*Résumé.* Le métabolisme des acides nucléiques des cellules du foie de souris de la souche $C_3H$ normales et atteintes d'une tumeur maligne, a été étudié à divers intervalles de temps avec de l'adénine marquée.

Dans le cas des souris normales, la proportion de RNA incorporée dans le noyau par rapport au cytoplasme se modifie fortement. Dans le cas des souris atteintes d'une tumeur maligne, ce rapport reste stable pendant la durée de l'expérience.

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*Chittaranjan National Cancer Research Centre, Calcutta (India), June 21, 1963.*

## Monocyte Participation in Connective Tissue Repair

### A Quantitative Study in Diffusion Chambers Using Sex-Chromatin as a Marker

1. *Introduction.* Fate and function of leucocytes that have migrated into a field of inflammation induced by mechanical or chemical trauma present a large number of unsolved problems.

Leucocytes in tissue culture have been shown to proliferate and differentiate into macrophages on the one hand and into fibroblast-like cells on the other (ALLGÖWER[1], BLOOM[2], HULLIGER and ALLGÖWER[3]). The origin of these fibroblast-like cells developing in buffy coat cultures was investigated by comparing thoracic duct lymph containing 99% small and medium sized lymphocytes in the rabbit with blood leucocytes in tissue culture (HULLIGER[4]). In thoracic duct cultures, fibroblasts developed only very rarely. Large mononuclear cells (monocytes and large lymphocytes) were therefore suggested to be the stem-cells for the fibroblasts developing in blood cultures. Similar observations have been made by SHELTON[5] comparing rabbit lymph and blood cells in diffusion chambers.

Large mononuclear cells in human blood have also been shown to take up tritiated thymidine (BOND et al.[6]) thus demonstrating their reproductive capacity.

The fibroblastic nature of the spindle cells developing in tissue culture has been proved by their capacity to form collagen precursors as measured by hydroxyproline formation (ALLGÖWER and HULLIGER[7]). SHELTON[5] demonstrated hydroxyproline by leucocytes growing in diffusion chambers as well.

On the basis of experiments done *in vitro* and *in vivo*, ALLGÖWER[1] has suggested that the leucocytes migrating into a field of wound-healing participate in the fibrocytic population of repair tissue.

To obtain more information on the quantitative aspects of the leucocytic contribution to fibroblastic repair, experiments with combined fibrocytic and leucocytic cell populations in diffusion chambers were planned; the diffusion chambers were chosen, since the reactive inflammation around them would provide an inflammatory milieu. Leucocytes were combined with fibrocytes to include possible reciprocal influences between the different cell types. To follow the fate of both cell types quantitatively, a cellular label was needed.

The use of a metabolic label such as tritiated thymidine to follow the fate of leucocytes was not considered suitable, since reutilization of leucocytic DNA as shown by DUMONT[8], FICHTELIUS and DIDERHOLM[9], and BRYANT[10] might interfere with the interpretation of the data.

Sex chromatin was chosen as a suitable marker for our experiments; its advantages have been discussed (HULLIGER et al.[11]).

A model type of granulation tissue was prepared by enclosing known numbers of fibrocytes and of leucocytes of opposite sex in diffusion chambers. The diffusion chambers were implanted into the peritoneal cavity of rabbits, where an inflammatory exudate formed around the chambers and small blood vessels covered the outer membranes. After 2–3 weeks the percentage of fibrocytic nuclei deriving from leucocytes was counted, using sex chromatin as a marker.

2. *Material and Methods.* (a) *Tissue.* Subcutaneous connective tissue was excised from the abdomen of rabbits weighing 2–3 kg. The tissue was chosen from the thin, almost avascular connective tissue sheet between skin and muscle. The tissue sheets were spread and cut into square pieces of 0.5–1 mm side length. One piece was explanted in a diffusion chamber either alone or together with a piece of buffy coat. At the same time 4 to 6 randomly chosen pieces were explanted in a plasma clot on coverslides and immediately fixed in 95% alcohol to determine the cell types being present in the explant.

Buffy Coat: Blood was obtained by carotid artery cannulation from a rabbit of sex opposite to that of the donor of the connective tissue. Buffy coat was prepared by centrifugation at 2000 RPM for 8 min in a refrigerated centrifuge using siliconized tubes. The supernatant plasma was pipetted off without disturbing the buffy coat. The remaining blood with buffy coat was centrifuged a second time at 2000 RPM, and then incubated at 37° until clotting of the buffy coat was complete. The buffy coat was cut into small pieces whose sides measured 1–2 mm. 4 to 6 randomly chosen pieces were used for the determination of the initial cell number.

(b) *Diffusion chambers* were prepared according to the technique described by ALGIRE[12] using two lucite rings of 18 and of 13 mm diameter that would fit into one another. Millipore[13] filters (pore size 0.45 μ) of the same size were glued to each ring with a special glue[14]. The rings with the filters were dried at room temperature for 1–2 h. The tightness of the filters on the rings was examined by dipping the rings into 70% alcohol, where places that were not glued tightly rolled up. The rings were then sterilized under an ultraviolet lamp overnight.

[1] M. ALLGÖWER, *The Cellular Basis of Wound Repair* (C. C. Thomas, Springfield 1956).
[2] W. BLOOM, Arch. exper. Zellforsch. *5*, 269 (1928).
[3] L. HULLIGER and M. ALLGÖWER, Schweiz. Med. Wschr. *91*, 1201 (1961).
[4] L. HULLIGER, Virchows Arch. ges. Physiol. *329*, 289 (1956).
[5] E. SHELTON, Xe Congr. int. Biologie, Paris (1960).
[6] V. P. BOND, E. P. CRONKITE, T. M. FLIEDNER, and P. SCHORK, Science *128*, 202 (1958).
[7] M. ALLGÖWER and L. HULLIGER, Surgery *47*, 603 (1960).
[8] A. E. DUMONT, Ann. Surg. *150*, 799 (1959).
[9] K. E. FICHTELIUS and H. DIDERHOLM, Acta path. microbiol. scand. *52*, 11 (1961).
[10] B. J. BRYANT, Exp. Cell Res. *27*, 70 (1962).
[11] L. HULLIGER, H. P. KLINGER, and M. ALLGÖWER, Exper. *19*, 240 (1963).
[12] G. H. ALGIRE, XV. Congrès de la Soc. Int. Chir. Imprimerie (H. DE SMEDT, Bruxelles 1954).
[13] Millipore Filter Corp., Bedford, Mass, Filter type HA.
[14] Millipore Filter Corp., Bedford, Mass, MF cement No. 1.

Pieces of connective tissue and of buffy coat were explanted either singly or in combination in one chamber using a small drop of 50% rabbit plasma in Gey's solution. The chambers were closed by pushing the smaller ring with the filter downwards into the larger ring, taking care not to squeeze the cells. The slit between the two rings was closed with glue. During the whole manipulation the two filters were kept moist with Gey's solution. After $^1/_2$–1 h, when the glue had dried, 8–10 chambers were implanted into the peritoneal cavity of a rabbit of the same sex as the donor of the connective tissue.

14–21 days later the animals were bled by carotid artery cannulation and the chambers were taken out. Their localization and vascularization were recorded.

(c) *Histology of filters.* The outside of the filters was cleaned with a moist gauze. The whole chamber was then dipped into 95% alcohol for 2–3 h until the glue had dissolved and the filters would separate easily from the lucite rings. The filters were stained according to the Feulgen method. After dehydration filters were mounted in HSR resin under especially thin coverslides.

(d) *Quantitative analysis. Explanted material. Connective tissue pieces* on coverslides were stained with Schiff reagent according to Feulgen. The size of the tissue pieces was then drawn using a 10× objective and a 6× measuring eye-piece. The areas were measured with a planimeter. Cell nuclei were counted in the tissue pieces in 20 squares of the 6× eye-piece using a 50× oil immersion objective. The total number of cell nuclei was then calculated.

*Leucocytes*: Total cell number in buffy coat pieces was determined by incubating buffy coat pieces in 4% acetic acid for 24 h. After this time the fibrin clot within the buffy coat has usually dissolved and the leucocytes can be counted as single cells in a counting chamber without further dilution. Differential counts were made from blood smears stained with Giemsa.

After cultivation: Cells cultivated in diffusion chambers were examined with a 100× oil immersion objective and a 10× eye-piece and were differentiated according to their shape and chromatin structure into fibroblasts, histiocytes, macrophages and lymphocytes. In chambers containing buffy coat only, the relative proportion of the above cell types was determined by couting 100–300 cells in one chamber. In chambers containing connective tissue or connective tissue and leucocytes only, fibrocytic nuclei were counted and their sex chromatin was registered.

3. *Results.* 10 experiments were performed with a total of 98 chambers implanted.

(a) *Cell counts in explanted material* are summarized in Table I. Connective tissue explants contained 7800 to 25000 cells per explant. Buffy coat pieces contained from 500000 to 800000 cells; of these an average of 7% were large mononuclear cells (monocytes and large lymphocytes) which means 35000 to 56000 per explant. The ratio of fibrocyte to large mononuclear cell in the mixed chambers was between 1:5 and 1:2.

(b) *Cell populations in chambers after 2–3 weeks.* Chambers containing buffy coat: 22 out of 25 chambers were covered with a large amount of small newly built vessels, the remaining 3 chambers were not well 'vascularized' and cells within the chambers were dead. 10 chambers were analysed and cells were classified according to their nuclear structure and shape into different cell types (HULLIGER et al.[11]); their distribution is shown in Table II.

Chambers containing connective tissue: 22 out of 26 chambers were well embedded in vascularized sites. In 25 out of 26 chambers connective tissue did not grow at all, only the nuclei of the explanted material were recognizable in some of the chambers. In one chamber macrophages of the host were invading between the slit of the two lucite rings. In this chamber fibrocytes were growing abundantly.

Chambers containing connective tissue and buffy coat in combination: 41 out of 47 chambers were well 'vascularized', whilst the remaining 6 chambers had no or very few blood vessels on their surface and the cells inside these chambers did not grow. In the well vascularized chambers there was abundant growth of fibrocytes mixed with macrophages.

The distribution of sex chromatin positive (Figure 1) and sex chromatin negative (Figure 2) fibrocytes in 33 out of 41 chambers is recorded in Table IIIa and IIIb. The rest of the 'vascularized' chambers were discarded due to technical difficulties in the work-up. The average of sex chromatin positive fibrocytes in mixed populations of female leucocytes and male fibrocytes is 43% with a range from 2% to 93% (Table IIIa). If male leucocytes were mixed with female fibrocytes, 63% of the fibrocytes were sex chromatin negative with a range from 37–100% (Table IIIb). Therefore roughly 50% of the fibrocytes present in mixed cultures after 3 weeks' cultivation in diffusion chambers were of 'haematogenous' origin.

*Discussion.* In a recent review on wound repair process in mammals, RUSSELL and BILLINGHAM[15] stated: 'Although from the evidence considered above, it seems an

Table I. Tissue explanted

| No. | Connective tissue | Buffy coat | Number of cells implanted into the diffusion chambers | |
|---|---|---|---|---|
| | | | Connective tissue | Buffy coat large mononuclear |
| 1 | ♀ | ♂ | 8900 | 59000 |
| 2 | ♀ | ♂ | 11000 | 42000 |
| 3 | ♂ | ♀♂ | 6500 | 50000 |
| 4 | ♂ | ♀♂ | 15700 | 12700 |
| 5 | ♂ | ♀♂ | 13400 | 47000 |
| 6 | ♂ | ♀♂ | – | 35000 |
| 7 | ♂ | ♀♂ | 21500 | 38000 |
| 8 | ♂ | ♀♂ | 12400 | 23000 |
| 9 | ♂ | ♀♂ | 22900 | 30000 |
| 10 | ♀ | ♂ | 13500 | 38000 |

Table II. Cellular population of buffy coat chambers after 2–3 weeks cultivation

| Exp. No. | Total cells counted | Fibrocytes % | Macrophages % | Histiocytes % | Lymphocytes % |
|---|---|---|---|---|---|
| 1 | 300 | 15 | 16 | 10 | 59 |
| 2 | 300 | 23 | 33 | 37 | 7 |
| 3 | 300 | 15 | 14 | 26 | 45 |
| 4 | 300 | 23 | 11 | 63 | 3 |
| 5 | 300 | 6 | 41 | 15 | 38 |
| 6 | 100 | 24 | 62 | 2 | 12 |
| 7 | 300 | 16 | 33 | 15 | 36 |
| 8 | 300 | 8 | 43 | 20 | 29 |
| 9 | 300 | 21 | 16 | 63 | 0 |
| 10 | 300 | 26 | 12 | 57 | 5 |
| Average | | 17.7 | 28 | 30.8 | 23.4 |

[15] P. S. RUSSELL and R. E. BILLINGHAM in *Progress in Surgery*, Vol. II by M. ALLGÖWER, Karger Basel/New York 1962).

almost inescapable conclusion that the majority of fibroblasts in a wound are of *local* origin, the possibility that some contribution to their number is made by transformation of mononuclear cells of hematologic origin cannot be excluded, especially in the case of the cornea'. In previous publications, however, we have suggested that in a repair area the hematogenous elements might form a much larger part of the final fibrocytic population than hitherto admitted. We had no quantitative data indicating to what extent this might be true. The experiments described in this paper confirm our previous hypothesis that mononuclear cells from peripheral blood substantially contribute to the fibrocytic population if migrated into a field of inflammation or repair.

In the combined explants used in our experiments we have taken larger amounts of leucocytes because of circulating leucocytes, only large mononuclear cells (average 7% or 11000–63000 per explant) can be considered as stem cells for fibrocytes (HULLIGER[4]). According to an investigation in man (BOND et al.[6]) on the basis of thymidine incorporation, only 0.4–0.7% of the large mononuclear cells are capable of division.

Little information is available as to the percentage of fibrocytes capable of division. At the moment of explantation of connective tissue all the cells are pyknotic, but after 24 h of cultivation *in vitro* most of the cells have regained the shape of a fibroblast with nucleoli. Many mitoses can be seen after 3–4 days of cultivation *in vitro* (ALLGÖWER[1]), so that one can assume that in our connective tissue explant a large percentage of the fibrocytes are capable of division.

In view of these findings we can state that our initial inoculum contained most probably more connective tis-

Table IIIa. Combined cultures of female leucocytes and male fibrocytes

| No. chamber | Host | % Leucocytic origin = sex chromatin-positive | % Fibrocytic origin = sex chromatin-negative | % Leucocytic contribution to final fibrocyte population |
|---|---|---|---|---|
| 3 a | ♂ | 10 | 90 | |
| b | | 21 | 79 | |
| c | | 30 | 70 | 20 |
| 7 a | ♂ | 73 | 27 | |
| b | | 90 | 10 | |
| c | | 15 | 85 | |
| d | | 93 | 7 | 68 |
| 8 a | ♂ | 25 | 75 | |
| b | | 4 | 96 | |
| c | | 81 | 19 | |
| d | | 34 | 66 | |
| e | | 38 | 62 | |
| f | | 18 | 82 | |
| g | | 15 | 85 | |
| h | | 17 | 83 | |
| i | | 13 | 87 | 26 |
| 9 a | ♂ | 72 | 28 | |
| b | | 73 | 27 | |
| c | | 94 | 6 | |
| d | | 71 | 29 | |
| e | | 61 | 39 | |
| f | | 53 | 47 | |
| g | | 27 | 73 | |
| h | | 2 | 98 | 57 |
| | | Average | | 43% |
| | | Standard deviation | | 31 |

Table IIIb. Combined cultures of male leucocytes and female fibrocytes

| No. chamber | Host | % Leucocytic origin = sex chromatin-negative | % Fibrocytic origin = sex chromatin-positive | % Leucocytic contribution to final fibrocyte population |
|---|---|---|---|---|
| 1 a | ♀♂ | 97 | 3 | |
| b | ♀ | 37 | 63 | |
| c | ♂ | 55 | 45 | |
| d | ♀ | 72 | 28 | 65 |
| 2 a | ♀ | 88 | 12 | |
| b | ♀ | 38 | 62 | 63 |
| 6 a | ♀ | 100 | 0 | |
| b | ♀ | 52 | 48 | |
| c | ♀ | 37 | 63 | 63 |
| Average | | 64 | | 64 |
| Standard deviation | | | | 26 |

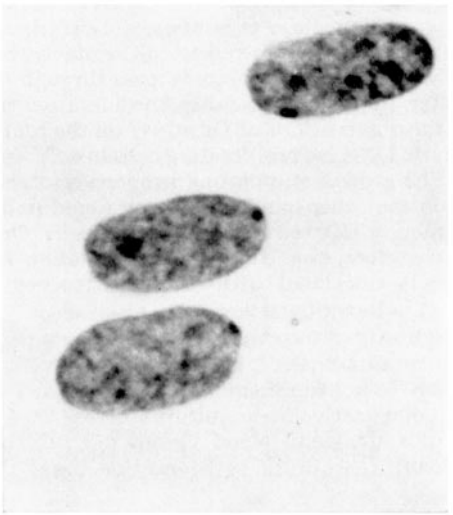

Fig. 1. Sex chromatin negative fibrocytic nucleus 1425 ×. Feulgen stain.

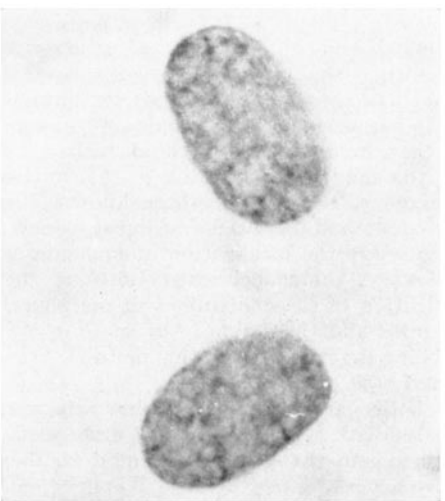

Fig. 2. Sex chromatin positive fibrocytic nucleus 1425 ×. Feulgen stain.

sue cells capable of reproduction than monocytes. We still find that 50% of the final fibrocytic population in our diffusion chambers is of monocytic origin.

In actual repair or inflammation, a continuous influx of hematogenous cells is likely to occur, whereas our 'model granulation tissue' only results from one initial cellular inoculum. Where no membrane hinders immigration of new monocytes, their participation in the final fibrocytic population should exceed that observed in our diffusion chambers.

The observation that fibrocytes did not grow if explanted alone in diffusion chambers is surprising and quite at variance with observations *in vitro*. The fact that infiltrating or added leucocytes induce abundant growth of the same tissue leads us to similar conclusions as CARREL[16]. On the basis of observations made on cultures *in vitro*, he suggested that leucocytes contain growth promoting substances called 'trephones', stimulating fibrocytic reproduction.

It was astonishing, anyhow, that the growth-promoting action of leucocytes surrounding the chamber was not transmitted through the Millipore filters with its pore-size of 0.45 µ. One has to assume, therefore, that any growth-promoting substance was present only in very low concentration and only active in cell-to-cell contact, or that its particles were of too large a size to pass through the pores of the filter. In this relationship consideration might be given to the observations of DUMONT[8] on the reutilization of leucocytic DNA by proliferating cells in areas of inflammation. The growth-stimulating properties of embryonic extract, on the other hand, have been found in a nucleoprotein fraction (KUTSKY and FEICHTMEIR[17]). One might assume, therefore, that the growth-promoting action of leucocytes is associated with reutilized leucocytic DNA transferred in larger particles.

One might argue that *in vivo* blood vessels will contribute a substantial part of the perivascular fibrocytes. This element is not represented in our combined cultures. In earlier comparative tissue culture studies (ALLGÖWER[1]). cultures of adult small blood vessels did not show any better growth than ordinary connective tissue. Furthermore, explants harbouring capillaries show no substantial difference in outgrowth from cultures taken in an avascular area.

On the basis of our investigations, we can conclude that the contribution of hematogenous monocytes to fibrocytic repair is considerable and appears to be in the order of magnitude of 50% or more of the final fibrocytic population[18].

*Zusammenfassung.* Monocyten des strömenden Blutes können *in vitro* die morphologischen und funktionellen (Hydroxyprolinbildung) Kriterien von Fibrocyten erwerben. Die quantitative Bedeutung dieses Phänomens für den reparativen Bindegewebsaufbau wurde mit folgender Versuchsanordnung geprüft: Vergleichbare Inokulate von Monocyten (enthalten in der Leucocytenhaut des zentrifugierten Blutes) und von Fibrocyten (aus subcutanem Bindegewebe) von Kaninchen verschiedenen Geschlechts, wurden in Millipore-Kammern eingeschlossen und für 14–21 Tage in das Abdomen eines Kaninchens implantiert. Die Auszählung des Sex-Chromatins in den fibrocytären Kernen am Ende der Züchtungsperiode ergibt, dass ca. 50% monocytären Ursprunges sind. Es wird gefolgert, dass dieses Phänomen bei der eigentlichen Wundheilung noch bedeutungsvoller sein dürfte, da der Zustrom teilungsfähiger Monocyten in einem Wundgebiet kontinuierlich vor sich geht und nicht auf ein initiales Inokulum beschränkt ist.

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[16] A. CARREL, J. exp. Med. *36*, 385 (1922).
[17] R. J. KUTSKY and T. V. FEICHTMEIR, Nature *194*, 1050 (1962).
[18] *Acknowledgment.* This work was supported by a grant of the Schweiz. Nationalfonds.

## Monoamines in Sympathetic Ganglia Studied with Fluorescence Microscopy

Biochemical and pharmacological studies have furnished evidence that the adrenergic transmitter, noradrenaline, is present in the sympathetic nervous system, not only in the peripheral terminals, but also in the cell bodies of the adrenergic postganglionic neuron[1]. Hitherto, however, the cellular distribution of this monoamine in the adrenergic neuron has not been known. Recently, a fluorescence method has been developed, which makes it possible to study the localization of monoamines at the cellular level[2–4]. Under different conditions, the content and localization of catecholamines in peripheral sympathetic neurons and the action of drugs on these neurons are now being investigated by this method. Some results are reported here.

Sympathetic ganglia of male albino rats were freeze-dried, treated with formaldehyde gas, embedded in paraffin, sectioned and the sections mounted for fluorescence microscopy according to FALCK[3].

By the formaldehyde treatment a specific green to yellow-green fluorescence developed in the perikarya of the majority of the ganglion cells, whereas the nucleus was non-fluorescent. The fluorescence was rather intense in a minority of the cells, whereas it was of medium or low intensity in most of them. The larger processes of the nerve cells exhibited a very faint fluorescence. A small number of cells were completely devoid of this type of fluorescence. These cells may be cholinergic[5]. In some prevertebral ganglia, adrenergic nerve terminals (FALCK[3]) with typical varicosities were observed. These terminals are not related to the blood vessels. Sometimes the fibres were seen to terminate in close contact with nerve cell bodies; and thus, in all probability, they represent synaptic terminals. In a study of the sympathetic ganglia of the cat, very numerous adrenergic terminals were observed in the inferior mesenteric ganglion[5].

[1] U. S. v. EULER, *Noradrenaline* (Ch. C. Thomas Springfield, Ill. 1956).
[2] A. CARLSSON, B. FALCK, and N.-Å. HILLARP, Acta physiol. scand. *56*, Suppl. 196 (1962).
[3] B. FALCK, Acta physiol. scand. *56*, Suppl. 197 (1962).
[4] B. FALCK, N.-Å. HILLARP, G. THIEME, and A. TORP, J. Histochem. Cytochem. *10*, 348 (1962).
[5] B. HAMBERGER, K.-A. NORBERG, and F. SJÖQVIST, Biochem. Pharmacol., in press.