°) indicates no significant difference.

No. of Animals		7	7	7	7	6	7	6	6	6	7	6
Time after Surgery		0	1d.	3d.	5d.	8d.	12d.	16d.	21d.	28d.	54d.	103d.
Uronic Acid (Carbazole)	Graft	.91°	1.01°	.83°	.73**	1.05°	1.46**	1.48**	1.47**	1.47**	.77°	.92°
		.03	.03	.06	.04	.07	.08	.06	.07	.08	.05	.04
	Skin	.86	.98	.86	.92	.93	1.12	1.03	1.10	1.02	.72	.88
		.04	.02	.04	.03	.03	.05	.05	.05	.04	.03	.03
Uronic Acid (Orcinol)	Graft	1.04°	1.09°	.99°	.89**	1.09°	1.54**	1.45**	1.52**	1.56**	1.14°	1.41°
		.05	.03	.06	.04	.03	.07	.05	.06	.08	.07	.09
	Skin	1.00	1.08	1.00	1.13	1.00	1.17	1.11	1.18	1.09	1.04	1.26
		.08	.02	.02	.04	.03	.03	.06	.05	.05	.05	.05
Carbazole to Orcinol Ratio	Graft	.88°	.93°	.84°	.81°	.96°	.95°	1.02°	.97°	.94°	.68°	.66°
		.03	.02	.03	.02	.04	.02	.04	.03	.04	.03	.04
	Skin	.88	.91	.86	.82	.93	.95	.94	.94	.94	.70	.71
		.05	.01	.03	.02	.03	.02	.03	.02	.03	.05	.03

TABLE II. Levels of Uronic Acid, and Carbazole to Orcinol Ratio in Skin and Autologous Skin Grafts of Rats.

Figures represent mean and s.e.m. values.
Uronic acid content is given in μg/mg dry, fat-free tissue. Symbols as in Table I.

equilibrium is obviously disturbed. One manifestation of this is the lowered content of hydroxyproline and uronic acid in the grafts 5 days after surgery. Generally, varying contents of hexosamine are considered to reflect alterations in the content of acid mucopolysaccharides. The figures presented here indicate that this is not true of skin autografts during the early period after operation. Actually, the values of "genuine" acid mucopolysaccharides related to the grafted connective tissue may be even lower than demonstrated in this study. The results include acid mucopolysaccharides invading the graft with the exudate from the wound bed. In human serum(14,15) and leucocytes(16), acid mucopolysaccharides have been demonstrated, and evidence has been presented that rat serum contains sulfo-mucopolysaccharides(17).

Once the blood supply has been restored, *i.e.*, about 7 days after grafting, reflected by a rise in the water content, a series of changes in the grafted connective tissue takes place. The content of acid mucopolysaccharides increases. In 28-day-old grafts it reaches a level which is about 50% higher than in control skin. Later, the connective-tissue biochemistry returns toward that of uninjured skin as to water content and concentrations of hexosamine, uronic acid and hydroxyproline. The alterations in mucopolysaccharides are similar to what has been found previously in repair tissue(18,19).

Chondroitin sulfate B contains as the major hexuronic acid component L-iduronic acid, which, compared to D-glucuronic acid, gives considerably less color by the carbazole determination, but slightly more by the orcinol determination(20). The ratio of carbazole to orcinol values for hexuronic acid in chondroitin sulfate B has been reported to be about 0.5(4, 20), and for heparin sulfate about 2.0 (21). The ratio of less than one found for hexuronic acid in the present study might indicate that chondroitin sulfate B is a constituent of acid mucopolysaccharides in the dermis and skin autografts of rats. Any definitive conclusion based on a comparison between the carbazole to orcinol ratios in grafts and in skin with regard to qualitative alterations in the acid mucopolysaccharides cannot be drawn from the values obtained. The increase in uronic acid content in the grafts may be explained in part by an increased content of hyaluronic acid. This presumption is supported by the strong water-binding capacity of hyaluronic acid(22,23) and the high water content found in the grafts 8, 12, 16, and 28 days after surgery.

The data presented lend further evidence to the concept of the response of connective tissue to injury(24). This response is characterized primarily by edema, then by mucinous and fibrous organization. They further parallel and supplyment Medawar's histologic observations on skin autografts(1).

Summary. The biochemistry of connective tissue in autologous skin grafts and intact skin of rats was studied. During the early period after surgery an increase in water and hexosamine content was observed in the grafts, together with a simultaneous decrease in concentration of hexuronic acid and hydroxyproline. The next period was characterized by a considerable increase in the content of acid mucopolysaccharides, and 54 and 103 days after grafting, the condition approached that of control skin. The alterations are considered part of the connective tissue response to injury.

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Characterization of Three New Rhinovirus Serotypes.* (30304)

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Although the rhinoviruses have only recently been recognized as a subgroup of the picornaviruses(1,2), a large number of apparently distinct serotypes has been isolated in various laboratories(3-11). The rhinoviruses have been noted to be small RNA viruses of approximately 15-30 mμ in size, ether stable, acid labile, and to grow best when rolled at 33°C(2). Three new serotypes were isolated in Houston, Texas, during a recent study involving a student population(12)and have been submitted to the World Health Organization collaborative study group as candidate rhinoviruses. Their properties are described in this report.

Materials and methods. Tissue culture. Primary African green monkey kidney (GMK), rhesus monkey kidney (RMK), human diploid (WI-38), and human aorta (A-39) cells were employed. The growth and maintenance of GMK(13) and of WI-38(14) have been described. The aorta cell lines have recently been described from this laboratory(15). All cultures were prepared in disposable constricted tubes(16) and grown stationary at 37°C until inoculation, after which they were rolled at 33°C.

Antisera. Antisera for the 3 new serotypes were prepared in baboons (*Papio doguera*) according to the method recently described (17). Antigens used for preparation of these antisera were purified by terminal dilution passages in A-39 and WI-38 (Table I). Thirty-one additional types of rhinovirus antisera were supplied by Dr. Maurice Mufson of the WHO International Reference Centre for Respiratory Viruses, National Institute for Allergy and Infectious Diseases, Bethesda, Md., and by Dr. Dorothy Hamre of the University of Chicago. Enterovirus antisera and antiserum for echovirus 28 (rhinovirus 1) were obtained from the stocks of the WHO International Reference Centre for Enteroviruses. The rhinoviruses used in the reciprocal neutralization tests were kindly supplied by Drs. Mufson and Hamre.

Acid lability test. This test and all additional tests performed in characterizing the candidate rhinoviruses were performed in continuous cell cultures of human origin (WI-38 or A-39). The method followed for determining acid lability has been described(18).

Ether sensitivity. Viruses were mixed with 20% fresh diethyl ether and incubated for 18 hours at 4°C. Viruses mixed with Eagle's medium containing 0.15% $NaHCO_3$ were treated in a similar manner. After incubation, ether was removed by evaporation at 33°C for 30 minutes and infectivity titer was determined in roller tube cultures.

Nucleic acid determination. Indirect evidence for determining the type of nucleic acid present was obtained by noting the sensitivity

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