Improvement of Chediak-Higashi Leukocyte Function by Cyclic Guanosine Monophosphate

By Laurence A. Boxer, Manfred Rister, John M. Allen, and Robert I. Baehner

The addition of cholinergic agents and cyclic 3′,5′-guanosine monophosphate (cGMP) to polymorphonuclear leukocytes in vitro from a patient with Chediak–Higashi syndrome corrected the impaired release of the lysosomal enzyme, β-glucuronidase, to normal. Coinciding with the improvement in degranulation, the bactericidal capacity was enhanced to normal. Similar concentrations of cholinergic agents potentiated chemotaxis to control values. On the other hand, the phagocytic rate of lipopolysaccharide-coated paraffin-oil droplets was not altered by the cholinergic agents. The improvement in Chediak-Higashi syndrome polymorphonuclear leukocyte function by the addition of cholinergic agents and dibutyryl cGMP suggested disturbed intracellular cyclic nucleotide levels.

The Chediak-Higashi Syndrome (CHS) is an autosomal recessive disorder occurring in man and several mammalian species.1,2 It is characterized by partial oculocutaneous albinism, recurrent pyogenic infections, peripheral granulocytopenia, and giant lysosomes in monocytes, polymorphonuclear leukocytes (PMN), and eosinophils. In addition, in many of the human subjects afflicted with the disorder, a lymphomalike phase of the disease eventually develops. Coincidental with the striking morphological changes in leukocytes are several aberrations in leukocyte function. Impaired leukocyte movement has been observed.3 Although phagocytosis proceeds unrestricted, there is a delay in delivery of lysosomal contents into the phagosome.4 The specific activities of several lysosomal enzymes are also diminished.5 Thus, the reduction in activity of the lysosomal enzymes as well as their delivery into phagocytic vesicles may explain the defective bactericidal capacity of these cells.

Oxidative-related activity, including H2O2 production, hexose monophosphate shunt activity, and iodination, has been reported not to be compromised.6 Since agents which increase intracellular cyclic 3′,5′-guanosine monophosphate (cGMP) levels potentiate degranulation,7 we studied the effects of cGMP on leukocyte function obtained from an infant with CHS. We were encouraged by the correction of the abnormal cap formation induced by concanavalin A in CHS mouse PMN following the use in vitro of cGMP and cholinergic agents.8

MATERIALS AND METHODS

Case Report

An 11-mo-old white female had been admitted to the hospital at age 2 mo because she had gained only 283 g beyond her birth weight of 3629 g. Physical examination at that time revealed...
ocular albinism and a liver palpable 1 cm below the right costal margin. The initial hemoglobin was 9.6 g/100 ml with a hematocrit of 28% and the white blood cell count was 8600/cu mm with 24% bands, 16% segmented neutrophils, 6% eosinophils, 49% lymphocytes, and 5% monocytes. Examination of the bone marrow and peripheral blood smear revealed giant lysosomes in all white cell lines. The patient subsequently gained weight, placing her currently in the third percentile for her age. She has had two episodes of otitis media and one each of bronchitis and lobar pneumonia. Her liver and spleen have not enlarged and liver function tests have remained normal. Although platelet counts were normal, platelet function was characterized by impaired nucleotide and serotonin release. Further studies have revealed an elevated serum lysozyme of 50 mg/ml (normal 2-10 mg/ml). Enzyme assays. Leukocytes were obtained from peripheral blood and PMN were isolated using a Ficoll-Hypaque gradient. Red cells were lysed by exposure to deionized water for 20 sec. Isotonicity was then restored by the addition of an appropriate volume of 3.5% saline. β-Glucuronidase was assayed with p-nitrophenol β-D-glucuronide, and alkaline phosphatase was measured using p-nitrophenol phosphate and a 50-mM glycine buffer adjusted to pH 10.0. Myeloperoxidase was measured by the peroxidation of guaiacol as previously described. All enzyme assays were carried out with the Gilford Model 2400 Recording Spectrophotometer. Total enzyme activity was obtained following lysis of purified PMN with Triton X-100. Degranulation. Degranulation of PMN was quantitated by measuring the release of β-glucuronidase upon exposure to opsonized zymosan. Red-cell-free mixed leukocyte suspensions or purified PMN were always adjusted to 4 x 10^6 granulocytes. After treatment with cytochalasin B (5 μg/ml) the cells were incubated with different test compounds in various concentrations at the times indicated in the figure legends. After resuspension in Krebs-Ringer phosphate buffer the cells were exposed to opsonized zymosan for 5, 15, and 30 min. At the end of the experiments the cells were centrifuged at 800 g at 4°C and the cell-free supernates were removed for the determination of β-glucuronidase. Degranulation was expressed as percent of total β-glucuronidase activity released by 0.2% Triton X-100. For determination of cell death cytoplasmic catalase activity was assayed. Bacterial killing test and paraffin-oil phagocytosis. A mixed leukocyte suspension or purified PMN were incubated with the different test compounds in various concentrations. After resuspension in Krebs-Ringer phosphate buffer 10^7 PMN were incubated with equivalent concentrations of Staphylococcus aureus 502A opsonized with AB negative human serum at 37°C for varying periods of time up to 90 min. At the indicated times and following the lysis of the cells, the viable bacteria were enumerated from agar pour plates. In some instances the removal of extracellular bacteria by phagocytosis was also determined by centrifuging the mixtures at 100 g for 5 min at 4°C and plating serial dilutions of the supernatant fluid. Ingestion of lipopolysaccharide-coated paraffin-oil droplets by purified PMN was analyzed as previously described. Chemotaxis. Leukocyte migration in vitro was measured with the modified Boyden chamber technique. From the peripheral blood 1.5 x 10^6 PMN were deposited with a cytocentrifuge (Shandon Scientific Co.) on a premoistened 5-μm Millipore filter (Millipore Corporation). The filter was then placed in a modified Boyden chamber and incubated for 3 hr at 37°C in a 10% CO₂-90% air incubator. A bacterial chemotactic factor was prepared from a culture filtrate of Escherichia coli. The number of PMN migrating through the filter in 10 high-power objective fields was enumerated, and the chemotactic index was determined. RESULTS There was no difference in the total amount of protein in CHS and control PMN. Purified CHS PMN contained only 21% ± 4% of total myeloperoxidase and 58% ± 10% of total β-glucuronidase activities compared to control. There was no difference in alkaline phosphatase activities between the CHS and control cells (Table 1). Degranulation of lysosomal enzymes from either mixed leukocytes or PMN was studied after rendering the phagocytic cells secretory with 5 μg/ml of cytochalasin β. Following addition of opsonized zymosan, the CHS mixed
EFFECT OF cGMP ON CHS

Table 1. Total Enzyme Activity in Normal and CHS PMN

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Normal Patient</th>
<th>Relative Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase†</td>
<td>3.50 ± 0.6</td>
<td>0.7 ± 0.14</td>
</tr>
<tr>
<td>β-Glucuronidase‡</td>
<td>120.00 ± 5.0</td>
<td>70.0 ± 12.00</td>
</tr>
<tr>
<td>Alkaline phosphatase§</td>
<td>53.70 ± 9.0</td>
<td>57.6 ± 4.00</td>
</tr>
</tbody>
</table>

*CHS enzyme activity expressed as per cent of control.
†Expressed as mmoles oxidized tetraguaiacol/mg protein/min.
‡Expressed as μmoles p-nitrophenyl-β-glucuronide/mg protein.
§Expressed as μmoles p-nitrophenyl-phosphate/mg protein.

leukocytes, containing 60%-70% lymphocytes, 20%-30% PMN, and 5%-15% monocytes, released only 8% ± 2%, 11% ± 2%, and 16% ± 1%, respectively, of the total β-glucuronidase activity (Fig. 1). When the mixed leukocytes were incubated for 10-30 min with 10^-6 M dibutyryl cGMP in order to potentiate the rate of degranulation, no effect was seen with the CHS cells. However, following incubation of the mixed leukocytes with 10^-2 M dibutyryl cGMP for 30 min, the rate of β-glucuronidase enzyme release by the CHS cells was increased to 25% ± 1%, 26% ± 2%, and 37% ± 1% at 5, 15, and 30 min, respectively, and became equivalent to the rate of the control. Incubation of the mixed leukocytes for 10 min with 10^-3 M carbamyl methylcholine chloride also potentiated β-glucuronidase release to 26% at 15 min, but enzyme release from CHS cells incubated with 10^-6 M carbamyl methylcholine chloride did not differ from untreated CHS cells. The release of β-glucuronidase from both CHS and control cells could not be attributed to cell lysis since the activity of cytoplasmic enzyme catalase in the extracellular media remained during the time course of degranulation at 6% of total cellular activity (Fig. 1). Furthermore, β-glucuronidase activity at 0 time was less than 1% in the extracellular media.

In comparison to mixed CHS leukocytes, purified PMN responded in a similar fashion following exposure to dibutyryl cGMP. Only 12% ± 2%, 17% ± 1%, and 19% ± 2% of the total β-glucuronidase activity was released by 5, 15, and 30 min, from CHS PMN compared to 20% ± 2%, 24% ± 1%, and 26% ± 2% of control without using dibutyryl cGMP (Fig. 2). Again, 10^-6 M dibutyryl cGMP had no effect on CHS PMN, while it potentiated release to 33% ± 1%
Fig. 2. β-glucuronidase release from PMN. (A) — normal PMN; — normal PMN preincubated with 10⁻² M dibutyryl cGMP for 5 min. (B) — CHS PMN; — CHS PMN preincubated with 10⁻² M dibutyryl cGMP for 5 min; — CHS PMN preincubated with 10⁻² M dibutyryl cGMP for 30 min. Recorded values are the mean ± SD of three experiments.

in the control at 30 min. On the other hand, following a 30-min preincubation with 10⁻² M dibutyryl cGMP, there was a marked increase in β-glucuronidase release to values similar to that of the control (Fig. 2). When 10⁻² M dibutyryl cGMP was preincubated with the PMN for 5 min, similar potentiation of enzyme release to 30% ± 1% at 5 min in control PMN was noted but no significant increase was seen with the CHS PMN. The total enzyme activity within the CHS PMN was not altered by exposure of the cells to dibutyryl cGMP or carbamyl methylcholine chloride. The exposure to various concentrations of dibutyryl cGMP did not lead to lysis of the CHS PMN. At 0 time there was less than 1% β-glucuronidase and catalase activity detectable. During degranulation catalase activity remained below 5% of total enzyme activity.

As seen in Fig. 3A, not until an interval of 40 min had passed were the CHS leukocytes able to kill bacteria. When the CHS leukocytes were preincubated with 10⁻² M dibutyryl cGMP for 10 min, the delayed bactericidal response was eliminated and the overall rate and extent of killing of the bacteria was comparable to that of the control.

At a concentration of 10⁻⁶ M dibutyryl cGMP there was no improvement in bactericidal capacity (Table 2). It was not until 10⁻³ M dibutyryl cGMP was utilized that the bactericidal function was normalized. The cholinergic agent, carbamyl methylcholine chloride, at 10⁻³ M, but not at 10⁻⁶ M, also restored activity. In order to assess viability of the CHS leukocytes during the exposure to the bacteria, both trypan blue exclusion and cell counts were determined. Over 95% of the PMN were able to exclude the dye at 100 min following exposure to the various test agents; furthermore, no difference in total cell number during incubation with the bacteria was noted between the control and CHS cells.

In order to verify that the phagocytic rate was not merely increased by exposure to the drugs, which also might improve the extent of killing, both the
EFFECT OF cGMP ON CHS

Fig. 3(A). Bactericidal capacity of mixed leukocytes: $10^7$ phagocytes were incubated with an equivalent amount of Staphylococcus aureus 502A. ■ CHS leukocytes; ▲ CHS leukocytes pre-incubated with $10^{-3} M$ dibutyryl cGMP. Recorded values are the mean ± SEM of three experiments. (B) Bactericidal capacity of purified PMN: $10^7$ PMN were incubated with an equivalent amount of Staphylococcus aureus 502A. Extracellular bacteria were removed at the indicated times by centrifugation. ■ CHS PMN; ▲ CHS PMN preincubated with $10^{-3} M$ dibutyryl cGMP; ○ CHS PMN preincubated with $10^{-3} M$ carbamyl methylcholine chloride; ◯ normal PMN.

extracellular bacteria and intracellular bacteria were enumerated. Following exposure to either $10^{-3} M$ carbamyl methylcholine chloride or $10^{-3} M$ dibutyryl cGMP, the rate of bacterial killing by purified CHS PMN improved to control values (Fig. 3B). On the other hand, the addition of the various test agents to the purified PMN did not alter the number of extracellular bacteria. No effect on the phagocytic rate as determined by lipopolysaccharide-coated paraffin-oil droplet uptake was seen in either the CHS PMN or control leukocytes following a 10-min treatment with $10^{-3} M$ dibutyryl cGMP or $10^{-3} M$ carbamyl methylcholine chloride (Table 3).

Neither $10^{-2} M$ butyrate nor $10^{-2} M$ cGMP affected degranulation or the bactericidal capacity of CHS leukocytes. In addition, $10^{-2} M$ dibutyryl cGMP
Table 2. Bactericidal Capacity of CHS Leukocytes Incubated With Increasing Concentrations of Dibutyryl cGMP or Bethanechol*

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>CHE Leukocytes</th>
<th>Normal Leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>$10^{-6}$ M cGMP</td>
</tr>
<tr>
<td>0</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>20</td>
<td>12.50</td>
<td>10.90</td>
</tr>
<tr>
<td>40</td>
<td>42.80</td>
<td>10.00</td>
</tr>
<tr>
<td>60</td>
<td>6.26</td>
<td>—</td>
</tr>
<tr>
<td>80</td>
<td>4.60</td>
<td>5.19</td>
</tr>
</tbody>
</table>

Leukocytes incubated with the respective agents for 10 min and then resuspended in buffered media. Values expressed are $10^9$ viable bacteria.

*Carbamyl methylcholine chloride.
Table 3. Phagocytic Rates and Chemotaxis of CHS PMN

<table>
<thead>
<tr>
<th></th>
<th>Ingestion*</th>
<th>Chemotactic Index†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.053</td>
<td>175 ± 10</td>
</tr>
<tr>
<td>CHS</td>
<td>0.049</td>
<td>129 ± 22</td>
</tr>
<tr>
<td>CHS preincubated with 10⁻³ M dibutyryl cGMP</td>
<td>0.047</td>
<td>—</td>
</tr>
<tr>
<td>CHS preincubated with 10⁻³ M carbamyl methylcholine chloride</td>
<td>0.046</td>
<td>220 ± 16</td>
</tr>
</tbody>
</table>

*Initial rate of ingestion of E. coli lipopolysaccharide-coated paraffin-oil droplets opsonized with fresh homologous serum as mg paraffin oil and ingested 10⁷ PMN/min. The incubation time with the drugs was 10 min. Results are the mean of two separate experiments.
†Chemotactic index: Number of PMN in 10 random fields; number of PMN (× 10⁶) in 0.4 ml delivered to the starting side of the filter.

had no effect on the growth of the bacteria themselves during a 100-min exposure.

The CHS PMN had impaired chemotaxis before treatment with 10⁻³ M carbamyl methylcholine chloride compared to normal controls (Table 3). Following a 10-min exposure to the drug and resuspension in autologous serum, the chemotactic index of the CHS PMN was increased by 59% over the initial values.

**DISCUSSION**

Patients with CHS, like patients with chronic granulomatous disease, suffer pyogenic infections. The infections observed in CHS, however, are not as frequently life-threatening as those seen in patients with chronic granulomatous disease. Unlike chronic granulomatous disease, there is no difficulty in generating H₂O₂, but the impaired function of the CHS cells appears to relate to the striking morphological and functional abnormalities of the lysosomes.¹⁷ There is peripheral neutropenia associated with markedly elevated serum muramidase activity and increased numbers of granulocyte precursors in the bone marrow, which may reflect increased intramedullary destruction of granulocytes.¹⁸

The granulocyte function of an infant with CHS was improved in vitro by exposure of the cells to dibutyryl cGMP or the cholinergic agent, carbamyl methylcholine chloride, when compared to control cells obtained from healthy adults. Others have shown that the phagocytic function of neonates differed from that of adults, but, within a week of age, there was no difference in oxidative metabolism or bactericidal capacity between infant and adult granulocytes.¹⁹ Therefore age-matched controls were not sought. Recently the abnormal cap formation characteristic of CHS PMN was corrected in vitro by these agents in two patients.²⁰ In addition, peripheral blood monocytes isolated from these two patients as well as fibroblasts obtained in CHS mice when grown in the presence of carbamyl methylcholine chloride contained significantly fewer abnormal lysosomes.²¹ Thus the improvement in chemotaxis, degranulation, and bacterial killing described in this study correlated with the change in morphology of CHS cells treated with cholinergic agents in vitro.

Although the total enzyme activity of β-glucuronidase and myeloperoxidase
within the CHS PMN was not altered by treatment of the cells with dibutyryl
cGMP or carbamyl methylcholine chloride, the release of the lysosomal en-
zyme, β-glucuronidase, increased to control values. Concomitantly, the known
delay in bacterial response was also eliminated and the bactericidal capacity of
the CHS leukocytes was restored to normal. The enhancement of the intracell-
ular killing exhibited by the CHS cells did not appear simply due to augmented
phagocytosis since the ingestion rates of lipopolysaccharide-coated paraffin-oil
droplets by CHS leukocytes or normal PMN was not changed by the choliner-
genic agents. The improvement in bacterial killing by CHS PMN coincided
with the correction of lysosomal degranulation.

Degranulation of phagocytic cells is partially mediated by cellular micro-
tubules. It has been suggested that microtubular function, in turn, is regulated
by cyclic nucleotides, microtubular assembly being inhibited by cyclic adenosine
monophosphate (cAMP) and potentiated by cGMP. Recently, we have quan-
titated cGMP and cAMP levels in the CHS infant's purified PMN and have
found cGMP and cAMP to be 0.27 and 23.60 pmoles/10⁷ PMN compared to
control values of 0.70 ± 0.07 and 3.5 ± 0.07 pmoles/10⁷ PMN, respectively.

Thus, the abnormally elevated concentrations of cAMP observed in the infant’s
PMN may be directly impairing the motility of the granules by inhibiting micro-
tubular assembly. Several lines of evidence indicate that the two cyclic nucleo-
tides, cAMP and cGMP, may produce functionally opposite effects in cells.
Dibutryl cGMP and the cholinergic agent may have corrected the patient’s
leukocyte function in vitro by antagonizing the effect of the excessive cAMP
concentration.

Chemotaxis is also believed to depend upon microtubules as well as micro-
filaments. Addition of cAMP to normal leukocytes will impair their move-
ment, whereas, the effect of cGMP is to potentiate movement. The diminshed
chemotaxis in CHS may also be caused by impaired microtubular function. Abnormal cyclic nucleotide levels, in turn, may be altered by exposure of the
cells to cholinergic agents with restoration of normal leukocyte function.

It is also possible to alter the lysosomal morphology in CHS mice in vivo by
treating the animals with cholinergic agents. If tested, it is likely that PMN
function would also be improved. Another approach to treating CHS success-
fully might entail lowering PMN cAMP levels. This approach has been tried
by oral administration of ascorbic acid to the infant. cAMP levels returned
nearly to control values with improvement of PMN function following the ad-
ministration of ascorbic acid.

Whatever the role of cyclic nucleotides are in CHS PMN, manipulating the
cyclic nucleotides with drugs may favorably improve PMN function. Future
observations will be required to determine if the clinical course of the patients
will also be favorably affected.

REFERENCES

1. Blume RS, Wolff SM: The Chediak-Higashi syndrome: Studies in four patients and
a review of the literature. Medicine 51:247-280, 1972

2. Padgett GA: The Chediak-Higashi syn-

3. Clark RR, Kimball HR: Defective granu-
locyte chemotaxis in the Chediak-Higashi

4. Stossel TP, Root RR, Vaughn M: Phago-
cytosis in chronic granulomatous disease and
EFFECT OF cGMP ON CHS


Improvement of Chediak-Higashi leukocyte function by cyclic guanosine monophosphate

LA Boxer, M Rister, JM Allen and RL Baehner