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The Functions of Polymorphonuclear Leukocytes Emigrating into the Skin

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The functions of polymorphonuclear leukocytes (PMNs) emigrating into the skin and of others separated simultaneously from the circulation were determined and compared in healthy individuals. The PMNs emigrating into the skin were separated with a skin chamber technique. The C3 rosette forming capacities of the skin migrating and the circulating PMNs were similar. The chemotactic responsiveness was evaluated using three different chemoattractants: zymosan-activated serum, casein and lymphocyte-derived chemotactic factor. The skin PMNs gave practically no chemotactic response in the Boyden chamber, irrespective of the chemoattractant used. On the other hand, the skin PMNs exhibited higher plastic surface adherence, nitroblue tetrazolium reduction and *Candida albicans* killing activities. The data indicate that some functional activities of PMNs undergo alteration during in vivo emigration into the skin. The alterations, overall, may be of importance in the physiological protective function of the skin. *Key words: Chamber technique; C3 receptor; Chemotaxis; Adherence; NBT reduction; Killing.* (Received May 15, 1984.)

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Polymorphonuclear leukocytes (PMNs) play an important role in the protective function of the skin, due to their tissue invasive ability. After leaving the peripheral blood, the leukocytes traverse numerous physiological cellular and basement membrane barriers before arriving at the skin, where they exert their defensive function. A large number of data are known about the functional activities of PMNs in the circulation, but hardly any are available about the cells which have already emigrated into the skin. Two methods are employed in the main to investigate in vivo leukocyte emigration into the skin. The skin window technique described by Rebuck & Crowley in 1955 (1) is used to investigate the qualitative composition of the cellular exudate and to monitor the kinetics of leukocyte migration. This procedure permits little further functional study of the skin migrating cells. The skin chamber technique in which the PMNs are collected in a chamber fluid after detachment from the traumatized skin surface, allows better functional chracterization of the emigrating leukocytes.

In this paper we present a functional comparative study of circulating PMNs and of others emigrating into the skin in healthy individuals. Five functions of the circulating and the emigrating PMNs were simultaneously tested and compared: the C3 receptor activity (evaluated via rosette formation with complement-coated zymosan beads), the chemotactic responsiveness, the plastic surface adherence, the nitroblue tetrazolium (NBT) reduction and *Candida albicans* killing activities.

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PATIENTS AND METHODS

After giving informed consent, 25 healthy volunteers (all male, aged 18–59 yr) were included in the study.

Circulating PMNs were separated by dextran sedimentation of heparinized fresh venous blood (2). The red cell contamination was hemolysed. After double washing with TC 199 medium, a cell suspension containing more than 92–93% PMNs was obtained. The viability, as determined with the trypan blue exclusion test, was higher than 98% in every case.

The leukocytes emigrating into the skin were separated by the method described by Dubertret et al. (3). Briefly, a 1.5 cm^2 blister was created on the forearm by vacuum suction (about -450 g/cm^2), in order to separate the epidermis from the dermis. After removal of the blister roof, a sterile siliconized skin chamber (volume: 3.0 ml) was put over the denuded area and fixed in place with a watch-strap. Two ml serum free TC 199 medium containing 60 μg/ml penicillin and 60 μg/ml streptomycin was introduced into the chamber. After 20 h the contents of the chamber were drawn off. The cell exudate was washed twice with TC 199 medium. By this means a cell suspension containing more than 95% granulocytes could be obtained. The viability of the PMNs was higher than 90% in every case.

C3 rosetting test

The C3 rosetting test was carried out with complement-coated zymosan beads as described earlier (4). Granulocytes which bound at least 3 zymosan beads were regarded as rosettes. Zymosan beads sometimes found intracellularly were also considered to be bound.

Chemotaxis assay

The chemotaxis assay was carried out via a slight modification of that described earlier (5). Briefly, the Boyden chamber (blind type) was divided into two compartments with a cellulose nitrate membrane filter (Sartorius, pore size: 3.0 μm). Two hundred μl zymosan-activated pooled healthy donor serum, diluted to 50% with TC 199 medium was placed in the lower compartment of the chamber, while 400 μl granulocyte cell suspension (4×10^6 cells) was pipetted into the uper compartment. Casein (2 mg/ml PBS) (8 cases) and lymphocyte-derived chemotactic factor (LDCF) (5 other cases) were used as chemoattractants in place of zymosan-—activated serum. (LDCF was generated by stimulation of peripheral blood mononuclear cell cultures (3×10^6 cells) with 30 μg concanavalin A/ml. The stimulation was followed by a 24 h period of culturing. Subsequently, the cells were centrifuged and the supernatant was used as chemoattractant in the Boyden chamber.) The chamber was incubated for 1.5 h at 37°C. After staining, the cells which bad migrated to the lower side of the filter were counted microscopically and the result was designated the chamotaxis index.

Adherence assay

One ml granulocyte cell suspension (10^7 PMN/ml TC 199 medium supplemented with L-glutamine, 10% autologous serum and 10% AB pos mixed human serum previously heated at 56°C for 30 min.) was pipetted into a plastic well (Linbro, tissue culture multi-well plate, area per well approx. 9.62 cm^2, Flow General Inc.) (6). The plates were incubated for 45 min. at 37°C in a humidified atmosphere. The incubation was followed by mild shaking of the plates and the supernatant was drawn off into a tube. After gentle washing of the bottom of the wells twice with PBS, the cells which failed to adhere were counted. The percentage of adherent granulocytes was calculated via the formula:

$$\%\ \text{PMN adherence} = \frac{\text{total cell number—number of non-adherent cells}}{\text{total cell number}} \times 100$$

Nitroblue tetrazolium (NBT) reduction assay

A quantitative NBT reduction assay was carried out as described earlier (2, 6). The zymosan-stimulated NBT reduction capacity of the PMNs was measured. By means of a calibration plot obtained via the chemical reduction of NBT with ascorbic acid the results were calculated in units of fmol formazan/phagocyte.

Candida albicans killing assay

The *Candida albicans* killing assay was accomplished by a modification of the method of Lehrer & Cline (7), as described in detail earlier (8). Briefly, in the test system 1 ml PMN suspension (2×10^7 cells), 1 ml yeast cell suspension (4×10^7 *Candida albicans* cells/ml, strain 0656 CBS Delft) and 1 ml pooled human serum were incubated for 1 h in a water-bath at 37°C. The Candida cells were extracted from the PMNs and the yeast was subjected to staining with 0.1% methylene blue solution (1 h, 4°C).

The percentage of uniformly stained (only the unviable cells became stained) Candida cells was determined microscopically and designated the Candida killing index.

The tests were carried out in duplicate. Investigations of the circulating and the skin PMNs were always performed simultaneously.

The statistical comparison of the circulating and into the skin emigrating PMNs was carried out with the Student's paired *t*-test.

RESULTS

The results of the experiments are summarized in Table I.

The percentage of the skin migrating PMNs capable of forming rosettes with complement-coated zymosan beads did not differ from that in the case of the circulating cells: 71.27±2.37% of the circulating PMNs and 70.5±4.80% of the skin migrating leukocytes proved to be rosette forming.

A striking difference was observed in the chemotaxis studies. The leukocytes which had emigrated in vivo into the skin were practically unable to migrate in vitro in the Boyden chamber. The chemotactic activity of the circulating PMNs was 112.09±26.42 when zymosan-activated serum was placed in the Boyden chamber, 107.50±20.24 with casein and 67.60±27.44 with LDCF as chemoattractant. In contrast, the chemotaxis indices of the skin PMNs were 14.81±14.82 (zymosan-activated serum), 35.25±23.32 (casein) and 10.40±10.71 (LDCF), respectively. The differences were statistically significant ($p<0.02$).

The plastic surface adherence of the skin PMNs was significantly higher than that of the circulating leukocytes. 85.80±8.23% of the skin PMNs adhered to the plastic surface of the culture plates, while 41.40±5.86% of the circulating cells adhered to the walls of the dishes ($p<0.001$).

The NBT reduction activity and the Candida killing index are related, since both are indicative, at least partly, of the metabolic activation, i.e. the oxidative burst of the

Table I. *C3 rosette formation, chemotaxis, adherence, NBT reduction and Candida albicans killing activities of PMNs emigrating into the skin and of others separated simultaneously from the circulation in healthy individuals*

	Polymorphonuclear leukocytes			Statistical significance
	In skin	In circulation	Number	(p)
C3 rosette formation	70.54±4.80	71.27±2.37	11	NS
Chemotaxis				
ZAS	14.81±14.82	112.09±26.42	11	<0.01
Casein	35.25±23.32	107.50±20.24	8	<0.01
LDCF	10.40±10.71	67.60±27.44	5	<0.02
Adherence	85.80±8.23	41.40±5.86	5	<0.01
NBT reduction activity	21.19±5.01	17.15±5.49	25	<0.05
Candida killing activity	35.05±4.08	30.95±4.31	21	<0.05

C3 rosette formation designates the percentage of granulocytes capable of rosette formation with complement-coated zymosan beads. The chemotaxis indices refer to the mean number of granulocytes counted microscopically on the lower side of the filter in the Boyden chamber (ZAS: zymosan-activated serum, LDCF: lymphocyte derived chemotactic factor). The adherence is expressed as the percentage of plastic surface adherent leukocytes. The NBT reduction activity is given in units of 10^{-15} mol (i.e. fmol) formazan/phagocyte. The Candida killing index means the percentage of killed yeast cells (those uniformly stained with methylene blue) counted under a light microscope. The tabulated data are the mean±SD. Student's paired *t*-test was used for statistical calculation. NS statistically not significant.

granulocytes. Regardless of whether the NBT reduction or the Candida killing index was considered, the skin PMNs exhibited consistently higher activities than the circulating neutrophils. The NBT reduction of the skin PMNs was 21.19±5.01 fmol formazan/cell while that by the circulating PMNs 17.15±5.49 fmol formazan/cell ($p<0.05$). The Candida killing index was 35.05±4.08 for the skin PMNs and 30.95±4.31 for the circulating granulocytes ($p<0.05$).

DISCUSSION

As far as the technique presented here is concerned, we agree with Dubertret et al. (3) that the method is suitable for the separation of PMNs which have emigrated into the skin in vivo. The granulocyte suspension collected in the chamber is almost pure, and more than 90% of the PMNs are viable and intact. By this method the functional characterization of the cells can readily be achieved besides quantitative estimation of the in vivo cell emigration.

The capacity of the skin migrating PMNs to form rosettes with complement-coated zymosan beads did not differ from that of the leukocytes separated simultaneously from the circulation. The data indicate that the C3 receptor expressivity and/or avidity of the PMNs is not altered during the in vivo emigration into the skin. The unchanged receptor activity of the skin PMNs may correspond to the observation of Dubertret et al. (3), who found an unaltered phagocytosis of opsonized *Staphylococcus aureus* by PMNs emigrating into the skin chamber.

Once they had emigrated into the skin in vivo the PMNs were unable to exert chemotactic responsiveness in vitro in the Boyden chamber. The leukocytes were challenged with 3 different chemoattractants: zymosan-activated serum, casein and LDCF. The chemotactic unresponsiveness of the skin PMNs was observed with each of the chemoattractants used. In vivo locally generated chemotactic factors may account for the loss of in vitro chemotaxis by skin PMNs. Complement split products, for instance, have recently been detected in the suction blister fluid of healthy individuals (9). Preexposure of granulocytes to chemotactic factors is known to increase PMN adhesiveness, thereby limiting their capacity to migrate (10, 11). As the plastic surface adherence of the skin PMNs was significantly higher, we assume that the increased stickiness of the skin PMNs is responsible for their poor locomotion in the Boyden chamber.

The NBT reduction and Candida killing activities of the skin PMNs were higher than those of the leukocytes, separated from the circulation. As both of these tests are related to the metabolic state of the granulocytes, the data suggest that a metabolic activation takes place during the in vivo emigration into the skin. Our observation is similar to that of Dubertret et al. (3) who also detected a consistent increase of the bactericidal activity of the skin migrating PMNs. During the in vivo emigration into the skin the PMNs are subjected to chemotactic stimuli known to induce not only chemotaxis but also degranulation, superoxide anion production, chemiluminescence and activation of the hexosemonophosphate shunt (12, 13, 14). This metabolic stimulation may account for the increased NBT reduction and *Candida albicans* killing activities of the skin PMNs.

Our results show that some functional activities of the PMNs undergo alteration during the in vivo emigration into the skin. The cells which have an unchanged capacity to form rosettes with complemen-coated zymosan beads lose their chemotactic responsiveness and become metabolically activated. This phenomenon, overall, may be of major importance in the physiological protective function of the skin. The PMN which has emigrated to the site of an injury adheres to the wounded surface and loses its further chemotactic ability to destroy the invading agents with the products of its activated metabolism.

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