Cytochemical and Immunohistologic Investigations on the 
Source and the Functional Changes of Mononuclear 
Cells in Skin Window Exudates

By F. Schmalzl, H. Huber, H. Asamer, K. Abbredersis and H. Braunsteiner

The large mononuclear cells, which after 6–8 hours appear 
in the cellular exudate of skin windows may be derived from either 
lymphocytes, or monocytes, or tissue histiocytes. Various investigators have 
considered each of these cell types as the main source of the skin window 
mononuclear cells.1-7 Braunsteiner, labelling rat blood cells with 3H-thymi-
dine, proved that in the rat the mononuclear cells and the macrophages of 
skin windows are emigrated blood cells.8 As shown in other experiments 
on humans, these cells were capable of transferring delayed hypersensitivity 
in man.9,10 Based on cytochemical findings Leder4 and Wulff5 concluded 
that the typical large mononuclear cells in the skin windows are derived 
from blood monocytes, whose emigration from blood vessels into skin lesions 
has been extensively studied by Leder.11 Similar conclusions were reached 
by Trepel and Begemann6 after evaluating the phagocytic potentials of the 
migrated cells.

By injecting labelled cells of various origins into inbred animals, Volkman 
and Gowans12 observed that the large mononuclear cells of the skin windows 
are derived from monocytoid cells that have developed in the bone marrow. 
Similar observations about the origin of macrophages in inflammatory proc-
esses were made by Spector and colleagues13 and Everett and Tyler.14

A cytochemical procedure for identifying human blood monocytes has been 
published by Fischer and Schmalzl,15,16 which allows the distinction of 
monocytes from cells of the reticulohistiocytic system. The technic is based 
on the inhibition of the esterase activity, present in monocytes, by sodium 
fluoride (1.5 mg./ml.). Using substrates of the naphthol-AS-group and two 
different diazonium salts, we were able to demonstrate NaF-sensitive and 
resistant esterases on the same slide.16,18 Various other enzymes were also 
studied by means of cytochemical methods and their pattern correlated with

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This investigation was supported in part by funds of the Austrian Cancer Campaign, 
"Kampf dem Krebs."

First submitted October 2, 1968; accepted for publication March 27, 1969.

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the morphologic changes in these mononuclear cells. As there is no suitable cytochemical method for detecting lysozyme, this enzyme was investigated by immunohistologic methods.\(^{19}\)

**MATERIALS AND METHODS**

Following the method of Rebuck,\(^1\) skin window studies were carried out on ten healthy individuals; four patients with marked symptomatic monocytosis (945−1,650 monocytes/mm\(^3\)); three patients with acute myeloblastic leukemia; and three patients with acute monocytic leukemia. The leukemic monocytoid cells of these patients corresponded in their NaF-sensitive naphthol-AS-esterase activity to normal monocytes. As discussed elsewhere, this criterion is particularly useful in detecting monocytic leukemias.\(^{20}\)

Simultaneously, six coverslips were placed on six lesions on the thigh and removed after 1,2,3,5,7,9,12,24,27,32 and 35 hours. One coverslip was stained with May-Grunwald-Giemsa, the other five were stained with one of the following cytochemical reactions:

Nonspecific esterase using naphthol-AS-LC-acetate with or without the addition of 1.5 mg/ml NaF to the 0.1 M phosphate buffer solution.\(^{16}\) Occasionally a double incubation technic was employed for the demonstration of NaF-sensitive and resistant esterase,\(^{17}\) using fast blue BB salt e.c. (HOECHST) as coupler for the demonstration of esterase activity in the presence of and fast red violet LB salt in the absence of NaF.\(^{16}\) Using this procedure monocyes, characterized by a strong NaF-sensitive esterase, stained red; whereas, all other cells stained blue-violet to black (Fig. 1).

The presence of acid phosphatase was demonstrated according to the method of Barka and Anderson\(^{21}\) with naphthol-AS-Bl-phosphate and hexazotized pararosanlin (Fig. 2).

The naphthylamidase reaction was performed with L-leucyl-\(\beta\)-naphthylamide and fast garent GBC salt at pH 6.5\(^{18}\) (Fig. 3).

Sudanblack B-stain was employed according to the method of Sheehan and Storey.\(^{22}\)

The coverslips were counterstained depending upon the resulting color of the cytochemical reaction either with the Feulgen reaction or with acid Mayer’s hemalum.

Purified human lysozyme, kindly provided by Dr. E. F. Osserman, New York, was used for preparing antisera against this protein in two rabbits, employing complete Freund’s adjuvant. After adsorption with purified human IgG,\(^{28}\) the antiserum was monospecific against lysozyme as tested by conventional Ouchterlony technic.

Standard methods were used for obtaining the fluorescent Ig-antibody preparation. Coverslips with the exudate cells were air dried, fixed in 96 per cent ethanol, washed in 0.15 M phosphate-buffer (pH 7.2) and incubated with the labelled antibody preparation in a moist chamber at room temperature for 30 minutes. Simultaneously, other coverslips were treated with rabbit fluorescein-labeled anti-egg lysozyme. This antiserum does not cross react with human lysozyme and consistently gave a negative reaction. The positive reaction with anti-human lysozyme was strongly inhibited by prior treatment of the coverslip with unconjugated anti-human lysozyme (Fig. 4).

**RESULTS**

I. **Identification of the Mononuclear Cells**

Almost all of the mononuclear cells appearing after the first wave of neutrophil migration showed uniform strong NaF-sensitive esterase activity, which is characteristic for monocytes. A small number of cells were lymphocytes with weak esterase and acid phosphatase activity. The neutrophils also showed a weak, NaF-resistant esterase. Forms intermediate between lymphocytes and mononuclears were not present and there was no evidence for a transformation of lymphocytes into large mononuclear cells. Depending upon the manner in which the cells were arranged on the coverslips, the shape
and chromatin structure of the nucleus in the mononuclear cells varied greatly. Mononuclear cells and neutrophils in the central portions of the cell layers contained denser nuclei, exhibited intenser enzyme activity, and appeared smaller than comparable cells on the peripheral portions of the layers which showed more extended cytoplasm.

II. Transformation of Monocytes into Large Macrophages

During the first three hours the acid phosphatase activity in the few monocytes present on the coverslips appeared considerably lower than that of blood monocytes. Subsequently the activity of this enzyme became more prominent appearing initially only in an area close to the nuclear hilus. After 12–16 hours the characteristic strong acid phosphatase activity was present in the mature macrophages.

Monocytes contain a naphthylamidase, which was also present in the mononuclear skin window cells. Variations in the distribution of this enzyme during macrophage development were comparable to those of acid phosphatase. Fine sudanblack B-positive lipid granules were detected on the nuclear hilus after 4–12 hours. In the later periods macrophages contained varying amounts of sudanblack-positive substances.

When a fluorescein labelled antiserum against human lysozyme was used for the demonstration of this enzyme, monocytes and skin window macrophages stained specifically; whereas, lymphocytes were consistently negative.
Fig. 2.—Skin window, normal control. (A and B) Acid phosphatase localized in the nuclear hilus of mononuclear cells (fifth and twelfth hour). (C and D) Strong acid phosphatase activity in mature macrophages containing vacuoles (twenty-fourth hour). × 1800.

The intensity of the label varied markedly, and its changes were closely comparable to those of the other lysosomal enzymes studied by cytochemical methods. Moreover, after 3 to 7 hours the fluorescence was also confined to the nuclear hilus.

III. The Cellular Exudate in Patients with Monocytosis, Acute Myeloblastic and Acute Monocytic Leukemia

The cell sequence in skin windows of patients with symptomatic monocytosis was comparable to that of normal humans, the relative number of monocytes and related cells being somewhat increased. The transformation of blood monocytes into skin window macrophages in these four patients and the increase of acid phosphatase and naphthylamidase activity in these cells were also similar to normal persons.

In one patient with myeloblastic leukemia the exudation was apparently reduced; the other two patients showed a normal cellular migration sequence. The cytochemical features of the mononuclear cells in these patients underwent changes comparable to that of normal individuals.

In all three patients with acute monocytic leukemia the exudate was more cellular than that observed in normal persons and in patients with myeloblastic leukemia, and the predominant cell type during the first hours were not granulocytes but esterase-positive leukemic monocytes. Towards the third to sixth hour the number of monocytes on the coverslips decreased, but re-
Fig. 3.—Skin window mononuclear cells, 7 hours after the onset of the experiment. Localization of naphthylamidase (A) and sudanblack B-positive phospholipids (B) in the nuclear hili (arrows). X 1200.

Fig. 4.—Lysozyme localization in human skin window cells. (A) Strong positive immunofluorescence in the nuclear hilus of a mononuclear cell (seventh hour); (B) The cytoplasm of mature macrophages is labelled (twenty-seventh hour). Neutrophils are also positive (A). X 1400.
### Table 1.—Relevant Hematological Data

<table>
<thead>
<tr>
<th>Patients</th>
<th>WBC (mm$^3$)</th>
<th>Monocytes %</th>
<th>Lymphocytes %</th>
<th>Granulocytes %</th>
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<tbody>
<tr>
<td>Symptomatic Monocytosis</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1. Cs.</td>
<td>3600</td>
<td>29</td>
<td>8</td>
<td>63</td>
</tr>
<tr>
<td>2. Pl.</td>
<td>4500</td>
<td>21</td>
<td>15</td>
<td>64</td>
</tr>
<tr>
<td>3. Ha.</td>
<td>7500</td>
<td>22</td>
<td>18</td>
<td>60</td>
</tr>
<tr>
<td>4. Ob.</td>
<td>6300</td>
<td>19</td>
<td>23</td>
<td>58</td>
</tr>
<tr>
<td>Monocytic Leukemia</td>
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<tr>
<td>5. Pl.</td>
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<td>20</td>
<td>44</td>
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<tr>
<td>6. Wo.</td>
<td>18700</td>
<td>93</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>7. Au.</td>
<td>12000</td>
<td>67</td>
<td>17</td>
<td>16</td>
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<tr>
<td>Myeloblastic Leukemia</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Ko.</td>
<td>49000</td>
<td>1</td>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td>9. Po.</td>
<td>15000</td>
<td>2</td>
<td>10</td>
<td>88</td>
</tr>
<tr>
<td>10. Gl.</td>
<td>4500</td>
<td>1</td>
<td>12</td>
<td>87</td>
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Fig. 5.—Exudation of blood cells 1 hour after the onset of skin window experiments in patients with various hematologic disorders. (A) Patient with symptomatic monocytosis (29 per cent monocytes), (B) acute myeloblastic leukemia, (C and D) monocytic leukemia. May-Grunwald-Giemsa stain. A, C, and D X 1800; B X 1400.

mained largely above the normal range. After the sixth to eighth hour, the number of monocytes increased again and resembled in this respect the normal mononuclear migration.

The esterase activity of circulating, as well as exudate monocytes, and macrophages was decreased in two patients by about one third as compared with normal monocytes and skin window macrophages; in the third patient the leukemic cells and macrophages exhibited a normal esterase activity. In all cases the esterase activity of monocytes and macrophages was inhibited by NaF.

In the three cases studied almost all of the leukemic monocytes underwent the morphologic and functional transformations into macrophages. However, the increase in acid phosphatase, naphthylamidase and lysozyme appeared to be delayed and slightly reduced. In only a few cells during the early periods the characteristic hilar or paranuclear localization of these three enzymes was detectable.

**DISCUSSION**

I. Characterization of Macrophages

Under a variety of in vitro and in vivo conditions, lymphocytes transform into blast-like cells\(^{24,25}\) and show some features earlier considered as characteristic of macrophages and related cells (e.g., lysosomal-like structures
and functions related to these organelles). Some investigators, in contrast to others, even claimed that they observed in vitro the transformation of lymphocytes into typical macrophages. A similar controversy exists concerning the origin of skin window macrophages.\textsuperscript{1,3,4,7,10,18} There is some indication that the poorly defined morphologic distinction between lymphocytes and cells of the monocyte series explain some of these controversial results. In this and in other publications we reported on two criteria which distinguish clearly between these two cell types. Macrophages derived from monocytes in culture,\textsuperscript{26-28} and those in skin window lesions, contain an active non-specific esterase, whose presence may be demonstrated by the use of either α-naphthyl-acetate or naphthol-\textsc{as}-\textsc{lc}-acetate.\textsuperscript{4,5,16,18} When the latter substrate is used, the esterase in monocytes and macrophages (derived from monocytes) is almost completely inhibited by 1.5 mg./ml NaF.\textsuperscript{16,18} By contrast, lymphocytes and immature cells of these series show only low, NaF-resistant esterase. Moreover, monocytes and macrophages contain an IgG-receptor with subclass specificity, whose presence has not been proved in circulating lymphocytes.\textsuperscript{29-31} A variety of other circulating and tissue cells were studied, but excepting macrophages, only monocytes were positive in respect to both criteria.\textsuperscript{31}

\section*{II. Origin of Skin Window Mononuclear Cells and Macrophages}

With the aid of the double incubation method for the demonstration of NaF-resistant and sensitive esterase, the identification of monocytes in granulomata, in various exudates and in the walls of abscesses, as well as in pus, was possible.\textsuperscript{16} Likewise monocytes and monocytic precursors can be demonstrated in the bone marrow.\textsuperscript{32} Leukemic infiltrates from monocytic leukemia also are easily distinguished from other cellular infiltrates.\textsuperscript{16,33} Using this cytochemical method, the mononuclear cells and macrophages appearing in skin window exudates are unequivocally identified. Our experiments suggest strongly that circulating monocytes are not only related to, but in fact are, the precursors of skin window macrophages. There was no indication of a de novo development of this NaF-sensitive esterase. Despite the profound metabolic changes in lymphocytes during phytohemagglutinin stimulation with concomitant increase in esterase activity, the NaF-sensitive variant of esterase was not found present.\textsuperscript{34}

These experiments and our previous results on the myelogenous origin of monocytes\textsuperscript{32} agree with the findings of Volkman and Gowans\textsuperscript{12} and other workers on experiments carried out on animals, which also indicate a myelogenous origin of inflammatory mononuclear cells.\textsuperscript{13,14}

\section*{III. Intracellular Events Associated with Macrophage Formation}

The hydrolytic and oxidative enzyme activities of mature macrophages differ markedly from those of monocytes.\textsuperscript{18,25,35,37} The fine structural and biochemical events occurring during the transformation of the monocytes into macrophages have been studied by various means.\textsuperscript{28,35,36} Characteristically the Golgi zone, lysosomal-like structures, and enzymes related to these organelles increase in size or activity during transformation.\textsuperscript{23,36}
The decrease of acid phosphatase, naphthylamidase, and lysozyme at the onset of the skin window exudation may correspond to the degranulation of the monocytic cytoplasm noted by other authors. Subsequently, these enzyme activities are localized in the nuclear hilus, where the Golgi zone is situated. Electron microscopic findings, especially those in connection with autoradiography, strongly suggest that these hydrolytic enzymes are not the result of phagocytosis of extracellular enzyme proteins, but are developed inside the cell. Phagocytized particles—mainly granulocytic cytoplasm—of various sizes are seen frequently in the skin window mononuclear cells. Their localization, however, does not correspond to the enzyme activities situated primarily in the hilus.

Thus it may be concluded that transformation of human monocytes into skin window mononuclear cells and macrophages is associated with a marked increase in lysosomal enzymes, which appear first in the nuclear hilus or in paranuclear regions—corresponding to the Golgi zone.

Various enzymes of the Krebs cycle and some other dehydrogenases and diaphorases were studied by means of cytochemical methods by Wulff. He was able to demonstrate increased enzyme activities associated with the development of the mononuclear cells, which suggest an activation of these energy-yielding pathways. In earlier studies on mononuclear cells Wulff found an enhanced glycogen synthesis and an increased phosphorylase activity; recently these findings were confirmed by Rebuck et al.

IV. Exudative Response in the Skin Windows of Patients with Acute Monocytic Leukemia

It is now generally accepted that immature cells do not or only rarely appear in the cellular exudate of skin windows. In acute myeloblastic leukemias the exudates show a normal cellular composition, and in severe cases the number of emigrated cells is reduced.

The findings in three patients with acute monocytic leukemia differed in some instances from the results in other leukemia cases and in normal individuals. In the first hours of the experiments monocytic cells appeared in large numbers. At this time the monocyte to granulocyte ratio ranged between 1:1 and 1.5:1 in 5 experiments. By contrast a pattern comparable to that found in normal persons was found in patients with symptomatic monocytosis. In these patients only the relative number of monocytes in the skin window exudates was slightly increased, mainly in the latter periods of the experiments. Thus the early exudation of monocytes appears as a specific feature of monocytic leukemia and as such may be of some diagnostic value.

In the patients with acute monocytic leukemia almost all of the monocytoid cells in the skin windows transformed to typical mononuclear cells and macrophages. An increase of lysosomal enzymes also occurred but it was not as marked as in the controls. In the two patients with a reduced esterase content of circulating monocytoid cells, the mononuclear cells at the skin lesions also showed reduced esterase activity differing thus from normal monocytes. This finding suggests that leukemic cells and not residual “normal” monocytes form the cellular exudates in these patients.
These results show, therefore, that leukemic monocytes—even if immature and of atypical enzyme pattern—share at least some functions with normal monocytes and differ characteristically from cells of other types of acute leukemias.

**Summary**

The cellular exudates in skin windows of 10 normal controls, 3 patients with myeloblastic leukemia, 3 patients with acute monocytic leukemia and 4 patients with symptomatic monocytosis were evaluated by cytochemical methods (naphthol-AS-esterase, acid phosphatase, naphthylamidase, sudan-black B) and by immunofluorescence using a specific antiserum against human lysozyme. Monocytes and derived cells were identified by their content of characteristic NaF (1.5 mg./ml.) -sensitive naphthol-AS-esterase, whose presence was demonstrated by a double incubation method. After an initial decrease, corresponding to degranulation, the acid hydrolases appeared again in the Golgi zone. Macrophages with NaF-sensitive esterase and strong lysosomal enzyme activities became apparent after 12–16 hours, and there was no indication of a development of these cells from lymphocytes. In myeloblastic leukemia and symptomatic monocytosis the cellular exudate was comparable to that of normal humans. In monocytic leukemia an early exudation, predominantly of monocytoid cells, (instead of granulocytes) was a consistent finding. The transformation of monocytoid cells to typical macrophages with an increase of the lysosomal enzymes occurred with enzyme activities remaining lower than in normals.

**SUMMARIO IN INTERLINGUA**

Le exsudatos cellular in fenestras cutanee de 10 normal subjectos de controlo, 3 patientes con leucemia myeloblastic, 3 patientes con acute leucemia monocytic, e 4 patientes con symptomatic monocytosis esseva evaluare per medio de methodos cytochimic (naphthol-AS-esterase, phosphatase acide, naphthylamidase, nigro sudan B) e per le metodo immunofluoroscentic con le uso de un antisero specific contra lysozyma human. Monocytes e cellulas derivate esseva identificate per lor contenuto de caracteristic naphthol-AS-esterase sensibile a NaF (1,5 mg/ml). Le presentia de iste naphthol-AS-esterase esseva demonstrate per un metodo a duple incubation. Post un declino initial, correspondentemente a degranulation, le hydrolases acide reappareva in le zona de Golgi. Macrophagos con esterase sensibile a NaF e forte activitate de enbyma lysosomal deveniva apparente post 12 a 16 horas. Esseva notate nulle indication de un disveloppamento de iste cellulas ab lymphocytes. In leucemia myeloblastic e monocytosis symptomatic, le exsudato cellular esseva comparable a illo de humanos normal. In le patientes con leucemia monocytic, un precoce exudation—predominantemente de cellulas monocytoid (in loco de granulocytes)—esseva un constante observation. Le transformation de cellulas monocytoidic ad in macrophagos typic con un augmento del enzymas lysosomal occurreva con nivellos de activitate enzymatic infra le nivellos normal.

**REFERENCES**


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