Degranulation of leukocytes in chronic granulomatous disease

Robert L. Baehner, …, Morris J. Karnovsky, Manfred L. Karnovsky


Quantitative chemical analyses of the subcellular distribution patterns for acid and alkaline phosphatase, beta glucuronidase and peroxidase were obtained for human peripheral blood leukocytes of four patients with chronic granulomatous disease (CGD). Five young adults with acute infections served as controls. The observations were made on fractions obtained by homogenization and centrifugation of leukocytes previously incubated with or without particles for ingestion. Distributions in resting CGD and normal cells were very similar for acid and alkaline phosphatase and peroxidase, but the proportion of beta glucuronidase in the granule fraction of CGD cells was depressed, with an increased proportion in the soluble fraction. Release of granule-bound enzymes during phagocytosis of a variety of particles was the same for CGD and control cells, except that release of beta glucuronidase was less marked in CGD cells. Total enzymatic activity of CGD cells for the hydrolases studied was normal. The data indicated that granular enzymes are released in a normal fashion in phagocytizing CGD cells. Supportive evidence of release of enzymes into the phagocytic vacuole of CGD cells was obtained by an electron microscopic study of myeloperoxidase.

Find the latest version:

http://jci.me/105967/pdf
Degranulation of Leukocytes in
Chronic Granulomatous Disease

ROBERT L. BAEHNER, MORRIS J. KARNovsky, and MANFRED L. KARyovsisky

From the Departments of Biological Chemistry, Pathology, and Pediatrics,
Harvard Medical School, Boston, Massachusetts 02115, and the Division of
Hematology of the Department of Medicine, Childrens Hospital Medical
Center, Boston, Massachusetts 02115

A B S T R A C T  Quantitative chemical analyses of the
subcellular distribution patterns for acid and alkaline
phosphatase, beta glucuronidase and peroxidase were
obtained for human peripheral blood leukocytes of four
patients with chronic granulomatous disease (CGD).
Five young adults with acute infections served as con-
trols. The observations were made on fractions obtained
by homogenization and centrifugation of leukocytes pre-
viously incubated with or without particles for inges-
tion. Distributions in resting CGD and normal cells were
very similar for acid and alkaline phosphatase and per-
oxidase, but the proportion of beta glucuronidase in the
granule fraction of CGD cells was depressed, with an
increased proportion in the soluble fraction. Release of
granule-bound enzymes during phagocytosis of a variety
of particles was the same for CGD and control cells,
except that release of beta glucuronidase was less marked
in CGD cells. Total enzymatic activity of CGD cells for
the hydrolases studied was normal. The data indicated
that granular enzymes are released in a normal fashion
in phagocytizing CGD cells. Supportive evidence of
release of enzymes into the phagocytic vacuole of CGD
cells was obtained by an electron microscopic study of
myeloperoxidase.

INTRODUCTION

Chronic granulomatous disease (CGD) is a genetically
determined disorder in which peripheral blood leuko-
cytes fail to destroy certain bacteria during phago-
cytes. The patients are afflicted with chronic and recur-
cent supplicative infections, usually due to Staph-

ylococcus aureus. Holmes, Quie, Windhorst, and Good
(1) first established that leukocytes from these patients
could ingest but could not kill the infecting organisms.
Kaplan, Laxdal, and Quie (2) subsequently showed that
leukocytes from CGD patients could kill streptococci
normally. At the metabolic level, Sbarra and Karnovsky
(3, 4) previously had established that respiration and
oxidation of glucose via the hexose monophosphate path-
way are stimulated during phagocytosis by normal
leukocytes from guinea pigs. In the CGD cells, Holmes,
Page, and Good (5) and Baehner and Nathan (6) noted
a lack of these oxidative reactions during phagocytosis,
although normal human cells are comparable to those
from the guinea pig. The defective cells, initially thought
to manifest their abnormalities as a result of an X
chromosome-linked trait, and the cells from the female
carriers of the gene for CGD, can be identified by their
total or partial inability, respectively, to reduce nitro
blue tetrazolium (NBT) during phagocytosis (7).

Cohn and Hirsch (9) have shown that granule-
associated hydrolytic enzymes shift toward the soluble
fraction after phagocytosis in rabbit peritoneal leuko-
cytes, and Zucker-Franklin and Hirsch (10) using
electron microscopic techniques verified this release as
being into the phagocytic vacuole. Also using electron
microscopic techniques, Quie, White, Holmes, and Good
(8) reported diminished autophagic and phagocytic
vacuole formation in the CGD granulocytes after phago-
cytosis of heat-killed bacteria. The latter observation
suggested that degranulation was impaired.

The purpose of our study was to quantify by chemical
methods the release of granule-associated enzymes into
a soluble form by leukocytes from human controls and
CGD patients after phagocytosis. An electron micro-
scopic study of peroxidase release was also made
because of the sensitivity and specificity of the reactions
involved in visualizing that cellular process. Our findings

The Journal of Clinical Investigation Volume 47 1968 187
are that the release of granule-associated enzymes into soluble compartments is quantitatively and qualitatively similar in control and CGD leukocytes.

**METHODS**

*Isolation and preparation of human leukocytes.* 50 ml of peripheral blood was collected by venipuncture from five control human subjects and four patients with CGD, identified by the quantitative NBT test (7). Control subjects were young adults with acute bacterial infections. The proportion of granulocytes was similar in all cases, circa 80% of the total white blood-cell count (WBC). The blood was collected in plastic disposable syringes previously rinsed with sodium heparin.1 The syringes were inverted and the red cells were sedimented at 37°C for 1 hr. 3 volumes of 0.87% NH₄Cl were added to the leukocyte-rich supernatant, mixed, and centrifuged at 250 g for 5 min. The leukocyte pellet was washed twice and suspended in Krebs-Ringer phosphate buffer at pH 7.4 at a concentration of 15% (v/v).

*Preparation of particles for ingestion and ingestion technique.* Zymosan,2 starch prepared from *Amaranthus caudatus* seeds (11), polystyrene spheres,3 or *E. coli* K-12 (cultured on Difco antibiotic medium No. 3, washed twice, then heat-killed at 70°C for 30 min), were suspended in Krebs-Ringer phosphate, pH 7.4. The particles were incubated for 30 min at 25°C with saline containing 10% (v/v) fresh human serum, and the serum was decanted after centrifugation. Control tubes containing buffered normal saline without particles were treated similarly. Incubations were carried out in siliconized 125-ml Erlenmeyer flasks labeled “resting” and “phagocytizing” and contained a final volume of 15 ml Krebs-Ringer phosphate buffer, with a final concentration of glucose, 10 mM, leukocytes, 2–4% by volume, and serum-treated buffered saline or particles at a final particle to cell ratio of 20–40 to 1. Incubations were performed for 30–60 min in a Dubnoff metabolic shaker water bath with an excursion of 3 cm at a speed of 50 excitations/min.

*Preparation of subcellular fractions.* The incubation medium and cells (see above) were transferred to chilled 40-ml siliconized glass conical tubes and centrifuged for 10 min at 4°C and 250 g. The cell button was then washed once with cold 0.34 M sucrose (buffered to pH 7.4 with bicarbonate) and centrifuged at 250 g for 10 min. The supernatant fluids were combined and the exact volume determined. The cell button was suspended in cold 0.34 M sucrose and homogenized in a 50 ml glass cylinder4 with a Teflon pestle for 5 min at 4°C using a motor-driven homogenizer5 at a speed of 10 g. The homogenate was centrifuged at 10 min at 250 g and 4°C, the supernatant removed, and the button again homogenized in sucrose using the same technique. The whole homogenate was then combined, the volume recorded, and an aliquot removed for analysis to determine recovery of the enzyme in the various subcellular fractions. The homogenate was centrifuged for 10 min at 250 g, 4°C, and then the button was washed twice in 0.34 M sucrose and again centrifuged. The residual button contained nuclear debris, cellular membranes, and rare unbroken cells. This button was resuspended in 0.34 M sucrose and labeled “debris.” The supernatant fluids were combined and centrifuged for 1 hr at 100,000 g in a Spinco ultracentrifuge at 4°C. The supernatant fraction was decanted and the granule fraction was resuspended in 0.34 M sucrose; the volumes of the supernatant, granule, and debris fractions were recorded. Enzyme specific activities were expressed as units min⁻¹ mg⁻¹ protein, and total activities were calculated from the total protein content of each fraction. Thus four fractions were obtained, i.e., external medium, debris, sedimentable granules, and supernatant fluid.

*Enzyme determinations.* Acid and alkaline phosphatase and beta glucuronidase were assayed with the appropriate p-nitrophenyl substrate, (p-nitrophenyl phosphate or p-nitroph- enyl glucuronide) in a final volume of 1 ml in the presence of 0.1% (w/v) Triton X 100.6 Acid phosphatase and beta glucuronidase incubations were carried out at 37°C in sodium acetate buffer, 50 mM, pH 5.0, and the reaction was stopped with 2 ml sodium hydroxide 0.1 M. Alkaline phosphatase incubations were carried out in glycine buffer 0.25M, pH 10.0 and magnesium chloride 10 mM; the reaction was stopped with 2 ml sodium hydroxide 0.08 M. Under the conditions used, the enzymatic determinations were linear with respect to time and protein concentrations. Peroxidase was assayed by the method of Maehly (12). Catalase was determined by the method of Chantrenne as modified by Baush and New, Rahman-Li, Sellinger, Watiax, and de Duve (13).

*Ultrastructural localization of peroxidase activity.* White cells were prepared, and ingestion studies were performed as described above except that the medium contained 10% (v/v) fresh human serum. Ingestion was allowed to proceed for 15 min. After spinning down the cells at 250 g and decanting the medium, the cells were resuspended in the fixative, which consisted of glutaraldehyde 2%, buffered to pH 7.2 with 0.067 M cacodylate-HCl containing CaCl₂ 1 mM. Fixation was at room temperature for 1 hr. After washing three times in the cacodylate buffer containing sucrose (120 mM), the cells were transferred to the incubation medium for the ultrastructural localization of peroxidase activity by the method of Graham and Karnovsky (14). Incubation was for 20 min at room temperature. After washing three times in Tris-HCl buffer, 0.05 M, pH 7.6, the cells were postfixed in buffered osmium tetroxide, and prepared for electron microscopy by standard methods previously described (14).

**RESULTS**

Since Cohn and Hirsch (9) noted maximal release of granular enzymes into the soluble fractions between 30 and 60 min after initiation of phagocytosis, differences in maximal granule release should be evident by that time. To obviate differences due to the nature of the particle itself, we obtained results using zymosan, starch, and polystyrene particles, and heat-killed *E. coli*. As shown in Figs. 1 and 2 there was a loss of acid and alkaline phosphatase, beta glucuronidase, and peroxidase; from the granules into the soluble fractions during phagocytosis by human CGD leukocytes as well as by control leukocytes.

Scrutiny of the actual numbers represented by the

---

1. Liqueam sodium “10,” Organon, Inc., West Orange, N. J.
2. Nutritional Biochemicals Corporation, Cleveland, Ohio.
6. Sigma Chemical Co., St. Louis, Mo.
bars in Figs. 1 and 2 revealed no significant differences between the control and CGD cells, although the differences between resting and phagocytizing cells of each category were indeed significant at $P$ values of 0.05 or less. One interesting feature that emerged was that the distribution of beta glucuronidase was different in resting CGD cells from that in normal leukocytes. The granules in the latter contained twice as much of the total enzyme as those of the former, and the net release in the case of phagocytizing CGD cells was therefore less than for control cells. The proportion of granule-bound enzyme decreased comparably during phagocytosis in both categories of cell. Finally, in this context, we noted that for none of these enzymes did the CGD cells exhibit a lower total activity than normal cells.

By "soluble fraction" in Figs. 1 and 2 we mean the combined extracellular incubation medium and the supernatant fraction from the homogenization. Table I shows the distribution of soluble enzyme between these two compartments. Catalase, 90% of which is normally in the soluble fraction of resting cells also shifted out of the cell during phagocytosis. We noted that during uptake of larger particles, there was a greater shift of enzyme into the extracellular phase than for smaller particles (Fig. 3), i.e. during phagocytosis of starch and zymosan (circa 3 $\mu$ diameter) more enzymatic activity appeared extracellularly than with E. coli and latex (circa 0.8 $\mu$ diameter).

Electron microscopic studies of the release of granule enzymes were confined to myeloperoxidase because of the specificity and sensitivity of the method available, as mentioned earlier. The pictures confirmed the release of this granular enzyme into the phagocytic vacuole 15 min after phagocytosis of zymosan particles in both control and CGD leukocytes (Fig. 4).

Both normal and CGD neutrophils exhibited numerous large granules, which stained strongly for peroxidase activity (Fig. 4a). Various other granules did not react. This is in accord with the cytochemical observations of others on normal human and rodent cells (14–17). After ingestion of zymosan particles had occurred, much peroxidase activity was observed within the phago-

Figure 1 Subcellular distribution of acid phosphatase and alkaline phosphatase. The hatched bars represent resting cells and the solid bars represent phagocytizing cells. The number of subjects studied is indicated at the top of each graph. Measurements on three controls and three CGD patients were carried out after 30 min incubation. The other measurements were made on cells from additional subjects, after 60 min of incubation. Since the data for the two time periods were very similar, they were combined. Starch or heat-killed E. coli were used for phagocytosis. No differences were noted with respect to release of enzymes from the granules. (However, see Fig. 3.) The percentage of enzyme activity recovered from the subcellular fractions compared to the whole homogenate is indicated at the right of each bar graph: resting cells on the left and phagocytizing cells on the right. The % of is indicated above each bar. See text for method of preparation of the debris (D), granule (G), and soluble (S) fractions.

Figure 2 Subcellular distribution of beta glucuronidase and peroxidase. Conditions were as indicated for Fig. 1.

Figure 3 Subcellular distribution of peroxidase 30 min after phagocytosis of smaller (E. coli K-12 ca. 1 $\mu$) and larger (starch ca. 3a) particles. The incubation medium is indicated by M and the intracellular soluble fraction is indicated by CS. The remaining fractions D and G indicate cellular debris and total sedimentable granules, respectively. Results include studies from two CGD patients and one control subject. Since no differences were observed, they are combined.

Degranulation of Leukocytes in CGD  189
FIGURE 4

R. L. Baehner, M. J. Karnovsky, and M. L. Karnovsky
Alkaline
Acid
external
medium
is
enzyme
Peroxidase
28 10 72 90 70 23 30 77
Catalase
79
in
present
* studies
the
bounding
phagocytizing
cells.
"P"
centrifugation
of
the
cells
(CS).
This
total
soluble
high-speed
was
almost
every
case
where
a
particle
had
cytoplasm
observed
between
normal
described
cytosis
fusing
with
peroxidase-containing
granules
lying
cytic
Franklin
and
Hirsch
controls
toplasm.
Particle
cytic
Neutrophil
and
3
have
an
entirely
similar
appearance.
N
lobe.
activity.
only
around
the
particle
74 43 26
curonidase
X
Myeloperoxidase
myeloperoxidase
particle.
contents
M,
around
11,000.
from
4
B.
CGD
patient.
When
H2O2
the
phagocytic
particle
are
discharged
granules,
and
many
particles
have
been
discharged
granules,
Particles
granules
in
the
cytoplasm.
Particle
3
has
not
yet
been
completely
ingested,
and
there
is
no
staining
for
peroxidase
around
it.
The
slight
density
around
the
particle
represents
the
capsule
of
the
zymosan
particle.
Compare
particles
1
and
3.
Cells
from
normal
controls
have
an
entirely
similar
appearance.
N =
nuclear
lobe.
× 11,000.  (C) Neutrophil
from
another
CGD
patient.
Particles
1, 2,
and
3
as
in
Fig. 4 B. At
arrows,
granules
are
apparently
fusing
with,
and
discharging
into,
the
phagocytic
vacuole.
N =
uclear
lobe.
× 8000.  (D) Neutrophil
from
same
CGD
patient
as
in
Fig. 4 C,
reacted
in
the
absence
of
H2O2.
Slight
density
in
phagocytic
vacuoles
around
particles
(1, 2, 3)
represents
contents
of
discharged
granules,
but
there
is
not
staining
for
peroxidase
activity.
A
number
of
granules
in
the
cytoplasm
have
slight
density,
but
are
also
negative
for
peroxidase
activity.
(Compare
with
Fig. 4 A-C).  N =
nuclear
lobe.
× 8000.

Degranulation
of
Leukocytes
in
CGD

This
quantitative
investigation
of
the
shifts
of
granule-
associated
enzymes
in
human
peripheral
blood
leukocytes
from
normal
and
CGD
subjects,
during
phagocytosis
of
a
variety
of
opsonized
particles,
indicated
that
the
disease
is
apparently
not
characterized
by
faulty
degranulation,
as
indicated
by
the
release
of
typical
hydrolases.
The
subcellular
distribution
patterns
of
leukocytes
from
either
human
controls
or
from
patients
with
chronic
granulomatous
disease
were
obtained
for
acid
and
alaka-
line
phosphatase
and
peroxidase
after
either
a
30
min
or
a
1
hr
incubation
at
37°C.
Both
the
distributions
and
the
patterns
of
release
were
found
to
be
very
similar
at
those
times.
On
the
other
hand,
beta
glucuronidase
was
proportionately
higher
in
the
soluble
fractions
of
the
CGD
leukocytes
at
rest
than
in
that
fraction
of
control
leukocytes
at
rest.
We
cannot
as
yet
assess
the
signifi-
cance
of
this
finding,
except
to
observe
that
it
suggests
heterogeneity
of
the
granule
population.

The
diminished
proportion
of
total
enzyme
activity
in
the
granule
fraction
after
phagocytosis
was
indeed
balanced
by
the
increased
proportion
of
total
soluble
enzyme
at
the
end
of
the
incubation,
and
the
overall
recoveries
were
generally
good.
The
shift
of
a
soluble
intracellular
enzyme
such
as
catalase
into
the
extracellular
media
during
this
period
implies
that
some
soluble,
as
well
as
granular
enzymes
leaked
out
of
the
cells.
The
movement
of
granular
enzymes
could
be
via
passage
into
incompletely
sealed
off
phagocytic
vacuoles.
It
may
be
noted
that
for
normal
cells
the
data
for
the
soluble
fractions
of
phosphatases
and
β-glu-
curonidase
were
very
similar,
while
peroxidase
exhibited
a
different
pattern.
This
again
suggests
heterogeneity
of
the
granule
popu-
lation.
This
distinction
between
the
hydrolases
and
peroxidase
was
not
noted
for
CGD
cells.
We
have
no
indication
as
yet
of
the
mode
of
exit
of
the
soluble
enzyme
catalase.

The
electron
microscopic
studies
therefore
confirm
the
release
of
peroxidase
into
the
phagocytic
vacuole
15
min
after
phagocytosis
of
zymosan
particles
in
both
control
and
CGD
leukocytes.

DISCUSSION

TABLE I
Percentage
of
Soluble
Enzyme
that
is
Intra-
or
Extracellular*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th></th>
<th>CGD</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>M</td>
<td></td>
<td>CS</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>R P</td>
<td>R P</td>
<td></td>
<td>R P</td>
<td>R P</td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>73 32</td>
<td>27 68</td>
<td></td>
<td>62 37</td>
<td>38 63</td>
<td></td>
</tr>
<tr>
<td>Alkaline</td>
<td>79 53</td>
<td>21 42</td>
<td></td>
<td>53 56</td>
<td>47 44</td>
<td></td>
</tr>
</tbody>
</table>
| Beta
glu-
curonidase| 74 43 | 26 57 |       | 81 56 | 19 44 |       |
| Peroxidase | 28 10 | 72 90 |       | 70 23 | 30 77 |       |
| Catalase | 79 61 | 21 39 |       | 59 39 | 41 61 |       |

* By "soluble enzyme" we mean that which is present in the external medium after incubation (M) plus that which is present in the supernatant fraction after homogenization and high-speed centrifugation of the cells (CS). This total soluble enzyme is represented by the bars labeled "S" in Figures 1 and 2. In Fig. 3 these two components of the total soluble enzyme are separately represented. "R" represents resting cells, and "P" phagocytizing cells.

Figure 4 Myeloperoxidase activity. (A) Neutrophil from blood of mother (heterozygotic carrier) of one of CGD patients reacted for myeloperoxidase activity. Large granules stain intensely. Other granule types do not stain. Neutrophils from normal controls and CGD patients are entirely similar in staining reaction and appearance. N = nuclear lobe. × 12,000. (B) Neutrophil from CGD patient. Two particles of zymosan have been ingested (1 and 2). Peroxidase is present in the phagocytic vacuole around particle 1, (arrow) and possibly around particle 2, although the density around the latter may represent only unstained contents of discharged granules (see Fig. 4 D). There are few free peroxidase-positive granules in the cytoplasm. Particle 3 has not yet been completely ingested, and there is no staining for peroxidase around it. The slight density around the particle represents the capsule of the zymosan particle. Compare particles 1 and 3. Cells from normal controls have an entirely similar appearance. N = nuclear lobe. × 11,000. (C) Neutrophil from another CGD patient. Particles 1, 2, and 3 as in Fig. 4 B. At arrows, granules are apparently fusing with, and discharging into, the phagocytic vacuole. N = nuclear lobe. × 8000. (D) Neutrophil from same CGD patient as in Fig. 4 C, reacted in the absence of H2O2. Slight density in phagocytic vacuoles around particles (1, 2, 3) represents contents of discharged granules, but there is not staining for peroxidase activity. A number of granules in the cytoplasm have slight density, but are also negative for peroxidase activity. (Compare with Fig. 4 A-C). N = nuclear lobe. × 8000.

Degranulation
of
Leukocytes
in
CGD
demonstrated clearly that this lysosomal enzyme could be found in intracellular phagocytic vacuoles, offering strong confirmation of the biochemical data.

These quantitative and qualitative studies would not support the suggestion (8) that one deficiency of the CGD leucocyte is its failure to degranulate in an hour after ingestion of bacteria. Under the conditions of our experiments, it seems clear that degranulation does occur normally after the ingestion of a variety of opsonized particles.\(^8\)

ACKNOWLEDGMENTS

This work was supported by U. S. Public Health Service research grants AI-03260 and AI-08173, and by a grant from the John A. Hartford Foundation.

Dr. Baehner is a U. S. Public Health Service Special Research Fellow of the National Institute of Arthritis and Metabolic Diseases.

REFERENCES


---

\(^8\) After submission of this manuscript, the paper of Kauder, Kahle, Moreno, and Partin appeared (18) in which the authors reach the same conclusion on the basis of morphologic data.