Bioluminescence, chemiluminescence

29

Pattern and functions (chemiluminescence, enzyme secretion) of alveolar macrophages and granulocytes in the posttraumatic course

A. Dwenger[1], C. Beychok[2], G. Schweitzer[1], and H. C. Pape[3]

[1] Abteilung für Klinische Biochemie,
Medizinische Hochschule Hannover,
Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61,
Federal Republic of Germany
[2] Albert Einstein College of Medicine, Bronx, N.Y., USA
[3] Unfallchirurgische Klinik der MHH, Hannover

In the recent years the essential role of granulocytes in the initiation and amplification of the pathomechanisms resulting in the adult respiratory distress syndrome (ARDS) has been documented [3, 5−7]. On the contrary, there is only little information about the participation of alveolar macrophages in the ARDS pathogenesis [4] and no report on alterations of macrophage functions during the development of the ARDS.

Methods

Bronchoalveolar lavage fluid (BALF) of 13 multiply traumatized patients (Injury Severity Score >30 points; 6 ARDS and 7 Non-ARDS patients) and of 7 controls (Co) was centrifuged at $400 \times g$, 10 min, $10°C$ [1, 2]. The supernatant was analysed for β-N-acetyl-glucosaminidase (β-NAG) spectrofluorimetrically and for elastase by enzymeimmunoassay [1]. The cell pellet was resuspended in phosphate buffered saline and the cell count was determined after staining with Türk's solution and the cell pattern by the use of a cytospin [1]. For the separation of granulocytes (PMNL) and alveolar macrophages (AMϕ) the cell resuspension was centrifuged on Percoll (1.058 g/ml) at $12000 \times g$, 30 min, $4°C$. From citrated blood granulocytes

were isolated by a two-step discontinuous Percoll gradient (1.064/1.095 g/ml) centrifugation at $350 \times g$, 20 min, $25°C$. The zymosan-induced and luminol-enhanced chemiluminescence response (CL) of granulocytes and macrophages, respectively, was determined with a six-channel Biolumat LB 9505 [1, 2]. Urea was determined enzymatically in the plasma and the BALF [1] and the concentration of proteins/cells in the epithelial lining fluid (ELF) was calculated according to

$$c_{ELF} = c_{BALF} \cdot c_{urea\text{-}Plasma}/c_{urea\text{-}BALF}$$

Statistical analyses were performed by the Student's non-paired t-test. For calculation and graphical presentation the results of each 2 days were combined.

Results

The posttraumatic courses of alveolar neutrophil and macrophage counts as well as the cell patterns of the bronchoalveolar lavage fluids for ARDS and Non-ARDS patients are listed in Table 1.
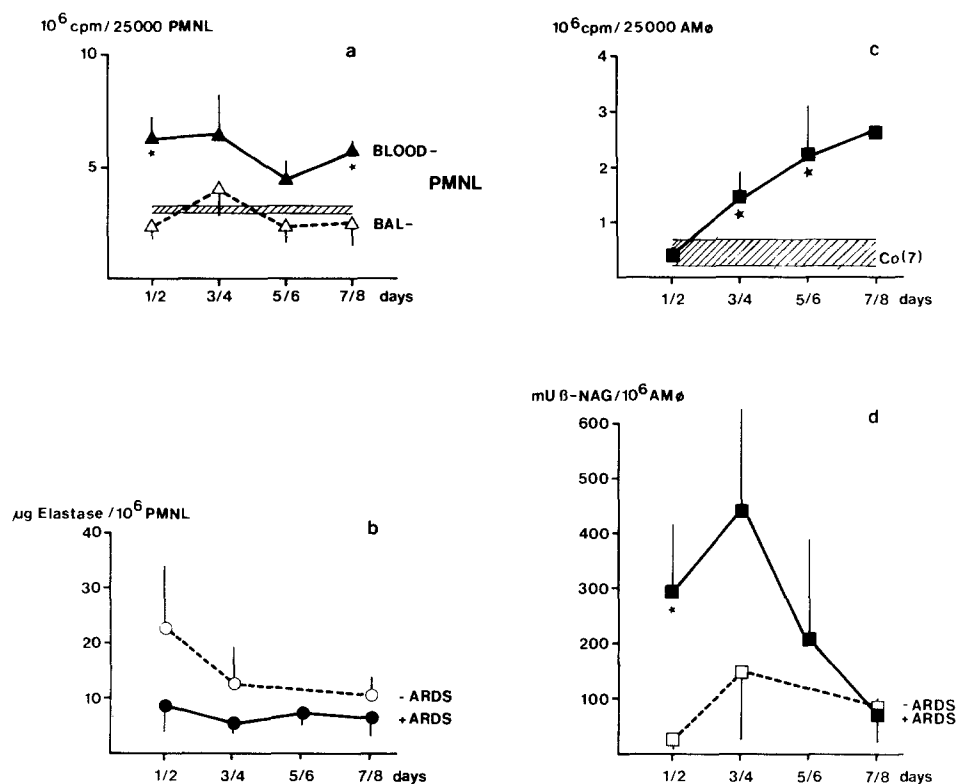
Figure 1 depicts the chemiluminescence response of isolated blood and alveolar neutrophils (a) and alveolar macrophages (c) of multiply traumatized patients as well as the secretion activity of alveolar neutrophils (b) and alveolar macrophages (d) for ARDS and Non-ARDS patients after multiple trauma. In the posttraumatic course the total alveolar cell count increased for ARDS patients and decreased for Non-ARDS patients for up to 8 days, predominantly caused by the neutrophil influx. There was an early (days 1/2) pathological elevation of the neutrophil fraction above normal values (<1% neutrophils) that further increased for ARDS patients and decreased for Non-ARDS patients, whereas the alveolar macrophage fraction (93% for normals) exhibited the corresponding inverse behaviour.

Concerning the chemiluminescence response against zymosan, blood neutrophils were hyperactive and BALF derived

Table 1. Posttraumatic courses of alveolar neutrophil and macrophage counts (as 10^6 cells/ml epithelial lining fluid) and cell patterns (% neutrophils, % macrophages) for ARDS and Non-ARDS patients

	Neutrophil count		Macrophage count		% Neutrophils		% Macrophages	
	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM
Days 1/2								
ARDS	5.45[a]	2.75	1.86	1.11	60.0	16.5	30.7	18.2
Non-ARDS	1.21	0.66	3.59	1.20	36.0	9.1	57.0	12.3
Days 3/4								
ARDS	3.26	0.32	0.22[a]	0.10	56.8	18.6	37.8	16.9
Non-ARDS	2.77	0.84	3.48	1.23	50.4	9.9	37.6	10.5
Days 5/6								
ARDS	5.81	1.64	0.56	0.22	88.0[a]	6.1	10.3[a]	5.8
Non-ARDS	−		−		23.0	0	70.0	0
Days 7/8								
ARDS	9.11[a]	2.47	1.57	0.94	77.0[a]	10.8	20.6[a]	10.8
Non-ARDS	1.72	0.65	2.14	0.37	33.0	15.0	66.0	14.0

[a] $p < 0.05$ ARDS vs Non-ARDS

Fig. 1 a — d. Posttraumatic chemiluminescence response ($\bar{x} \pm$ SEM) of blood (▲) and BALF (△) derived neutrophils **a** and alveolar macrophages **c** of multiply traumatized patients

    normal range of controls (Co)
$\bar{x} \pm$ SEM n = 43 (PMNL)
n = 7 (AM ϕ), * p < 0.05

Posttraumatic enzyme secretion activity ($\bar{x} \pm$ SEM) of alveolar neutrophils **b** as µg elastase/10^6 PMNL (calculated from µg elastase/l ELF/PMNL count/l ELF) and alveolar macrophages **d** as mU β-NAG/10^6 AM ϕ (calculated from mU β-NAG/l ELF/ AM ϕ count/l ELF) for ARDS and Non-ARDS patients

neutrophils were hypoactive from the beginning up to day 8 after trauma, whereas alveolar macrophages developed a hyperactive state not before days 3 or 4. On the contrary, alveolar macrophages were already active in enzyme secretion in the initial posttraumatic course (days 1/2), especially of ARDS patients. Alveolar neutrophils showed an initially increased enzyme secretion, especially in Non-ARDS patients. The secretory reactivity decreased for both cell types and for both patient groups up to 8 days after trauma.

Conclusion

After multiple trauma alveolar macrophages responded with different cellular reactions in different time courses, whereas alveolar neutrophils seemed to have lost most of their metabolic capacity before they invaded into the alveoli.

References

1. Dwenger A, Schweitzer G, Regel G (1986) J Clin Chem Clin Biochem 24:73–88
2. Dwenger A, Schweitzer G, Regel G, Funck M, Sturm JA, Tscherne H (1988) Fresenius Z Anal Chem 330:441–442
3. Regel G, Dwenger A, Seidel J, Nerlich ML, Sturm JA, Tscherne H (1987) Unfallchirurg 90:99–106
4. Steppling H (1986) Prax Klin Pneumol 40:226–228
5. Tate RM, Repine JE (1983) Am Rev Respir Dis 128:552–559
6. Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, Gadek JE (1986) Am Rev Respir Dis 133:218–225
7. Zimmerman GA, Renzetti AD, Hill HR (1983) Am Rev Respir Dis 127:290–300

30

Dose-dependent inhibition by prostaglandin E1 of oxygen radical production, adherence and enzyme release of stimulated polymorphonuclear leukocytes

A. Dwenger[1], G. Schweitzer[1], G. Röllig[1], and M. L. Nerlich[2]

[1] Abteilung für Klinische Biochemie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61, Federal Republic of Germany
[2] Unfallchirurgische Klinik der MHH, Hannover

Polymorphonuclear leukocytes (PMNL, neutrophils) hyperactivated by multiple trauma release oxygen derived radicals and lyosomal enzymes. These inflammatory mediators can damage endothelial structures of capillaries and, therefore, contribute to the development of multi-organ failure including the adult respiratory distress syndrome (ARDS) [5, 6]. One therapeutical approach to prevent the ARDS is based on the inhibition of neutrophil functions by prostaglandin E1 (PGE1) [4].

Methods

Citrated blood was obtained from donors, PMNL were isolated by Percoll gradient centrifugation [1, 3].

The oxygen radical production was measured by luminol (0.4 mmol/l test) and/or lucigenin (0.23 mmol/l test) enhanced chemiluminescence response (CL) (Biolumat LB 9505, Berthold) in absence or in presence of different stimuli, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, Sigma; $3.5 \cdot 10^{-6}$ mol/l test), zymosan A (Sigma; 3.5 mg/ml test), latex (Unisphere latex 22, 0.8 µm, Serva; 2 µl/ml test), lipopolysaccharide (LPS from E. coli serotype No. 055:B5, Sigma; 20 ng/ml test), 4β-phorbol 12β-myristate 13a-acetate (PMA, Sigma; $5 \cdot 10^{-6}$ mol/l test) and nylon fiber (from Leuko-Pak Leukocyte Filter, Fenwal, Travenol Laboratories; 5 mg/test) [1–3]. All parameters have been measured in dependency on the PGE1 concentration (Alprostadil, Schwarz Pharma AG).

The enzyme release was determined by the measurement of the intra- and extracellular elastase activity by the kinetical enzyme test with methoxysuccinyl-L-Ala-L-Ala-L-Pro-L-Val-p-nitroanilide (Bachem) as a substrate and of the β-N-acetyl-glucosaminidase (β-NAG) activity determined spectrofluorimetrically [1, 3]. Briefly, neutrophils were isolated from citrated blood and resuspended in Minimal Essential Medium
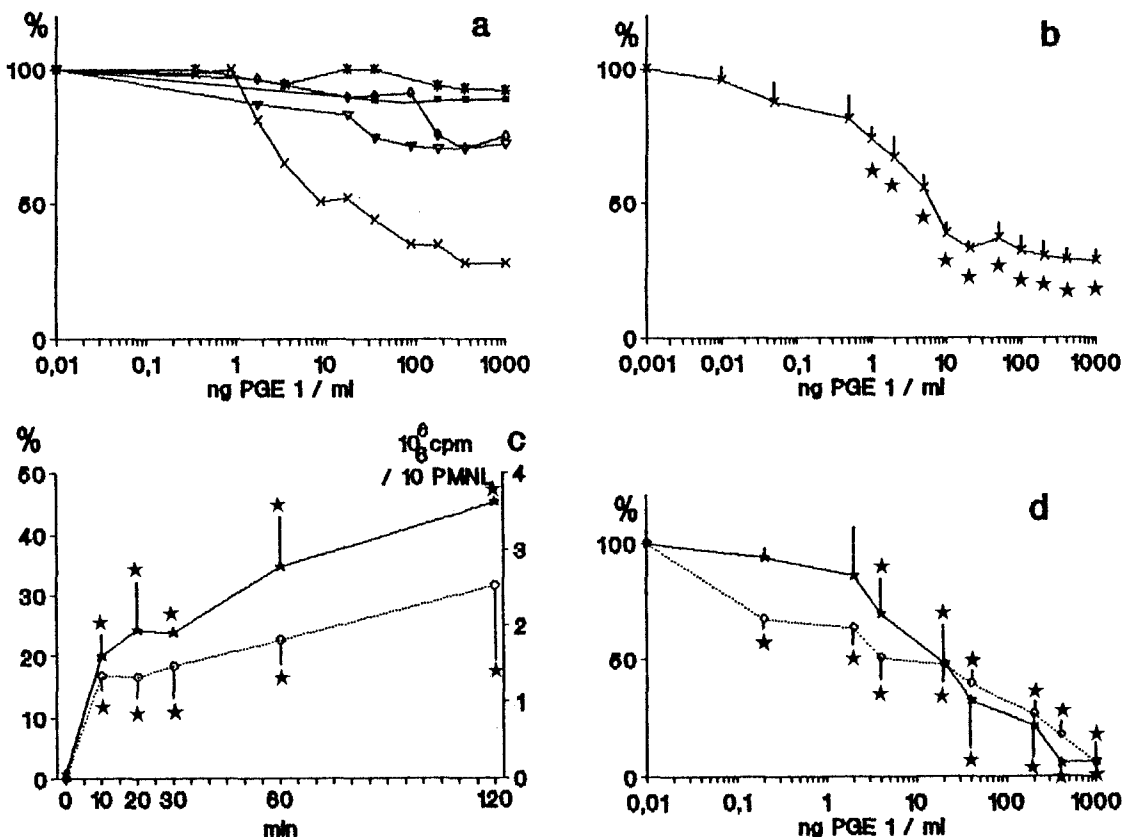
Fig. 1a–d. Inhibition by PGE1 of neutrophil CL response and adherence to nylon fiber. **a** Inhibition of CL response (%) of stimulated isolated neutrophils preincubated (10 min, 37° C) with different PGE1 concentrations. Stimuli: ×———× FMLP, *———* PMA, ◇———◇ zymosan, ■———■ latex (luminol-enhanced CL), ∇———∇ LPS (lucigenin-enhanced CL). Means of 3 experiments. 100% = CL response (cpm/25000 PMNL of the peak maximum) in the presence of the lowest PGE1 concentration. **b** Inhibition of the CL response (%; ×———×) of neutrophils in citrated blood preincubated (10 min, 37° C) with different PGE1 concentrations. Stimulus: FMLP (luminol-enhanced CL). $\bar{x} \pm$ SEM; $n = 6$. 100% = CL response (cpm/200000 PMNL of the peak maximum) in the presence of the lowest PGE1 concentration. ★ $p < 0.05$ *values vs* 100% *values.* **c** Adherence (%; *———*) and CL response (10^6 cpm/10^6 PMNL; ⊙·····⊙) of neutrophils in citrated blood in dependency on the incubation/adherence time. Stimulus: 5 mg of nylon fiber (lucigenin-enhanced CL). $\bar{x} \pm$ SEM; $n = 6$. ★ $p < 0.05$ values vs 0 min values. **d** Adherence (%; *———*) and CL response (%; ⊙·····⊙) of neutrophils in citrated blood in dependency on the PGE1 concentration. Stimulus: 5 mg of nylon fiber (lucigenin-enhanced CL). $\bar{x} \pm$ SEM; $n = 6$. 100% = CL response (10^6 cpm/10^6 PMNL of the peak maximum) in the presence of the lowest PGE1 concentration. ★ $p < 0.05$ *values vs* 100% *values*

(Boehringer) with 0.5% bovine serum albumin to 10^7 PMNL/ml. PGE1 was added (0; 2; 200, and 1000 ng/ml) and after 10 min at 37°C the stimulation was started with FMLP (3.5 · 10^{-6} mol/l test). After 45 min at 37°C the reaction mixtures were centrifuged and the supernatants were analysed for elastase and β-NAG. Additionally, an aliquot of the original PMNL suspension was lysed and the enzymes were determined [1, 3].

The neutrophil count in blood and of isolated cells was performed by the use of a Neubauer hemocytometer after staining with Türks solution [1, 3]. The significance between groups of values were tested by the tests according to Wilcoxon and Mann-Whitney, respectively, and accepted if $p < 0.05$.

Results

The dose-dependent inhibition by PGE1 of the CL response of isolated neutrophils after stimulation with different stimuli is shown in Fig. 1a, whereas Fig. 1b depicts the inhibition of neutrophils in blood after FMLP stimulation. The fraction of neutrophils adhered to nylon fiber as well as the corresponding CL response are shown in dependency on the incubation/adherence time in absence of PGE1 (Fig. 1c). The correlation coefficient of the linear regression analysis between adherence and CL values was $r = 0.975$.

The dose-dependent inhibition by PGE1 of adherence and CL response is shown in Fig. 1d. The correlation coefficient between adherence and CL values was $r = 0.945$.

The FMLP-induced enzyme release (elastase/β-NAG) was 12%/7.1% without PGE1; 8%/4% with 2 ng/ml; 1.6%/3.7% with 200 ng/ml; 0.5%/2.2% with 1000 ng PGE1/ml test volume (100% = total intracellular enzyme activities). The values represent the means of 6 experiments.

Conclusion

The production and the release of oxygen derived radicals and lysosomal enzymes from stimulated polymorphonuclear leukocytes were inhibited by prostaglandin E1 in a dose-dependent manner. Furthermore, regarding the nylon fiber system as an artificial but relevant model for endothelial cell adherence [2] the observed inhibition by PGE1 of adherence and CL response seems to be of marked pathophysiological importance. Since the contact activation/stimulation of neutrophils was inhibited and less inflammatory mediators were produced, less endothelial cell and tissue structure damage can be produced in an in vivo system. The good correlation of adherence and CL production in absence and in presence of PGE1 indicated that the oxygen radical production is nearly exclusively caused by adherence-mediated stimulation and can be inhibited by PGE1. As a result, PGE1 may be a drug to prevent inflammation-induced damage of capillary endothelial structures in different disorders, e.g. the adult respiratory distress syndrome.

References

1. Dwenger A, Schweitzer G, Regel G (1986) J Clin Chem Clin Biochem 24:73−88
2. Dwenger A, Schweitzer G, Funck M (1988) J Biolum Chemilum 2:35−39
3. Dwenger A, Tost P, Holle W (1986) J Clin Chem Clin Biochem 24:299−308
4. Holcroft JW, Vassar MJ, Weber CJ (1986) Ann Surg 203:371−378
5. Regel G, Dwenger A, Seidel J, Nerlich ML, Sturm JA, Tscherne H (1987) Unfallchirurg 90:99−106
6. Tate RM, Repine JE (1983) Am Rev Respir Dis 128:552−559

31

Effect of varying concentrations of prostaglandin E 1 on chemiluminescence response and endothelial cell damage during interaction between polymorphonuclear leukocytes and human endothelial cells

E. Jonas, A. Dwenger, and M. Funck

Abteilung für Klinische Biochemie,
Medizinische Hochschule Hannover,
Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61,
Federal Republic of Germany

The present study is based upon the theory that polymorphonuclear leukocytes (PMNL, neutrophils) play an important role in inflammatory reactions [4]. Further investigations hypothesize that affection of endothelial cells (EC), mediated by lipopolysaccharide (LPS)-stimulated neutrophils, is rather caused by oxygen derived metabolites than by lysosomal enzymes [5, 7]. Investigations, performed with endothelial cells, have shown a respiratory burst stimulation of neutrophils during adherence of neutrophils to EC and amplification of this process by previous LPS-priming of neutrophils [3].

The aim of the present study was to examine, if PGE 1 might influence injury to EC, caused by LPS-primed neutrophils, and furthermore, if this effect could be explained by a diminished oxygen radical production, measured by chemiluminescence (CL).

Methods

Endothelial cells. Human umbilical cord vein endothelial cells were harvested according to [2]. After reaching confluence in RM-medium containing 12% human serum, endothelial cells were trypsinized onto cover slips (Lux Scientific Corporation) for measurement of chemiluminescence response and were split onto microtiter wells (Greiner Co) for measurement of cell injury.

Neutrophils. Blood of healthy donors was preincubated for 20 min at 37°C with 20 ng LPS/ml blood (LPS: E. coli serotype 055: B5, Sigma Co). Neutrophils were prepared using Percoll density gradient centrifugation according to [1], and were resuspended in phosphate buffered saline. 70,000 neutrophils were added to 10,000 endothelial cells/test.

Prostaglandin preparation. Varying concentrations of prostaglandin E 1 (donation of Schwarz Pharma, Monheim, FRG) were prepared in 0.9% NaCl solution. Concentrations were 5, 10, 25, 50, 100 and 700 ng/ml test.

Chemiluminescence measurements. Production of oxygen derived metabolites by neutrophils was measured by lucigenin enhanced chemiluminescence, which has been measured simultaneously in a six channel Biolumat (LB 9505 C, Berthold, Wildbad, FRG). Chemiluminescence measurements (cpm of peak maximum) of LPS-primed neutrophils were performed in the absence and presence of varying concentrations of PGE 1.

Injury assay. The evaluation for EC damage was based upon ^{111}In-release from labeled endothelial cells, as described [6].

Statistical analysis. For statistical analysis U-test according to Mann-Whitney was used. Values represent mean +/- SEM of experiments performed in duplicate.
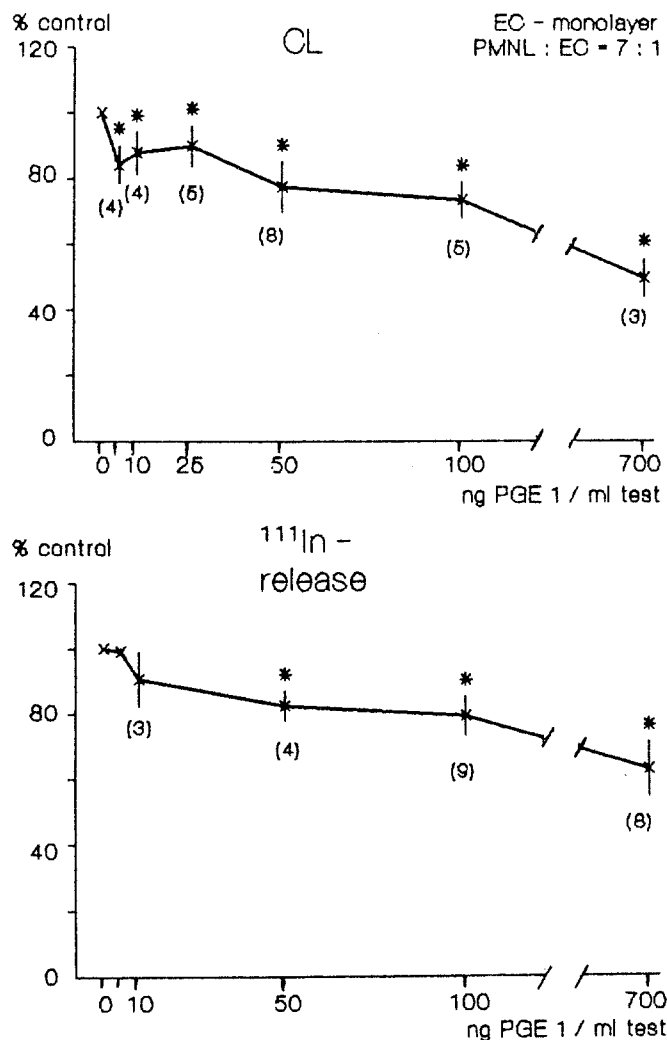
Fig. 1. Inhibitory effect of varying concentrations of PGE 1 on chemiluminescence response of neutrophils during interaction between neutrophils and endothelial cells (upper section) and on ^{111}In-release of labeled EC (lower section). Data are shown as $\bar{x} \pm$ SEM; values were considered significant when $p < 0.05$

Results

In Fig. 1 dose response relationship between varying concentrations of PGE 1 and inhibitory effect on chemiluminescence response (upper section) and inhibitory effect on ^{111}In release (lower section) is shown. Respiratory burst stimulation of neutrophils was already significantly different at a concentration of 5 ng/ml test in comparison to control. Incubation with 50 ng PGE 1/ml test resulted in a significant suppression of ^{111}In-release. The correlation coefficient between chemiluminescence response and ^{111}In-release was 0.67.

Conclusions

1. Respiratory burst stimulation of neutrophils, caused by EC and additionally by LPS-priming of neutrophils, could be diminished by PGE 1 in a dose dependent manner.

2. EC-injury measured by ^{111}In-release of labeled EC could be suppressed by PGE 1.

3. Therefore, these results support the hypothesis that oxygen derived metabolites might contribute to EC injury, as supposed for the pathogenesis of the Adult Respiratory Distress Syndrome.

References

1. Dwenger A, Schweitzer G, Regel G (1986) J Clin Chem Clin Biochem 24:73−88
2. Gimbrone MA, Shefton EJ, Cruise SA (1978) Tissue Culture Association Manual 4:813−817
3. Jonas E, Dwenger A, Lueken B (1988) Fresenius Z Anal Chem 330:421−422
4. Repine JR, Bowmann CM, Tate RM (1982) Am Rev Respir Dis 81:47−50
5. Shingu M, Yoshioka K, Nobunaga M, Yoshida K (1985) Inflammation 9:309−320
6. Smedly LA, Tonnesen MG, Sandhaus RA, Haslett C, Guthrie LA, Johnston RB, Henson PM, Worthen GS (1986) J Clin Invest 77:1233−1243
7. Wong C, Flynn J, Demling RH (1984) Arch Surg 119:77−82

32

Chemiluminescence response of whole blood and polymorphonuclear leukocytes following experimentally induced haemorrhagic-necrotising pancreatitis

T. Zimmermann[1], S. Albrecht[2], R. Schuster[1], G. Lauschke[1], and W. Jaroß[2]

[1] Klinik für Chirurgie
[2] Institut für klinische Chemie und Laboratoriumsdiagnostik der Medizinischen Akademie Dresden, Fetscherstrasse 74, DDR-8019 Dresden, German Democratic Republic

Introduction

The release of toxic oxygen metabolites from sensitised polymorphonuclear leukocytes is an important pathogenetic factor in a series of diseases (ARDS, MOF, myocardial infarction). Moreover, toxic oxygen metabolites seem also to play an important part in the formation of necroses in acute haemorrhagic-necrotising pancreatitis. Kelemen et al. [2] found excessive production of toxic oxygen metabolites (MDA) accompanied by loss of tissue antioxidative capacity (SOD, GSH) in experimentally induced haemorrhagic-necrotising pancreatitis in rats. Besides the liver, the pancreas is known to surpass all other organs with respect to radical formation and its antioxidative potential. However, to what extent toxic oxygen metabolites are responsible for the development of MOF as a consequence of acute pancreatitis has scarely been investigated as yet.

The present study is intended to clarify the question, whether the activation of granulocytes takes place in the pancreas and whether these activated granulocytes are capable of releasing toxic oxygen metabolites which substantially contribute to endothelial cell damage in the respective organs (lung, kidney, liver, small intestine, heart).

In addition, the antioxidative effect of MDTQ-DA was studied in a first in vitro test.

Methods

Following anaesthetization with pentobarbital (Nembutal®), acute haemorrhagic-necrotising pancreatitis was induced in dogs by injection of 0,5 ml/kg BW of autologous bile. Catheters for selective blood sampling were placed in the portal vein (via splenic vein) and in the coeliac artery (via femoral artery). Samples were taken within 15 min after placing the catheters, at the moment of bile injection and within 1, 2, 4, and 24 h after the injection of bile. At these times CL response was determined in whole blood and separated granulocytes from the portal vein (i.e. after passage through the pancreas) and the coeliac artery
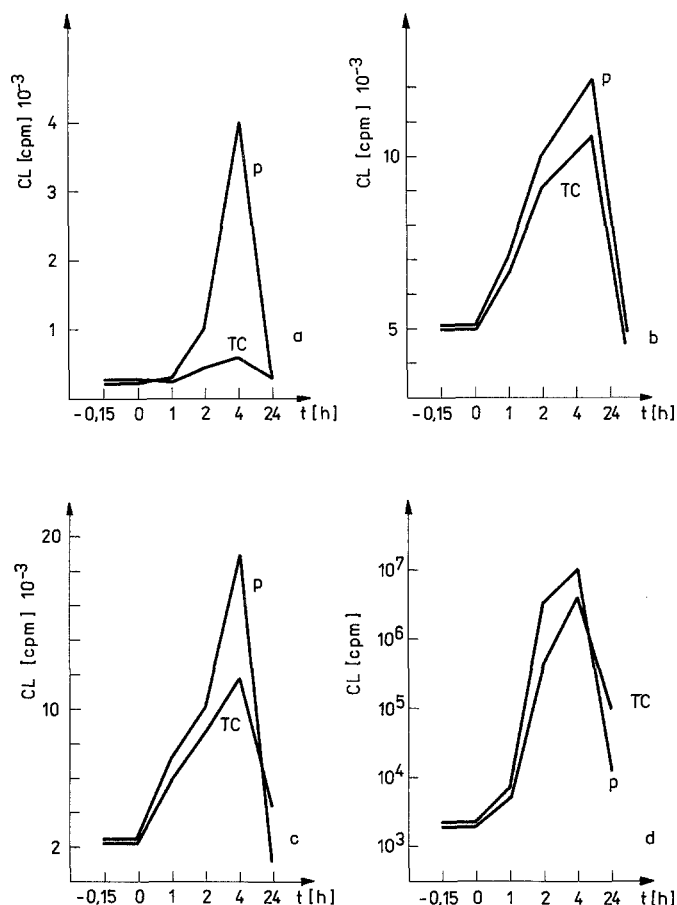
Fig. 1 a−d. CL response of whole blood and separated granulocytes from the portal vein (P) and the coeliac artery (TC): **a** spontaneous CL of whole blood; **b** zymosan-activated whole blood; **c** spontaneous CL of granulocytes; **d** zymosan-stimulated granulocytes

(i.e. before passage through the pancreas). In addition, various complement components (CH_{50}, AH_{50}, C_{5A}, $C_{5A\ INH}$) were determined and tissue samples taken for immunohistochemical detection of complement deposits in the pancreas (C_{3B}). For the separation of granulocytes we modified the technique inaugurated by Gazit and Gil [1]. The suspension contained 98% polymorphonuclear leukocytes, 95−99% of which were viable. Samples of 10 μl of zymosan activated whole blood and of 10^6 separated granulocytes stimulated with opsonized zymosan and suspended in 1 μl of a nutrient solution as measured in a Clinilumat LB 9502 (Fa. Berthold, FRG) after 30 min of incubation. 100 μl of luminol (10^{-4} in PBS) were added as sensitiser to each sample.

Results

As appears from Fig. 1, whole blood and especially separated granulocytes from the portal vein (to a much lower degree also from the coeliac artery) show a sharp increase in spontaneous (only luminol-enhanced) CL already within 1 h after injection of bile. The maximum is reached within 4 h after injection, with CL response being much higher in blood and granulocytes from the portal vein than in samples taken from the coeliac artery. Within 24 h after injection of bile CL response of granulocytes from the coeliac artery surpasses that of granulocytes from the portal vein. There was no difference in CL response between whole blood from the portal vein and the coeliac artery after 24 h. Stimulation with zymosan-activated plasma results in a 10-fold increase in CL response of whole blood and a 1,000 – 10,000-fold increase in CL response of granulocytes. At the same time we found massive activation of complement in the blood, accompanied by the formation of complement split products and their deposition throughout the pancreas. Addition of 0.1 μg of radical trap (MDTQ-DA) to each zymosan stimulated whole blood sample reduced CL response by 70 – 90 p.c.

Discussion

Following induction of haemorrhagic-necrotising pancreatitis, high quantities of toxic oxygen metabolites are released from pancreatic tissue, contributing to the development of MOF. Granulocytes are obviously sensitised by activated complement deposited in excessive quantities in pancreatic parenchyma. Later on, the release of toxic oxygen metabolites from activated granulocytes persists in a systemic circulation level and becomes independent of the inflammatory process in the pancreas. Stimulating agents, such as zymosan, zymosan-activated plasma, or endotoxin drastically increase the release of toxic oxygen radicals from sensitised granulocytes, causing an even greater damage to the organs. That is why chemiluminescence response of granulocytes is much higher in systemic blood than in blood from the portal vein after 24 h. Our study gives rise to the assumption that complement-induced activation of granulocytes and the release of toxic oxygen metabolites are essential pathogenetic factors in the development of MOF as sequela of acute pancreatitis. We believe that the prognosis of this disease can be considerably improved by therapeutic use of antioxidants.

References

1. Gazit E, Gil R (1983) Tissue Antigens 21:9 – 13
2. Kelemen D et al. (1987) XIth Hungarian Congress of Experimental Surgery, A 42, Szeged, Hungary

---

33

Evaluation of macrophage activation stage by measurement of lucigenin-enhanced chemiluminescence using a 96-well microtiter system

V. Kaever, E. Schmitz, and K. Resch

Institute of Molecular Pharmacology,
Department of Pharmacology and Toxicology,
Medical School Hannover, D-3000 Hannover 61,
Federal Republic of Germany

Macrophages are crucially involved in the regulation of various immune reactions. They represent a heterogeneous group of cells not only due to their different tissue origin but even more attributable to distinct activation stages during which they acquire additional receptors, metabolic functions and capacities. Reactive oxygen intermediates (ROI) such as the superoxide anion radical (O_2^-) or hydrogen peroxide (H_2O_2) contribute to the development of cytotoxic and antimicrobial activities and also play an important role in inflammatory processes [4]. The amount of ROI production strongly correlates with macrophage activation where-at fully activated cells are the most effective producers after addition of appropriate stimuli [3, 5]. ROI decay leads to the emission of small amounts of light but this chemiluminescence (CL) is enormously intensified in the presence of chemical amplifiers such as luminol and lucigenin [1]. In the report we describe a method for the determination of lucigenin-enhanced CL of resident or *in vivo* preactivated mouse peritoneal macrophages using a 96-well microtiter system (Amerlite research luminometer).

Materials and methods

Resident macrophages were obtained by peritoneal lavage of untreated DBA/2 mice [2]. Elicited or fully activated cells were
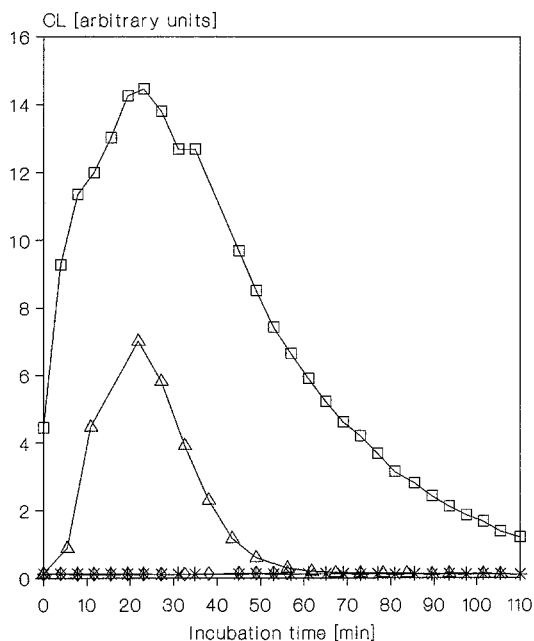
Fig. 1. 5×10^5 resident ($\Diamond$), thioglycolate-elicited ($\triangle$), or *Corynebacterium parvum*-activated ($\square$) macrophages were stimulated with 1 mg/ml zymosan or incubated without stimulus ($*$), respectively. Lucigenin-amplified CL was repeatedly measured as described in the experimental section. Means of triplicates are shown

induced by i.p. injection of 2 ml sterile thioglycolate broth (Becton Dickinson) or 1.4 mg formalin-killed *Corynebacterium parvum* (Wellcome) 6 or 14 days prior to cell harvest, respectively. The peritoneal exsudate cells were washed twice and resuspended in Hank's balanced salt solution (HBSS). In a total

volume of 250 μl 5×10^5 cells were preincubated in white 96-well microtiter plates (MicroFluor^R, Dynatech) with lucigenin (10,10′-dimethyl-bis-9,9′-acridinium nitrate) (Sigma) at a concentration of 10^{-4} mol/l for 30 min at 37°C. After addition of 1 mg/ml of the phagocytic stimulus zymosan (Sigma) CL was repeatedly measured in an Amerlite research luminometer (Amersham Buchler) in the scan only program with a dwell time of 5 s for each sample.

Results and discussion

Figure 1 shows that by use of Amerlite research luminometer the ROI production of differentially preactivated macrophages could easily be measured. Where-as thioglycolate-elicited or *Corynebacterium parvum*-activated cells exhibited a maximal response to zymosan after about 20 min no difference to background CL was observed with resident macrophages.

Addition of superoxide dismutase (300 U/ml) during the preincubation time totally inhibited the zymosan-induced CL response (data not shown) indicating that the lucigenin-amplified CL predominantly resulted from O_2^--production by NADPH oxidase.

The applied system seems suitable to study CL as a correlate of the macrophage activation stage. Additionally, enhancing or inhibitory properties of drugs can effectively be determined in up to 96 samples running in parallel with only small amounts of cells required.

References

1. Müller-Peddinghaus R (1984) Int J Immunopharmacol 6: 455
2. Pfannkuche H-J, Kaever V, Resch K (1986) Biochem Biophys Res Commun 139:604
3. Schleupner CJ, Glasgow LA (1978) Infection Immunity 21:886
4. Till GO, Johnson KJ, Kunkel R, Ward PA (1982) J Clin Invest 69:1126
5. Weinberg JB, Misukonis MA (1983) Cell Immunol 80: 405

34

Application of peroxyoxalate chemiluminescence in analytical biochemistry

S. Albrecht[1], H. Brandl[2], W.-D. Böhm[3], R. Beckert[4], E. Köstler[5], M. Menschikowski[1], and W. Jaroß[1]

[1] Institut für Klinische Chemie und Laboratoriumsdiagnostik der Medizinischen Akademie „Carl Gustav Carus", Fetscherstrasse 74, DDR-8019 Dresden, German Democratic Republic
[2] Gymnasium Kaltenkirchen, Neuer Weg 9, D-2358 Kaltenkirchen, Federal Republic of Germany
[3] Abteilung Urologie der Zentralen Hochschulpoliklinik der Medizinischen Akademie „Carl Gustav Carus", Fetscherstrasse 74, DDR-8019 Dresden, German Democratic Republic
[4] Sektion Chemie der Friedrich-Schiller-Universität, Lessingstrasse 1, DDR-6900 Jena, German Democratic Republic
[5] Hautklinik des Bezirkskrankenhauses Dresden-Friedrichstadt, Friedrichstrasse 41, DDR-8010 Dresden, German Democratic Republic
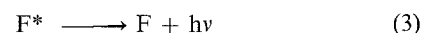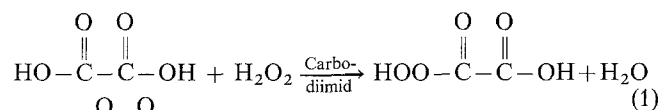
Introduction

Chemiluminescent systems based on luminol, acridine, phenanthridine, and lophine derivatives as well as singlet oxygen generating hypohalogenite−peroxy compound systems have been widely used in analytical biochemistry during the last two decades. In contrast to this, there are only few reports on the application of the currently most efficient chemiluminescent system, the peroxyoxalate chemiluminescence (CL) with CL quantum yields up to 0.34 Einstein/mol in aprotic solvents. Oxalic acid esters or oxamides which are poorly soluble in protic environment and/or susceptible to solvolysis are predominantly used in micellar or reversed micellar systems for the detection (also as HPLC-detector) of H_2O_2 or fluorescent compounds [6]. Moreover, there are attempts at using specially substituted dioxetanes as labels for CL-Immunoassay technique [5]. One of the most interesting recent papers describes the peroxyoxalate CL as an extraordinarily favourable alternative to the haematoporphyrin sensitized phototherapy for tumours without light in animal experiments [7]. In the following the applicability of the CL system oxalic acid/fluorescer/dehydrator/peroxy compound is described with regard to biochemical analysis in protic solvents.

Reaction principle

In 1965, Rauhut described bright, strongly visible CL of an oxalic acid/carbodiimide/fluorescer/H_2O_2 system in aprotic solvents [8]. Our own experiments showed intensive short-time CL at pH = 1 with a maximum within 0.4 s after the start of reaction also in protic environment (ethanol/H_2O). Monoperoxyoxalate is formed as an intermediate which, in the presence of a fluorescer, decays accompanied by photoemission via several intermediate compounds (e.g. dioxetanedione) into CO_2 and H_2O (equation 1−3):

$$HO-\overset{\overset{O}{\|}}{C}-\overset{\overset{O}{\|}}{C}-OH + H_2O_2 \xrightarrow{\text{Carbo-diimid}} HOO-\overset{\overset{O}{\|}}{C}-\overset{\overset{O}{\|}}{C}-OH + H_2O \quad (1)$$

$$HOO-\overset{\overset{O}{\|}}{C}-\overset{\overset{O}{\|}}{C}-OH + F \longrightarrow H_2O + 2\,CO_2 + F^* \quad (2)$$

$$F^* \longrightarrow F + h\nu \quad (3)$$

(F^* = fluorescer in excited electronic singlet or triplet state).

The quantification of oxalate, fluorescer and peroxy compound with the above-mentioned system is described in the following.

Chemiluminometric measurements

All chemiluminometric measurements were made on a Clinilumat LB 9502 (Berthold, FRG). Dosed by injector, 300 μl of a solution of bis(cyclohexyl)-carbodiimid (DCC) in abs. ethanol (25 g/l) were added to 100 μl of aqueous sample adjusted to pH = 1. H_2O_2 was, if necessary, admixed to the sample (10 μl of a 0.2 mol/l aqueous solution of H_2O_2). The fluorescer was, depending on what was to be determined, either added to ethanol (9,10-diphenylanthracene (DPA) in case of determina-
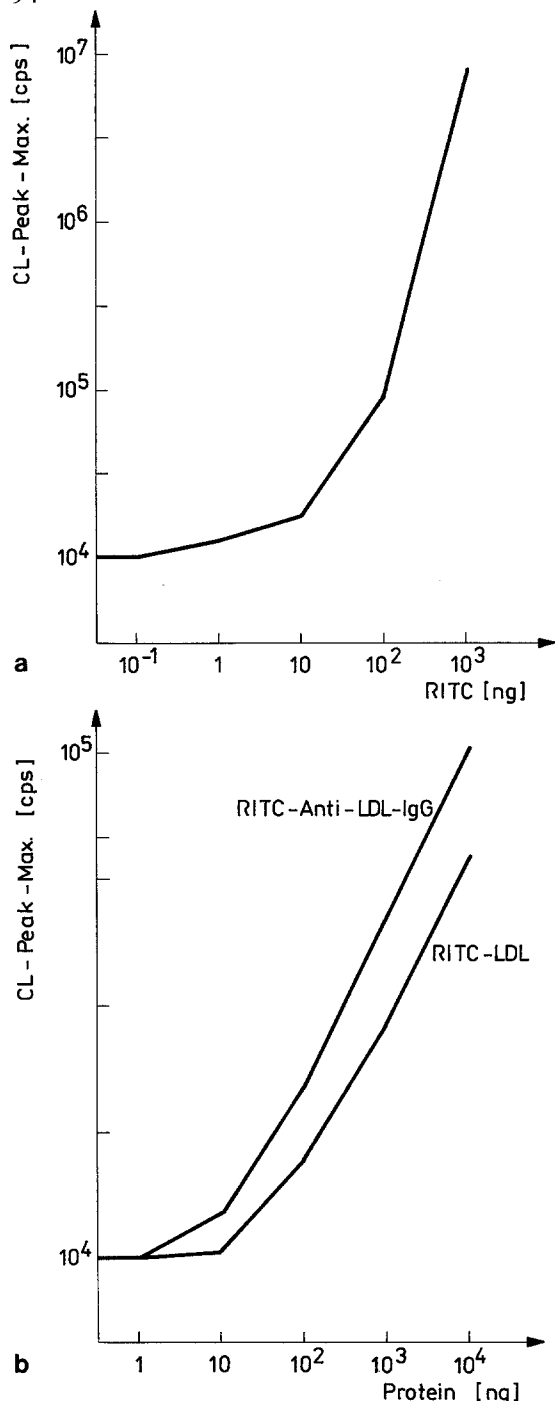
Fig. 1a, b. Sensitization of the chemiluminescent system oxalic acid/DCC/H_2O_2 by a free and b protein-bound rhodamineisothiocyanate contained in 100 µl of sample (1 mol/l oxalate solution, pH 1)

tion of oxalate and H_2O_2) or used together with the oxalate solution as sample. Measurement was started immediately after injection and continued for 10 s.

Results and discussion

Quantification of oxalate in urine. We previously reported on chemiluminometric quantification of oxalate in urine after precipitation of calcium oxalate by means of a LKB Luminometer 1250 [1]. As further studies with the Clinilumat LB 9502 have shown, oxalate concentration can be directly determined in native urine under optimized preanalytic conditions, concentration of reagents and timing of measurements, because all in-

terfering organic substances in urine are inferior to oxalate by several orders of magnitude with regard to their reaction kinetics and CL quantum yield.

Adaptation of the system to the quantification of the fluorescer. A 1 mol/l oxalic acid or alkali oxalate solution (adjusted with HCl to pH = 1) used as sample, a sensitization of the system by 2 to 3 orders of magnitude depending on the concentration of fluorescer can be achieved regarding the CL measuring signal. However, many of the common fluorescent dyes cannot be employed because of insufficient fluorescence quantum yield at pH = 1. Apart from polycondensed carbohydrates (e.g. diphenylanthracene), brillant sulfoflavine, rhodamine and porphyrins (except complexes of metals with several stable valence states, e.g. iron and cobalt) are, among others, known as excellent sensitizers.

Covalent bounding to protein of the fluorescer will decrease CL quantum yield in comparison to an adequate quantum of free fluorescer. Yet, few nanogrammes per ml can still be detected.

We labeled human low density lipoprotein (LDL) and anti-LDL-IgG (sheep) with rhodamineisothiocyanate (RITC), the limit of detection for proteins appearing from Fig. 1. This offers the possibility of a CL immunoassay with a fluorescent dye instead of luminogen used as label.

First in vitro studies as to the interaction between RITC-LDL and isolated human leucocytes admit of the conclusion that this peroxyoxalate system might also be suitable for the detection of cell receptors.

Brandl [3] reported on chlorophyll-sensitized peroxyoxalate chemiluminescence producing bright and strongly visible light in ethyl acetate, when aryloxalates, namely bis(2,4-dinitrophenyl)-oxalate (DNPO), were used. On the basis of DNPO we developed a qualitative peroxyoxalate-CL-test for the determination of porphyrins in urine [2]. Its simplicity and comparable sensitivity makes it an useful alternative to the porphyrine fluorescence talc test [4], particularly since an analytical quartz lamp is not needed. The system oxalate/DCC/H_2O_2 now also permits a quantitative analysis of porphyrins in urine down to the concentration of about 250 µg/l, with some problems of standardization of the procedure remaining to be solved. The use of a suitable photodetector with a maximum sensitivity within a narrow range of the fluorescence (chemiluminescence) maximum of porphyrins (about 630 nm) is a basic requirement for sufficient high sensitivity or further enhancement of sensitivity.

Determination of H_2O_2. Using DPA as fluorescer in a concentration of 150 mg/l in ethanolic DCC-solution and in the presence of 1 mol/l oxalic acid solution, determination of H_2O_2 can be achieved down to limiting concentration of 10^{-8} mol/l at pH = 1, with peak maximum within 0.4 s and the reaction being completed to 80 p.c. within 2 s after start. This shows that this procedure can also be used for the determination of enzymes or substrates which are in direct relation to H_2O_2 (e.g. systems catalyzed by oxidase or peroxidase).

References

1. Albrecht S, Beckert R, Boehm WD (1989) J Clin Chem Clin Biochem 27:451−454
2. Albrecht S, Brandl H, Koestler E (1989) Z Klin Med 44:2071−2073
3. Brandl H (1986) Chem unserer Zeit 20:63−66
4. Doss M (1979) Krankenhausarzt 52:787−791
5. Hummelen JC (1986) Meth Enzymol 133 B:531−537
6. Monzir S, Guilbault GG (1988) Anal Chem 60:2671−2674
7. Phillip MJ, Maximuke PP (1989) Oncology 46:266−272
8. Rauhut MM, Sheehan D, Clarke RA, Semsel AM (1965) Photochem Photobiol 4:1097−1110

35

A rapid and sensitive chemiluminescent DNA probe system (HPA) for detection of amplified HIV and HBV DNA

D. Kacian, T. Lawrence, M. Sanders, J. Putnam, M. Majlessi, S. McDonough, and T. Ryder

Gen-Probe, Incorporated, 9880 Campus Point Dr., San Diego, CA 92121, USA

Acridinium esters are highly chemiluminescent molecules with high quantum yield and rapid reaction kinetics [5]. DNA probes can be labeled with acridinium esters using alkylamine linker arms to approximately the same specific activity as the free ester. We have identified conditions in which acridinium ester linked to unhybridized probe is hydrolyzed to a non-chemiluminescent form, while ester linked to hybridized probe is protected [1]. We have incorporated chemiluminescent labeled probes into a homogeneous DNA probe assay referred to as the Hybridization Protection Assay (HPA) and applied the assay to the detection of Hepatitis B (HBV) and Human Immunodeficiency Virus (HIV) DNA sequences. Rapid and sensitive detection methods for screening large numbers of samples in a simple format are needed for clinical diagnoses as well as basic research endeavors, particularly in clinical syndromes in which currently available tests or serological tests cannot be used to follow the course of infection. HIV and HBV are present in levels too low to allow consistent detection by direct methods. Detection of these low levels of virus is made possible by specific amplification of viral nucleic acids by enzymatic methods including the polymerase chain reaction or PCR [4] or transcription-based amplification methods [2]. Current methods for detection of the specific amplification products include visualization after gel electrophoresis and hybridization in solution or to immobilized targets. These methods require many steps and several hours to days to complete, and often involve radioisotopic DNA probes. We demonstrate that HPA is a rapid and sensitive method for detection of HIV and HBV DNA amplified by PCR.

Materials and methods

DNA probes were labeled as described in Arnold et al. [1]. Chemiluminescence was detected following the addition of hydrogen peroxide under basic conditions. The reaction proceeds through a cyclodioxetane-like intermediate with the production of an excited acridone which emits light upon collapse to ground state. Purified cloned DNA was amplified with Taq polymerase under conditions recommended by the enzyme supplier (Cetus) for 30 or 35 cycles in a Perkin-Elmer Cetus thermocycler. Ten microliters of the PCR reaction were denatured at 95° C and hybridized to AE-labeled probe at 60° C, followed by a differential hydrolysis step at the same temperature. After differential hydrolysis, remaining chemiluminescence was a direct measure of the amount of hybrid formed. Detection of chemiluminescence was performed with a Leader I luminometer. The results were given as a numerical reading in Relative Light Units (RLU), allowing quantitation of the amount of target present. The hybridization assay required less than 30 min to complete.

Results and discussion

The differential hydrolysis of hybridized and unhybridized probe forms the basis of a homogeneous DNA probe assay referred to as the Hybridization Protection Assay (HPA). The HPA format was used to detect HIV and HBV DNA amplified by PCR. Purified cloned HBV DNA was amplified by PCR with primers from conserved regions within the HBV genome. A single band was seen on EtBr-stained agarose gels, confirming the specificity of the primer sequences. When dilutions of HBV cloned DNA were amplified and then analyzed by HPA, reactions containing as few as 10 copies of input HBV DNA gave chemiluminescent signals significantly above background, even when only 10% of the sample was analyzed. The quantitative capabilities of HPA were demonstrated by analyzing serial dilutions of HBV+ serum. The assay showed a linear response over three logs of target dilution. One HBsAg+ serum was positive by HPA even when diluted 10^7-fold prior to amplification. We have also applied HPA to specifically detect HIV-1 DNA amplified by PCR using gag-region primers described in Ou et al. [3]. Amplifications containing less than 10 copies of input HIV-1 DNA gave signals significantly above background. HPA provides a rapid and sensitive technique, which should be useful in studies involving epidemiology, diagnosis, prevention and treatment of viral diseases.

References

1. Arnold et al. (1989) Clin Chem 35:1588
2. Kwoh et al. (1989) Proc Natl Acad Sci USA 86:1173
3. Ou et al. (1988) Science 239:295
4. Saiki et al. (1985) Science 230:1350
5. Weeks et al. (1983) Clin Chem 29:1471

36

Determination of benzo(a)pyrene in micellar medium

J. J. Santana Rodríguez[1], **Z. Sosa Ferrera**[1], **A. Afonso Perera**[2], **and V. González Díaz**[2]

[1] Department of Chemistry, Faculty of Marine Sciences, University of Las Palmas de Gran Canaria, Spain
[2] Department of Analytical Chemistry, University of La Laguna, Tenerife, Spain

The micellar media and the fluorescence techniques have been widely used in the study of the structure and dynamics of biological systems [1]. Analytically, the micellar media present a great interest, specially to improve the sensibility and selectivity of many determinations [2, 6].

In the present communication, we report the micellar enhanced spectrofluorimetric determination of a polynuclear aromatic hydrocarbon (PAH), benzo(a)pyrene (B(a)P) of great toxicological interest and for its significance in pollution studies [3].

Experimental

Reagents. The standards PAH were ontained from Sigma Chemical Co. and used as received. The surfactants: sodium dodecylsulfate, Triton X-100, bencyldimethyltetradecylammonium chloride, hexadecyltrimethylammonium bromide and cetylpyridinium bromide were obtained from Aldrich Chemical Co. The stock solutions of PAH were prepared in ethanol.

Apparatus. All fluorescence measurements were made with a Perkin-Elmer MPF-44A recording spectrofluorimeter equipped with a 150-W Osram XBO xenon arc lamp, a DSCU-1 corrected spectra unit (0.5‰ Rodamine B in ethylene glycol as the
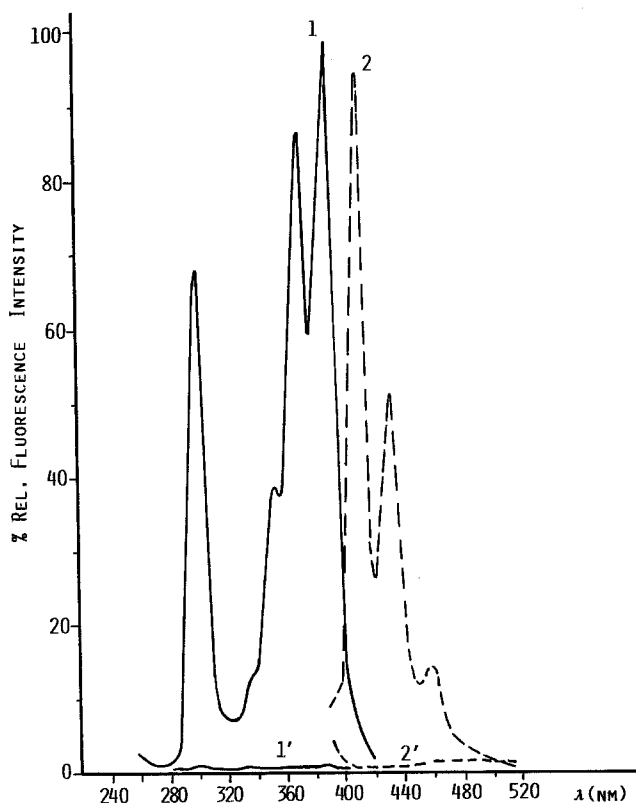

Fig. 1. Fluorescence behaviour of benzo(a)pyrene in the presence of Triton X-100, *1* (EXC), *2* (EM) and aqueous medium, *1'* (EXC), *2'* (EM)

reference), a UDR-3 digital read-out, a Selecta Frigitherm ultrathermostat and 1-cm quartz cells. The emission intensity measuring system of the spectrofluorimeter was calibrated daily by using the Perkin-Elmer set of fluorescent polymer blocks.

General procedure for the determination of benzo(a)pyrene. To an aliquot, containing 2.5 ng—12.6 μg of benzo(a)pyrene, in a 25 ml calibrated flask add 5 ml solution of Triton X-100 10^{-2} mol/l, and dilute to volume with deionised water. Measure the fluorescence at 406 nm using excitation at 386 nm. The calibration curves are obtained from solutions prepared in the same conditions.

Results

The behaviour of B(a)P in solvents of different dielectric constant and bipolar moment has been studied as well as in presence of different surfactants. The fluorescence spectra do not present significant changes at the excitation and emission wavelengths maxima, neither in the ratio of emission bands intensities [4]. However, the fluorescence intensity suffers important changes in cationic and neuter micellar media.

Figure 1 shows a great increase of the fluorescence intensity of B(a)P in solutions with Triton X-100, due to an increase in the molar absorptivity and, above all, in the quantum yield of the hydrocarbon. Among the reasons which can justify that the non-radiative processes are found less favoured in micellar medium, could be mentioned the lower facility of movement of the fluorophore molecules and the reduction of quenching effects of oxygen or other species [7].

The fluorescence intensity changes sharply with the concentration of the surfactant when it is close to the critical micellar concentration. In concentrations of Triton X-100 higher than 10^{-3} mol/l remains practically constant. Increases in temperature as well as ethanol contents up to 5% (v—v), produce decreases in the fluorescence intensity.

For different intervals of concentrations: 0.1—5 ppb, 5—50 ppb and 60.5—505 ppb, exist a lineal relationship — with high coefficients of correlation — between the fluorescence intensity and the concentration of B(a)P in solutions 2.10^{-3} mol/l of Triton X-100. In eleven solutions containing 3.03, 30.3, and 302.8 ppb of B(a)P, relative errors of 2.21, 0.76, 1.52%, and relative standard deviations of 0.10, 0.34 and 6.81% have been obtained, respectively. The method shows a detection limit of 0.07 ppb [5].

Other PAH, as 1,12-benzoperylene, crysene and perylene, do not interfere the determinations up to ratios of 0.5/1, 200/1 and 200/1 with respect to B(a)P, respectively.

The method has been applied to the determination of B(a)P in sea water samples to which known hydrocarbon concentrations have been added. Recoveries oscillating from 93.4 to 105.7% have been obtained from ten samples containing between 3 and 400 ppb of B(a)P.

References

1. Gratzel M, Thomas JK (1979) Modern fluorescence spectroscopy. Plenum Press, New York 2:169—216
2. Hinze WL, Sing HN, Harvey NG (1984) Trends Anal Chem 3:193
3. Howard JH, Fazio T (1980) J Assoc Off Anal Chem 63:1077
4. Kalyanasundaram K, Thomas JK (1977) J Am Chem Soc 99:2039
5. Long GL, Winefordner JD (1983) Anal Chem 55:712A
6. Pelizzetti E, Pramauro E (1985) Anal Chim Acta 169:1
7. Sing H, Hinze WL (1982) Anal Lett 15:221