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Control of Cell Multiplication in Epidermis.* (30107)

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Experiments on wound healing have provided considerable data on the mechanisms of cell replication and tissue renewal(1). The repair of a mechanical injury to the skin consists of restoration of the epidermis by multiplication and migration of epithelial cells, accompanied by a non-specific reaction of the connective tissue. Following a wound, the rate of mitotic activity in the adjacent epidermis increases 5- to 10-fold until continuity and full thickness are restored. The experiments of Bullough and Laurence(2) on the response of mouse ear epidermis to superficial wounds have strongly suggested that the proliferative response observed is due to a release of the uninjured tissues from inhibition that is normally present. Their data support the concept of Mazia(3) that "the stimulation of division in multicellular systems is the removal of a block . . . the stimulus is no more than permission of the cell to do what it would have done if it had been left alone in the first place."

Bullough and Laurence showed that the gradient of augmented mitotic activity adjacent to a wound extended for about one millimeter, with the highest activity adjacent to the wound. In the mouse ear, which is only 0.15 mm thick, the gradient of increased mitotic activity extended to the epidermis on the uninjured surface opposite the wound. That is, removal of the epidermis and superficial dermis from the dorsum of the ear resulted in the appearance of a high mitotic count in the ventral epidermis opposite the wound as well as in the adjacent 1 mm perimeter of dorsal epidermis. When the dorsal wound was 3 mm square, a difference in mitotic rate was found opposite the center of the square rather than opposite the wound margins, with the highest activity being opposite the center. Since the wound was 3 mm square and the gradient of increased mitotic activity extended for only 1 mm, this observation cannot be explained by the release of a stimulatory substance or "wound hormone" from the injured marginal cells, but can be readily accounted for in terms of the removal of inhibition of mitotic activity normally present in the epidermis.

In the experiments to be described, additional evidence for the inhibitory action of normal epidermis on epidermal cell replication will be presented. It will be shown that replacement of the resected tissue by a graft of intact epidermis results in suppression of proliferative activity in the nearby epidermis.

Materials and methods. Adult male Syrian hamsters weighing 90-120 g were used. Their ears measure approximately 0.6 mm in thickness and consist of a continuous layer of superficial epidermis supported by thin layers of dermal connective tissue containing hair follicles and sebaceous glands and a central core of cartilage measuring about 0.1 mm thick. Under barbiturate anesthesia, identical wounds measuring 5 mm square were made in the dorsum of each ear using a scalpel and fine forceps. With the aid of magnifying lenses, the epidermis and upper portion of the dermis were excised, leaving the lower half of the dorsal dermis, cartilage, ventral dermis and epidermis intact. One ear served

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TABLE I. Numbers of Labelled Cells Opposite a Wound.

Time (hr)	Untreated wounds (1)	Wounds filled by graft: Graft with label (2)	Wounds filled by graft: Graft—no label (3)	% suppression (Wound-graft / Wound) (4)
24	45 ± (3.2)	47.2 ± (6.0)†	47 ± (2.8)	-5
48	77 ± (4.5)	47.9 ± (2.3)*	70.2 ± (3.6)	37.7
72	62 ± (2.6)	43.0 ± (2.0)*	75 ± (3.6)	30.6
96	75 ± (9.9)	20.0 ± (1.3)*	—	73.3
120	17 ± (1.6)	6.0 ± (.6)*	—	64.7

Data derived from a study of 106 ear skin wounds, of which 42 were left open and 64 were filled in by application of an autograft or homograft of skin. Normal baseline labelling in the 5 mm lengths of epidermis examined was 3-10 basal cells, with an average of 6. Each of values in Table represents an average derived from an examination of at least 5 ears, except for (†) when only 3 specimens were available. Total counts with standard deviations at each time after operation are shown. Values in Column 2 marked (*) differ significantly (P less than 0.05) from corresponding values in Columns 1 and 3, according to Student's T test.

as a control: the wound was left alone, or in a few cases was covered with a skin graft that was sutured in place and then immediately removed. The latter procedure yielded the same results as simple wounding and served to control the effects of the trauma of grafting. The other ear received a graft which was sutured in place at the 4 corners of the square and consisted of the tissue just excised, or a comparable piece of skin from the opposite ear or ear of another hamster. The animals were caged individually to protect the operative sites. Selection of individuals for study on a particular day after operation was random, as the wounds and grafts were not evaluated until histological preparations were obtained.

At various times after operation, tritiated thymidine (Schwarz Bioresearch, 1.9 C/mM) was given in a dose of one microcurie per gram of body weight in 0.25 cc of isotonic saline by subcutaneous injection in the back. The thymidine is incorporated by cells which are synthesizing DNA in preparation for division, and the tritium label serves to identify these cells by the local effects of its low energy beta emissions on photographic emulsions. An index of cell replication is thereby obtained by radio-autography(4). Ears were removed 4 hours after the thymidine injection, fixed in 10% buffered neutral formalin, and imbedded in paraffin. Sections were cut 6-7 μ thick and oriented to provide a section of the full thickness of the ear through the middle of the wound or graft including the adjacent "host" tissue. Following removal of the paraffin with solvents, liquid emulsion (Eastman Kodak NTB3) was applied to the slides according to the method of Messier and Leblond(5). The emulsion was exposed for one month and then developed (Eastman Kodak D-19B). The slides were then lightly stained with hematoxylin to show nuclei. For a cell to be counted, the number of silver grains above the nucleus had to be at least 5 more than in a comparable area of background and was generally 10 to 20 more. A single section was examined from each ear. With the aid of an ocular micrometer, the tissue was subdivided for convenience into 0.33 mm segments and, using the wound edge as a starting point, all labelled cells in the adjacent and opposite epidermis were counted and the totals compared.

Results. The presence of a skin graft in a wound suppressed the proliferative response in the epidermis opposite the wound. The data are shown in Table I (columns 1 & 2). With an untreated wound, the number of tritium-labelled cells increased to approximately 7 × baseline values by 24 hours, rose to 12 × normal at 48 hours, and subsided to 3 × normal by 120 hours. When a graft was placed in the defect, the number of labelled cells opposite the wound at 24 hours was approximately equal to that in the control, but by 48 hours there was a 37.7% reduction, which may be taken as the degree of suppression by the graft (Table I, column 4). A similar degree of suppression was ob-

TABLE II. Results of Grafting.

Time (hr)	(1) Total No. of grafts	(2) Total No. of intact grafts studied			(3) No. of grafts Discarded*
		Total	With label	No label	
24	15	13	3 (23%)	10 (77%)	2
48	27	16	8 (50%)	8 (50%)	11
72	22	12	7 (58%)	5 (42%)	10
96	16	14	14 (100%)	0	2
120	16	9	9 (100%)	0	7

* These grafts were discarded either because the grafts were necrotic or because the tissue sections were folded or otherwise unsuitable for study.

served at 72 hours, and a peak of inhibitory activity was reached at 96 hours, when the number of labelled cells opposite a graft was 73.3% lower than that opposite a wound. By 120 hours, the labelled cell count opposite the graft had returned to baseline levels, whereas opposite the wound the count was still 3 × normal levels. At this time and at 144 hours there was in most cases fusion between the graft and "host" epidermis. Only at these times was a different effect of wound and graft on responses in the adjacent epidermis seen. No suppression could be demonstrated in this area compared to the opposite side. The reasons for this will be discussed below. There was no difference between autografts and homografts either in the success of the grafts or in their effect on the host epidermis, so the two groups were combined.

The absence of any suppressive effect at 24 hours and development of maximum inhibition at 96 hours were unexpected findings. When the sections were reexamined in an effort to account for them, thymidine uptake was studied in the grafts, and it was found that there was a progressive increase with time in the number of grafts containing labelled cells (Table II). Of the total number of grafts that were in place and suitable for counting (*i.e.*, not necrotic or excessively inflamed, etc.), only 3 of 13 contained any labelled cells at 24 hours, whereas every graft was labelled at 96 and 120 hours, with progressive increase during this interval. Since animals were selected at random for examination at a particular time, and since the rate of discard of sections because of poor quality was distributed rather evenly among the various groups (Table II, column 3), these differences in the numbers of grafts containing labelled cells are thought to represent a real difference between grafts at different times after operation. For example, the amount of exudate separating the graft from the host circulation might determine whether or not the label reaches the graft cells. As time passes, those grafts which have remained in place are in closer contact with the underlying granulation tissue and have greater access to nutrients and other materials from the host circulation. By the same token, an inhibitor made by such grafts would have greater access to the nearby host epidermis. Another possible explanation of the progressively increasing percentage of labelling among grafts is that the trauma of excision and handling lasts for a variable period and DNA synthesis is temporarily halted following the procedure. With time, those grafts which are viable resume metabolic activity. When grafts at 24, 48 and 72 hours were divided into 2 groups according to the presence or absence of labelled cells, the data shown in Table I (columns 2 & 3) were obtained. At 24 hours there was no difference in uptake of label by "host" cells opposite the wound between the 3 grafts having a few labelled cells and the 10 which did not. At 48 and 72 hours, however, the differences were significant, with the unlabelled grafts having no suppressive action.

Discussion. The theory that tissues regulate their growth by production of diffusible substances which inactivate templates for self-replication was enunciated by Weiss(6). In terms of the principle of organ-specific inhibitors of cell multiplication, it has been useful in explaining the decline in rate of cell division of various tissues as they reach maturity,

the marked increase in mitotic rate when many tissues are removed from the body into artificial culture media, the compensatory hyperplasia that follows removal of parts of organs or one of two paired organs, and the proliferative response of remaining tissue after injury. Considerable support for this hypothesis was subsequently developed by Weiss(6) in tissue culture and embryonic tissue experiments and by Glinos and Gey(7), Glinos(8), Saetren(9), and Stich and Florian(10) using the mitotic response to partial hepatectomy as a model system.

Additional evidence for the thesis of autoregulation of growth is provided by Bullough's study of epidermal wounds(2) and by the current work which grows out of his highly suggestive data, as outlined in the introduction. The clear suppression of cell multiplication in the epidermis opposite a graft as compared to that opposite an untreated wound indicates that the epidermis normally provides some inhibitory influence on cell division which is lost when the tissue is removed. The mechanism of this inhibition appears to be related to metabolic activity within the graft, since those grafts which were intact and histologically viable but did not incorporate radioactive thymidine failed to inhibit cell replication in host epidermis. The uptake of thymidine by graft cells is taken as an indicator of metabolic activity and significant interaction between graft and host tissues, and this suggests that further experiments utilizing labelled amino acids and various antimetabolites might provide important information regarding the nature of the inhibitor. The different effects on host epidermis according to whether or not the grafts contained labelled cells helps to rule out a non-specific effect of grafting as an inhibitory influence on mitosis, such as the graft acting as a barrier to the exudation of nutrients or stimulatory substances present in the exudate, or as a barrier to the migration of cells from the wound edges. In each group of grafts, such effects are theoretically possible, yet only those grafts having labelled cells reduced the proliferative response in the host tissue.

The current experimental model provides an opportunity to evaluate the concept of a wound hormone being released by injured cells in areas of trauma and stimulating cell replication(11,12). Since wounds of equal size were made in each ear, the number of injured host cells at the margins was the same. The addition of a graft to one side provided another group of injured cells, those bordering the inserted tissue. If injured cells were responsible for release of a material that stimulates mitotic activity, the epidermis opposite a graft should have displayed even greater cell labelling than that opposite a wound. Yet, as shown, grafting suppressed the proliferative response.

Other influences on mitotic activity must be considered. There was no inhibition of epidermal cell division among host cells adjacent to a graft until host and graft epithelia fused. In this region local factors, such as decreased cellular adhesiveness and migration of cells into the defect, probably outweigh the potency of available inhibitor. Also, the cells which migrate and multiply may be less differentiated in terms of synthesis of inhibitor than the more typically squamous cells of the superficial layers. It has been shown(2) that removal of the superficial strata of epidermis by the application and removal of adhesive tape stimulated mitotic activity in the intact underlying basal layers. When a graft is placed into a wound, the full thickness of epidermis is provided, including both the less differentiated basal layer and the better-defined prickle cells, which might be the source of inhibitor.

Summary. Excision of a piece of skin, including epidermis and superficial dermis, from the dorsum of the ear of a hamster resulted in marked proliferation of cells in the ventral epidermis opposite the wound. Restoration of the excised tissue by grafting suppressed this hyperplastic response by 30 to 75% at various times, indicating that tissue-specific inhibition of mitotic activity occurs in epidermis. The suppression of host cell proliferation was correlated with the presence of tritium labelled thymidine within the graft cells. This suggests that metabolic activity within the graft, rather than the mere presence of a graft in the defect, is necessary for the

inhibition of cell multiplication in the host epidermis.

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Influence of Hydralazine on the Sulfhydryl Group in the Presence of Cupric Ion.* (30108)

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Two antirheumatic drugs, gold thiomalate (1) and chloroquine(2) have been reported to inhibit the reactivity of the sulfhydryl group. Sulfhydryl group reactivity in turn controls the extent of sulfhydryl-disulfide interchange reaction(3) which can denature proteins(4) such as human gamma globulin (5) by forming intermolecular and new intramolecular disulfide bonds. Fig. 1 illustrates one type of sulfhydryl-disulfide interchange reaction between 2 protein molecules. It is possible that such denaturation might render proteins antigenic and play a role in the inflammation associated with rheumatoid arthritis and systemic lupus erythematosus. This hypothesis would be supported if it were shown that drugs(6) reported to induce rheumatic complaints in patients, increase the reactivity of the sulfhydryl group and promote denaturation by sulfhydryl-disulfide interchange. A study was therefore devised to determine whether these rheumatogenic drugs can increase sulfhydryl group reactivity by removing cupric ion which suppresses sulfhydryl group reactivity(7). Normal human serum contains approximately 1.7 μM "free" non-ceruloplasmin copper(8).

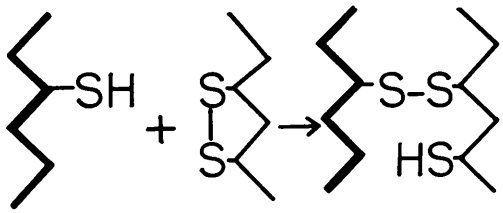

FIG. 1. Sulfhydryl-disulfide interchange reaction between 2 protein molecules.

Methods. Effect of hydralazine on reaction between bis(p-nitrophenyl)-disulfide and cysteine in presence of cupric ion. Bis(p-nitrophenyl)disulfide† reacts with cysteine by means of a sulfhydryl-disulfide interchange reaction to give rise to a yellow product that has been identified as p-nitrophenylsulfide(9). The concentration of this substance may be determined by its absorbency at 412 mμ. The parent compound bis(p-nitrophenyl)disulfide does not absorb significantly at this wave length. Reagents that react with the sulfhydryl group prevent the formation of the yellow color by reacting either with the sulfhydryl group of cysteine or the sulfhydryl group of p-nitrophenylsulfide(9). Accordingly, cysteine does not produce an increase in absorbency at 412 mμ when incubated with bis(p-nitrophenyl)disulfide in the presence of

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