Oxidative and non-oxidative activation of murine peritoneal macrophages by histone H1

G Vani, C N Deepa & C S Shyamala Devi

Department of Biochemistry, University of Madras, Guindy Campus, Chennai 600 025, India

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The present study aimed at assessing the role of histone H1 in activating macrophages. Histone H1, injected intraperitoneally at a dose of 1 mg/kg body weight as multiple regimens weekly, significantly increased the number of peritoneal macrophages post 21 days of injection. The oxidative and non-oxidative activation of peritoneal macrophages by histone H1 was assessed. For the assessment of oxidative activation the levels of superoxide radical and nitric oxide radical were assessed. The oxidative activation was evident from release of significantly high levels of superoxide and nitric oxide radicals liberated by macrophages of animals treated with histone H1 (P<0.001) than in untreated animals. In addition, the higher activities of superoxide dismutase indicated protective effect of histone H1, to keep away the macrophages from noxious effects of superoxide. The catalase activity was decreased significantly in macrophages of histone H1 treated animals. The levels of reduced glutathione were significantly lowered in treated animals, whereas the levels of lipid peroxides generated were non-significant. The non-oxidative activation was assessed from the activities of lysosomal enzymes released and also from cytosis of NO-insensitive L929 cells. The activities of lysosomal enzymes-acid phosphatase and β-glucuronidase released were significantly high in treated animals than in untreated animals (P<0.001). Histone H1 stimulated the cytosis of macrophages in L929 cells than in untreated animals. These results suggest that histone H1 stimulates macrophages by oxidative and non-oxidative mechanisms, which favor its future therapeutic prospects.

Keywords: Cytolysis, Glutathione, Histone H1, L929 cells, Lysosomal enzymes, Macrophages

Activated macrophages recognize and destroy neoplastic and bacterial cells. To eradicate individual cells in vivo, macrophages may rely upon expression of one or more cytotoxic effector mechanism some requiring cell-to-cell contact and others dependent on elaboration of soluble effector molecules such as peroxides, cytolytic proteases, NO or TNFα. While these killing mechanisms do not appear to be mutually exclusive, they may act synergistically. Although some details on biological effects of above mentioned cytotoxic effectors are known, present observations demonstrate that macrophages are not themselves immune from cytotoxic effects of NO. When stimulated to produce NO in culture, macrophages exhibit a pattern of metabolic inhibition including suppression of oxidative metabolism and protein synthesis that is similar to that observed in tumor cells exposed to NO and die prematurely. Activated macrophages exhibit enhanced phagocytosis. Phagocytic challenge causes subsequent depression of macrophage respiratory burst capacity and phagocytic function. Specific GSH transporter triggered by the release of H2O2 during respiratory burst induces uptake of GSH into the cell. Such a mechanism has potential to protect the phagocyte against oxidative damage.

Histone H1 is biologically active peptide that plays a vital role in suppressing growth of leukemic cells in vitro and in vivo. It is involved with homeostatic thymic hormone and hence in the regulation of immune homeostasis. Histone H1 as a component of homeostatic thymic hormone protects mice against radiation-induced leucosis. It has been considered as a potential therapeutic agent in immunotherapy. Histone is a potent activator of macrophages, enhances phagocytic index against gram positive and negative bacteria.

But so far, the mechanisms involved in the role of histone H1 in activating macrophages have not been studied. Assessment of the modifications in macrophages following exposure to histone H1 could prove fruitful in its use as a therapeutic agent.

Materials and Methods

Histone H1 (Calf thymus Type-III) and Ficoll-Histopaque were obtained from Sigma, USA. α-Naphthylethylene diamine and α-naphthyl
butyrate were obtained from SRL, India. All other reagents used for the study were of analar grade. L929- murine fibrosarcoma cells were obtained from National Center for Cell Science, Pune, India. $^{51}$CrCl$_3$ were obtained from BRIT, India. Male albino mice (25-30g each) were obtained from Tamil Nadu Veterinary and Animal Sciences University, Madhavaram, Chennai. Animals were housed in polystyrene cages and provided feed and water ad libitum. All guidelines for the use of experimental animals were adhered to and approved by Institutional Ethics Committee.

Animals were divided as follows:

Group I: Control animals treated with drug vehicle (PBS) only

Group II: Animals treated with histone H1 (1 mg/kg body weight in PBS).

These animals were subdivided as:

Animals administered ip with single dose of histone H1 on day 1.

Animals administered ip with two doses of histone H1 on day 1 and 8.

Animals administered ip with three doses of histone H1 on day 1, 8 and 15.

These doses were chosen, as this concentration of histone H1 acted as an effector dose that suppressed collagen-induced arthritis.

All animals were sacrificed at the end of experimental period of 21 days. Prior to 3 days of sacrifice, animals were injected intraperitoneally with 0.5 ml of 5% sodium caseinate and rested for 3 days.

Estimation of number of macrophages

Briefly, mice were sacrificed and skin peeled off. Modified Hank's solution was used to withdraw cell exudates from peritoneal cavity. The exudates were centrifuged at 1000 rpm for 10 min with Ficoll-Histopaque gradient. To remove RBC (if any) the gradient was centrifuged further at 800 rpm for 30 min. Macrophages were collected from interphase between the Hank's solution and gradient. The pellet was resuspended in Hank's solution. Aliquots of cell suspension were stained with α-naphthalene butyrate to count macrophages as described previously.

Disruption of macrophages

Macrophages were disrupted using lysis buffer containing 20 mM Tris pH 8.5, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100. Protein concentration was estimated, along with superoxide anion$^{19}$, nitric oxide$^{21}$, lipid peroxide as TBARS$^{22}$, glutathione$^{23}$ and activities of superoxide dismutase$^{24}$, catalase$^{25}$, acid phosphatase$^{26}$ and β-glucuronidase$^{27}$ were assayed.

Cytolysis of L929 cells

Cytolysis in L929 cells exposed to macrophages of experimental animals was assessed as described$^{28}$. L929 cells were expanded and maintained on Eagle's Minimum Essential Medium supplemented with 10% Fetal Calf Serum. The cells were grown at 37°C in a fully humidified atmosphere of 95% air and 5% CO$_2$. L929 cells were collected in logarithmic phase of growth and 10$^5$ cells were labeled in 100 μl of culture medium with 100 μCi of $^{51}$CrCl$_3$ for 2 hr at 37°C. The cells were washed extensively and 10$^5$ cells of macrophages collected as described above, were added into wells. Final volume was 1 ml. At indicated times, aliquots of supernatants were collected and counted for $^{51}$Cr using RIASTAR Gamma counter (Beckard). Total $^{51}$Cr content of cells was determined by lysis with 1% Triton X-100. Spontaneous release was determined in the absence of macrophages and was <15%. The percentage of specific $^{51}$Cr released was determined as follows:

$$\text{Percentage of } ^{51}\text{Cr release} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100$$

Experimental cpm refers to radioactivity in the presence of macrophages.

Statistical analysis

The results are expressed as mean ± SD and statistical analysis was done using Students' $t$ test.

Results

The peritoneal macrophage count and the protein content in peritoneal cells of experimental animals are presented in Table 1. The number of macrophages increased significantly in treated animals ($P<0.001$) when compared to control. The macrophage count increased with increasing doses of histone H1. The protein content in macrophages also increased in treated animals ($P<0.001$) when compared to control animals.

The levels of superoxide and nitric oxide radical released increased significantly ($P<0.001$) in peritoneal cells of animals treated with histone H1 when compared to control. The glutathione content of macrophages in histone H1 treated animals decreased progressively with increasing doses of histone H1,
with levels of TBARS were non significant in treated animals \( (P<0.001) \) (Table 2).

The activities of superoxide dismutase increased progressively \( (P<0.001) \) in animals treated with histone H1 at increasing doses, whereas activities of catalase decreased on treatment \( (P<0.001) \) (Table 3).

The non-oxidative activation as assessed from the activities of acid phosphatase and \( \beta \)-glucuronidase showed significant increases \( (P<0.001) \) in treated animals (Table 4). Macrophages from histone H1 treated animals induced significant cytolysis of \( >20\% \) in L929 cells, whereas in control animals the percentage of cytolysis was significantly low (Fig. 1).

To summarize, the results indicate that histone H1 treatment increased the macrophage cell count as evident from the increase in cell number and protein content in peritoneal cells. The oxidative activation was evident from the increased levels of superoxide radical and nitric oxide in peritoneal cells of histone H1 treated animals. Also the lack of involvement of lipid peroxidation was evident from the non-significant levels of lipid peroxidation products and maintenance of activities of antioxidant enzymes. The non-oxidative activation of peritoneal cells was evident from the increased activities of lysosomal enzymes released by peritoneal cells of histone H1 treated animals. Also peritoneal cells from treated animals showed enhanced cytolysis in L929 cells (Table 5).

**Discussion**

The increased cell number and protein concentration in histone H1 treated animals may be attributed to the activation of macrophages. Activation of macrophages by histone H1 may have resulted from its structural homology with tufstin\(^{28} \), a

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**Table 1** — Macrophage count and levels of total protein in peritoneal macrophages of experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
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</thead>
<tbody>
<tr>
<td>Peritoneal cell count ( \times 10^6 )</td>
<td>12.3±0.34</td>
<td>14.6±0.1***</td>
<td>15.8±0.13**</td>
<td>17±0.23***</td>
</tr>
<tr>
<td>Macrophage count ( \times 10^6 )</td>
<td>2.9±0.05</td>
<td>3.5±0.67***</td>
<td>4.3±0.11***</td>
<td>4.5±0.09***</td>
</tr>
<tr>
<td>Total protein mg/10^6 cells</td>
<td>4.85±0.12</td>
<td>4.7±0.05***</td>
<td>4.9±0.02***</td>
<td>5.4±0.04***</td>
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\( **P<0.01, ***P<0.001 \) vs group I

**Table 2** — Levels of glutathione and lipid peroxide in peritoneal macrophages of experimental animals

<table>
<thead>
<tr>
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<th>Group III</th>
<th>Group IV</th>
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<tbody>
<tr>
<td>Glutathione nM/10^6 cells</td>
<td>96±0.04</td>
<td>93.3±0.05***</td>
<td>90.5±1.1**</td>
<td>89.3±1.4***</td>
</tr>
<tr>
<td>Lipid peroxides nM/10^6 cells</td>
<td>1.85±0.12</td>
<td>1.58±0.05***</td>
<td>1.49±0.02***</td>
<td>1.31±0.04***</td>
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\( **P<0.01, ***P<0.001 \) vs group I

**Table 3** — Activities of superoxide dismutase and Catalase in peritoneal macrophages of experimental animals

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<tr>
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<th>Group IV</th>
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<tbody>
<tr>
<td>Superoxide dismutase ((U/10^6 cells))</td>
<td>194.8±0.68</td>
<td>227.8±0.31**</td>
<td>236.5±0.21**</td>
<td>241.8±0.5***</td>
</tr>
<tr>
<td>Catalase ((nM of H_2O_2/oxidized/min/10^6 cells))</td>
<td>9±0.3</td>
<td>7±0.2**</td>
<td>6±0.1***</td>
<td>5±0.3***</td>
</tr>
</tbody>
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\( **P<0.01, ***P<0.001 \) vs group I
tetra peptide that activates macrophages and has antitumor activity. These results are concurrent with previous studies that have shown the activation of macrophages by histone H1 as evidenced with enhanced phagocytosis that results from a reduction of electrostatic repulsion between polymorphonuclear cells and phagocytized particle with an increase in cell surface activity. The intracellular killing of microorganisms enhanced by histone H1 shows that they are active in cytoplasm and/or have direct influence on intracellular metabolic processes. Previous studies have shown that histone H1 alters mitochondrial membrane permeability, ion transport and oxidative phosphorylation. Oxidative status was assessed from the levels of superoxide and nitric oxide radical.

Generation of superoxide radical and nitric oxide radical testify the potential of histone I in activating macrophage by oxidative dependent mechanism. The present results concur previous studies that have shown generation of superoxide anion in leucocytes by histone. Several reasons can be proposed for generation of superoxide radical. Primarily, the role of histone H1 as an uncoupler disrupting the mitochondrial respiratory chain may have resulted in the generation of superoxide radical. Augmentation of cytokine IL1 by histone H1, releases TNF-α. The release of TNF-α could have resulted in the superoxide radical as it has been reported to disrupt mitochondrial transport.

Generation of nitric oxide (NO) indicates the activation of macrophages by histone H1 as a NO dependent activation. NO levels in macrophages of treated animals in particular even after 21 days suggests prolonged cytotoxic and cytostatic activity and long lasting effect of histone H1 in activating macrophages. The generation of NO may have resulted from the action of histone H1 on nitric oxide synthase (NOS) enzyme, an enzyme involved in synthesis of nitric oxide from superoxide radical. Histone H1 has been reported to activate nitric oxide synthase in neuronal cells.

Intracellular thiol-glutathione (GSH) levels were significantly reduced. Few studies have suggested that a fall in GSH level does not depend on export of GSH, but upon either a decrease in synthesis de novo or an increase in GSH consumption and that a fall in GSH in macrophages follows nitric oxide synthase induction. GSH synthesizing enzyme contains an active thiol site and it is subject to regulation by NO mediated S-nitrosylation reduction, linking macrophage activation with NO production. Alterations of macrophage GSH concentration to regulate NO output could be clinically important in pathological conditions. Therefore, it is possible that decreased levels of GSH in animals treated with histone H1 may play a vital role in the regulation of NOS induction.

The activities of superoxide dismutase increased in animals treated with histone H1. The higher activities in these macrophages point to the protective effect of histone H1 in keeping themselves away from noxious effect of toxic radicals generated during the respiratory burst in activated macrophages. As

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</tr>
</thead>
<tbody>
<tr>
<td>Superoxide</td>
<td>99±0.68</td>
<td>106.1±0.31**</td>
<td>113±0.21**</td>
<td>115±0.5***</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>90±0.46</td>
<td>95.1±0.54**</td>
<td>96.2±0.09***</td>
<td>97±0.03***</td>
</tr>
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**P<0.01, ***P<0.001 vs group I

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<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>0.12±0.22</td>
<td>0.26±0.14**</td>
<td>0.4±0.05**</td>
<td>0.66±0.05***</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>0.1±0.05</td>
<td>0.21±0.01**</td>
<td>0.27±0.01***</td>
<td>0.39±0.02***</td>
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**P<0.01, ***P<0.001 vs group I
monocytes mature to macrophages, their capacity to generate free radicals decline precipitously and at the same time have the specific activity of superoxide dismutase increased. The increased generation of superoxide radical necessitates higher activities of superoxide dismutase in treated animals. In control animals, lack of activation of macrophages results in the lack of induction of superoxide dismutase as a consequence of low superoxide levels.

The activities of catalase decreased significantly in histone H1 treated animals. The lowered activities of catalase shows the lack of lipid peroxide generation as lipid peroxides have been reported to induce catalase in vascular cells. Lack of lipid peroxidation was also evident from significant decrease in the levels of TBARS. The absence of lipid peroxidation may be attributed to the generation of NO. Generation of NO confers protection to activated macrophages as, apart from being a toxic radical, it also acts as an antioxidant.

L929 cells are NO resistant and are capable of detoxifying NO responsible for cell death. Macrophage mediated apoptosis in L929 explains that NO dependent cytotoxic mechanism is not operant in the induction of apoptosis. Instead TNFα production partially explains cytotoxicity in L929 cells. The enhanced cytosis of L929 cells by macrophages from animals treated with histone H1 shows that histone H1 also activates macrophages by a non-oxidative mechanism.

The increased activities of lysosomal enzymes signify the role of histone H1 in activating macrophages non-oxidatively. Results from the current study favor both oxidative and non-oxidative dependent mechanisms involved in the activation of macrophage by histone H1.

Acknowledgement

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