A chemiluminescent probe with a *Cypridina* luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, specific and sensitive for O_2^- production in phagocytizing macrophages

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When a *Cypridina* luciferin analog (the title compound) was added to a macrophage suspension in Hank's balanced salt solution (control), the system emitted a weak, but detectable light, which was not altered in the presence of superoxide dismutase. The same system, however, emitted a much stronger light, just after the addition of a trigger, opsonized zymosan. The luminescence was suppressed to the control level in the presence of superoxide dismutase, while it was only slightly influenced, if at all, by NaN_3, a scavenger of singlet oxygen and an inhibitor of myeloperoxidase. Some other results obtained also indicate the participation of O_2^- in the luciferin analog-dependent luminescence in macrophages during phagocytosis.

Macrophage *Superoxide anion* (Cypridina) *Luciferin analog* *Chemiluminescence*

1. INTRODUCTION

During the process of phagocytosis or chemical activation, macrophages (MCP), as well as polymorphonuclear leukocytes, undergo remarkable alterations in oxidative metabolism. One such change is the generation of O_2^- [1–3], which can be monitored by one-electron reduction of ferricytochrome *c* added to the system. The method of assay, however, requires a relatively large number of cells. Allen and Roose [4] first described the luminol-dependent luminescence in activated alveolar or peritoneal MCP, which could be involved in the oxidation of luminol to excited aminophthalate ion by O_2^-. This chemiluminescent probe is highly sensitive for the detection of O_2^-, but not specific for O_2^-. This report describes that a chemiluminescent probe with a *Cypridina* luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (CLA), is very useful for investigating the ability of O_2^- generation by MCP during phagocytosis.

2. MATERIALS AND METHODS

2.1. *Macrophages*

Mouse peritoneal or human peripheral blood MCP were prepared according to Kumagai et al. [5] and resuspended in Hank's balanced salt solution (HBSS).

2.2. *Enzyme and special reagents*

Milk xanthine oxidase (XO) and bovine erythrocyte superoxide dismutase (SOD) were purchased from Boehringer Mannheim and Sigma, respectively. The former was dialysed overnight against

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0.01 M phosphate buffer (pH 7.4) at 4°C. Opsonized zymosan (OZ) was prepared by the method of Johnston et al. [6] and its amount expressed as dry weight of zymosan used. CLA was synthesized according to [7]. 6 mg CLA was dissolved in 100 ml distilled water and stored at −20°C. The molar extinction coefficient of CLA in water was 8900 at 410 nm. Luminol from Aldrich (18.26 mg) was dissolved in a slightly alkaline solution which was then brought to pH 7.4 with HCl, the volume adjusted to 100 ml with water and stored at −20°C.

2.3. *Assays*

XO activity was determined as described by Roussos [8]. O_2^- production was monitored by the reaction of ferricytochrome *c* according to [9].

2.4. *Incubation*

The incubation mixture contained MCP-OZ or hypoxanthine-XO, CLA or luminol, and HBSS (1.8–1.9 ml) in a total volume of 2 ml. In some cases, 5×10^{-7} M SOD or 5×10^{-4} M NaN_3 was added to and one and two components excluded from the mixture. All components, except for OZ and CLA or XO and CLA, were preincubated for 3 min and the reaction initiated by the simultaneous addition of the other two components. CLA- or luminol-dependent luminescence was monitored with a luminescence reader (Aloka, BLD-101) as in [10]. During luminescence measurement, the incubation mixture was rotatively agitated at 37°C in the luminescence reader. Light yield (integrated light intensity) was determined as in [9–11].

3. RESULTS

No detectable emission was obtained in HBSS containing MCP or both OZ and MCP. When CLA in water was added to an MCP suspension (or none) in HBSS a detectable light was emitted which did not increase with time and was not altered in the presence of SOD (fig.1 and inset, CONT). On the other hand, the same system containing CLA and MCP in HBSS emitted a strong light, just after the addition of the trigger, OZ. Light intensity (counts/min) as a function of time increased, reached its maximum and decreased (fig.1 and inset, CLA). The CLA-dependent luminescence induced by phagocytosis (CLA minus CONT) was completely abolished by SOD (fig.1 and inset, CLA + SOD), while it was only slightly influenced, if at all, by NaN_3 (fig.1, CLA + AZ). The system containing luminol at the same concentration as that of CLA did not emit enough light during phagocytosis (fig.1 and inset, L). With a fixed CLA concentration, increasing the number

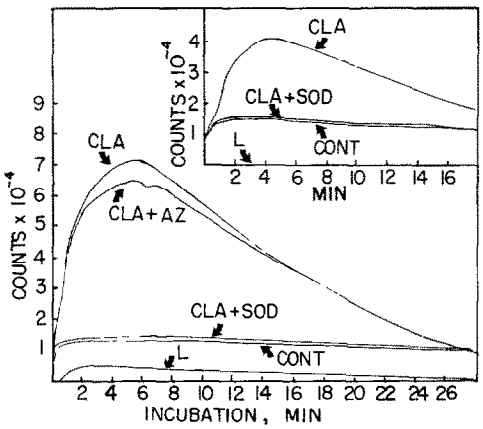

Fig.1. CLA-dependent luminescence during phagocytosis. The incubation mixture contained 1 μM CLA (CLA) or 1 μM luminol (L), 1×10^6 MCP (human peripheral blood), 2 mg OZ, 5×10^{-7} M SOD (CLA + SOD) or none and HBSS. NaN_3 (CLA + AZ) at 5×10^{-4} M was added to the incubation mixture instead of SOD. For control (CONT), both MCP and OZ or only OZ was omitted from the above system. Inset: incubation mixture and conditions essentially the same as above, save that 4×10^5 MCP (mouse peritoneal exudate) was added instead of human MCP.

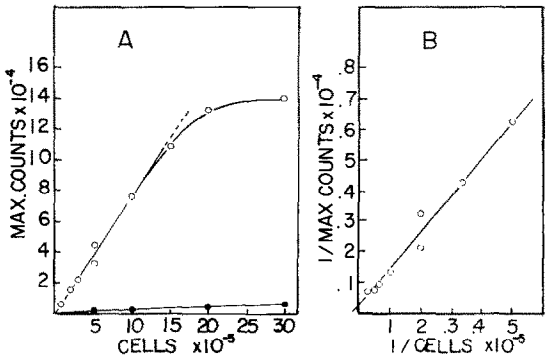

Fig.2. Effect of the number of cells on CLA-dependent luminescence (A,B). The incubation mixture contained 1 μM CLA (○) or 1 μM luminol (●), 2 mg OZ, MCP (human peripheral blood) and HBSS. Maximal light intensity (max. counts/min) was corrected for control excluding OZ.

of MCP resulted in a linear increase in the maximal light intensity (corrected for control) up to 1×10^6 cells/2 ml (fig.2A). With the data in fig.2A, the plot 1/max. counts vs 1/number of MCP yielded a straight line (fig.2B). To prove the participation of O_2^- in luminescence of CLA, CLA was exposed to a hypoxanthine-XO system, in which the number of enzyme units was varied and the luminescence detected. As shown in fig.3,

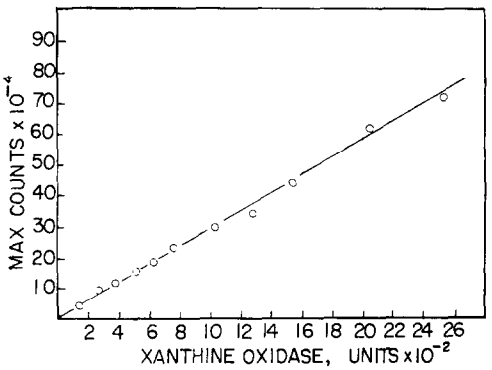

Fig.3. Luminescence of CLA in a hypoxanthine-XO system. The incubation mixture contained 1 μM CLA, 43 μM hypoxanthine, XO, and HBSS. Maximal light intensity was corrected for control excluding XO.

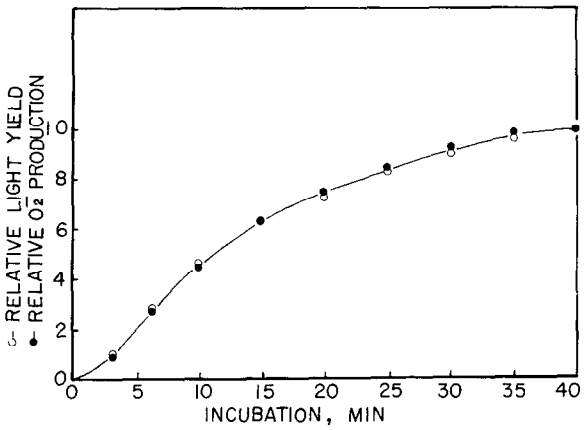

Fig.4. Comparison of light yield with O_2^- production during phagocytosis. The incubation mixture for the luminescent probe contained 15 μM CLA, 1.25×10^6 MCP (human peripheral blood) per ml, 1 mg OZ/ml and HBSS in a total volume of 2 ml. The same system, but excluding CLA and containing 0.1 mM ferricytochrome *c* in a total volume of 20 ml, was used for the measurement of O_2^- production. The values were corrected for controls excluding OZ.

maximal light intensity (corrected for control excluding XO) increased linearly with increasing number of XO units. Thus, one can derive the following experimental expression (eqn 1)

$$\text{max. counts/min} = 294 \times \text{XO unit} \quad (1)$$

In all cases, SOD completely abolished the enzyme-induced luminescence (not shown). Correlation of light yield with O_2^- production during phagocytosis is shown in fig.4. This clearly indicates that O_2^- production is parallel to CLA-dependent light yield. Thus the rate of O_2^- generation should be proportional to CLA-dependent light intensity during phagocytosis.

4. DISCUSSION

The luminescent probe with CLA was first applied to the quantitative determination of xanthine and XO activity [12]. CLA, however, becomes luminescent if the reaction mixture contains some oxidants such as H_2O_2 and a catalytic amount of Fe^{3+} [12]. A non-specific luminescence also appeared when CLA in water was mixed with HBSS. However, luminescence of this type is strikingly different from CLA-dependent luminescence in phagocytizing MCP, with respect to the intensity at, time course of and SOD effect on the luminescence. The results obtained with a well-known O_2^--generating system and the comparison of light yield with O_2^- production durng phagocytosis clearly indicate the participation of O_2^- in CLA-dependent luminescence. Singlet oxygen, O_2 ($^1\Delta g$ type), may also participate in the luminescence of CLA. Cadenas et al. [13] have reported the possible formation of $^1O_2$ in Con A-activated MCP. Furthermore, CLA also becomes luminescent when added to myeloperoxidase H_2O_2 in HBSS [14]. Even though myeloperoxidase activity in MCP is zero or much lower than that in polymorphonuclear leukocytes [15], the activity in MCP could be detected by a very sensitive method [16]. However, the involvement of $^1O_2$ or a myeloperoxidase-H_2O_2-Cl system in CLA-dependent luminescence during phagocytosis would be neglected, because of the slight effect, if any, of azide, an inhibitor of myeloperoxidase [17] and a scavenger of $^1O_2$ [18], on the luminescence response. Thus, the ability of O_2^- production by MCP during

phagocytosis can be simply expressed as XO units, by substituting the maximal light intensity measured in a phagocytizing system (corrected for control) into eqn 1.

REFERENCES

[1] Johnston, R.B. jr, Godzik, C.A. and Cohn, Z.A. (1978) J. Exp. Med. 148, 115–127.
[2] Murray, H.Y. and Cohn, Z.A. (1980) J. Exp. Med. 152, 1590–1608.
[3] Kitagawa, H.Y., Takaku, F. and Sakamot, S. (1980) J. Immunol. 125, 359–364.
[4] Allen, R.C. and Roose, L.D. (1976) Biochem. Biophys. Res. Commun. 69, 245–252.
[5] Kumagai, K., Itoh, K., Hinuma, S. and Tada, M. (1979) J. Immunol. Methods 29, 17–25.
[6] Johnston, R.B. jr, Keel, B.B. jr, Misra, H.P., Lehmeyer, J.E., Webb, L.S., Baemer, R.L. and Rajagopalan, K.V. (1975) J. Clin. Invest. 55, 1357–1372.
[7] Inoue, S., Sugiura, S., Kakoi, H. and Goto, T. (1969) Tetrahedron Lett. 1609–1610.
[8] Roussos, G.G. (1967) Methods Enzymol. 12, 5–16.
[9] Ushijima, Y. and Nakano, M. (1980) J. Appl. Biochem. 2, 138–151.
[10] Kobayshi, S., Sugioka, K., Nakano, H., Nakano, M. and Tero-Kubota, S. (1984) Biochemistry 23, 4589–4597.
[11] Nakano, M. and Sugioka, K. (1977) Arch. Biochem. Biophys. 181, 371–383.
[12] Goto, T. and Takagi, T. (1980) Bull. Chem. Soc. Jap. 53, 833–834.
[13] Cadenas, E., Daniele, R.P. and Chance, B. (1981) FEBS Lett. 123, 225–228.
[14] Nakano, M., unpublished.
[15] Gee, J.B.L., Vassallo, C.L., Bell, P., Kaskin, J., Basford, R.E. and Field, J.B. (1970) J. Clin. Invest. 49, 1280–1287.
[16] Seim, S. (1983) Acta Pathol. Microbiol. Immunol. Scand. C91, 123–128.
[17] Klebanoff, S.J. and Pincus, S.H. (1971) J. Clin. Invest. 50, 2226–2229.
[18] Hastry, H., Merkel, P.B., Raddich, R. and Keans, D.R. (1972) Tetrahedron Lett. 49–52.