PRO EXPERIMENTIS

Perifused alveolar macrophages. A technique to study the effects of toxicants on prostaglandin release[1]

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Summary. A technique was developed for perfusing free airway cells (FAC) obtained by guinea-pig bronchoalveolar lavage. 10×10^6 FAC (mostly macrophages) were placed on the filter of a Nuclepore chamber perfused with Tyrode's solution or with Eagle's Minimal Essential Medium (MEM); the effluent was collected at intervals and the release of prostaglandin E_2 by the perifused cells was used as an index of the inflammatory reaction to toxicants. Zymosan, asbestos fibers and soluble toxic agents stimulated the synthesis of prostaglandins while indomethacin abolished it. Our technique of FAC perifusion allows the sequential study of biochemical events involved in macrophage defense mechanisms as well as providing a simple test for the evaluation of various toxicants.

Among the 40 different types of lung cells, macrophages play a key role in pulmonary defense[2]. In the lung, these cells are responsible for the maintenance of sterility; for this purpose, they ingest bacteria, dead pulmonary cells, inhaled dusts, surfactant, erythrocytes, etc...[3-5]. Even though the primary function of macrophages is to protect the lung against intruders, they are also involved in the induction and expression of cellular immunity. As multifunction cells, macrophages possess a very important synthetic capacity; they secrete various enzymes such as collagenase, LDH and lysosomal enzymes, as well as interferon, prostaglandins, components of complement and a large number of stimulators and inhibitors of various cellular functions[2,5,6].

The exact nature and role of each of these products of macrophages are still unclear[7], but many of them are definitely involved in the complex processes called inflammatory reactions. In the evaluation of the physiopathology of lung disease, these secreted substances appear as reliable indices of lung defense[8].

Following stimulation by various pharmacological or toxic particulate agents, macrophages secrete substantial amounts of prostaglandins[9-13]. These biologically active fatty acids are synthesized by cyclooxygenase from arachidonate liberated from membrane phospholipids. Prostaglandin E_2 appears to be the major active metabolite of arachidonic acid in macrophages[14].

Since prostaglandins are believed to be very important mediators of inflammatory processes, the major aim of the present experiments was to develop a new system that would allow analysis of the time-course of release of prostaglandins from macrophages exposed to various pharmacological or particulate substances. Such analysis could help in understanding the very early events in the physiopathology of environmental lung disease. Part of this work has been presented in a preliminary report to the Physiological Society[15].

Materials and methods. 1. Bronchoalveolar lavage: The technique of bronchoalveolar lavage used in the present study was similar to that of Maxwell et al.[16]. Briefly, guinea-pigs of either sex weighing 300–500 g were killed by cervical dislocation taking care to avoid blood in the trachea. The peritoneal cavity was opened, the diaphragm was cut to permit access to the lungs and the trachea was dissected. A catheter connected to a 3-way stopcock with 2 20-ml syringes was inserted into the trachea. Aliquots of 10 ml of Tyrode's solution (pH 7.4; 37 °C) were injected via 1 syringe into the lung, which was massaged gently, and the washings were withdrawn by the 2nd syringe. 8 successive bronchoalveolar lavages were pooled and kept on ice.

2. Preparation of cells: Only siliconized glassware or plastic vessels were used in the preparation of cells. The lavage fluid was centrifuged (10 min.; 350 xg) and the supernatant was discarded. When red blood cells were present, the pellet was resuspended in 2.5 ml of Tyrode solution and an osmotic shock was performed by adding 7.5 ml of distilled water for 30 sec. The cell suspension was then made up to 50 ml with Tyrode and recentrifuged to remove cellular membrane debris. The pellet was resuspended in 5 ml of Tyrode and 100 µl were withdrawn and mixed with 20 µl of a solution of Trypan blue (0.4% w/v) for counting and estimation of viability.

3. Perifusion system: An aliquot of the cells (1×10^7 cells) was placed in a Nuclepore filter holder (25 mm diameter) and perifused (1.1 ml/h) by a Harvard Syringe pump with either Tyrode's solution containing gelatin (0.1%) or with Eagle's Minimal Essential Medium (MEM) with Hank's salts and heat-inactivated foetal calf serum (2%). Both solutions were adjusted to pH 7.4 before use. A diagram of the perifusion system is shown in figure 1. The medium also contained penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml) and amphotericin B (250 ng/ml) to prevent bacterial and fungus growth. Up to 4 separate Nuclepore chambers immersed in a water bath at 37 °C were perfused simultaneously. In each experiment, cells pooled from 1 or more guinea-pigs were distributed

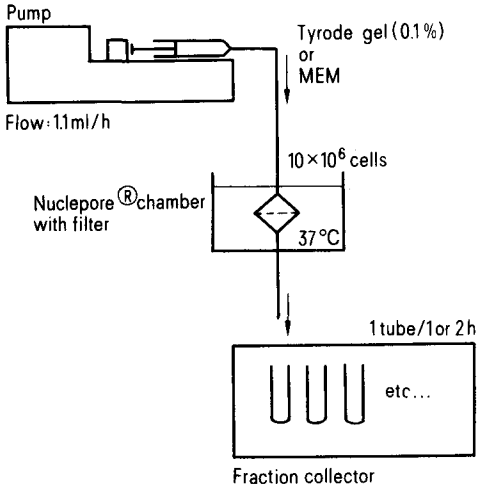

Figure 1. Macrophage perifusion system: the medium (either MEM or Tyrode-Gel) is perfused with a Harvard Syringe pump (Model 940) (50 ml syringe) through a Nuclepore chamber with filter in which 10×10^6 cells are introduced an kept at 37 °C. Several separate chambers could be perfused simultaneously. The effluent (1.1 ml/h) is collected with a fraction collector at a rate of 1 tube/h or 1 tube/2 h.

into the various chambers, of which one served as a control. The effluent was collected at 1- or 2-h intervals with a fraction collector. Either Metricel cellulose acetate filters (pore size 1.2 µm) (Gelman Inst. GA-3) or nylon filters (Nitex HD-3-1) (1 µm pores) were used.

4. *Prostaglandin extraction and determination:* At the end of the perifusion period, each collected fraction (1 or 2 ml) was extracted and bioassayed according to Sirois and Gagnon[17].

5. *Drugs used:* The following drugs were used: atropine sulphate, gelatin-type 1, gentamicin sulphate and endotoxin from *Salmonella minnesota* (Sigma Chem. Co.); Canadian chrysotile B (UICC); zymosan (ICN Pharm.); Eagle's medium (MEM), amphotericin B; penicillin-streptomycin and foetal calf serum (Gibco); the following drugs were supplied as generous gifts: methysergide hydrogen maleate (Sandoz Ltd); propranolol hydrochloride (Ayerst Lab.); phenoxybenzamine hydrochloride (SKF); diphenhydramine hydrochloride (Parke, Davis & Co.); indomethacin (Merck Frosst Lab.); prostaglandin E_2 (Dr J.E. Pike of Upjohn Co.).

Results. 1. Cell identification: Guinea-pig bronchoalveolar lavages yielded 19.2 (± 8.1) $\times 10^6$ ($n=31$) free airway cells per animal as counted with a hemacytometer following Trypan blue coloration; cell viability was above 95%. By the usual morphologic criteria, 85% of the cell population were macrophages, the remainder being lymphocytes and eosinophils.

2. Effect of toxicants: Free airway cells perifused for 20 h without any added stimuli released approximately 3 ng of PGE_2-like activity per aliquot of 10^7 cells. The release over the first 2 h of perifusion averaged 0.5 ng PGE_2. The concentration in the perifusion fluid increased slightly for 2-6 h and then diminished towards the end of the incubation.

Figure 2 shows the typical effect of 2 particulate substances on prostaglandin E_2-like activity released by perifused macrophages. When a suspension of zymosan (1 mg) was introduced into the Nuclepore chamber at the beginning of the experiment, a large output of prostaglandins was observed in the perifusion medium. As shown in figure 2, maximal amounts of the mediator are released at the beginning of the incubation, with a return to control values after approximately 6 h. This 1st stimulatory period may correspond to a very active phase of phagocytosis of the particles. The release of prostaglandins from the same cell

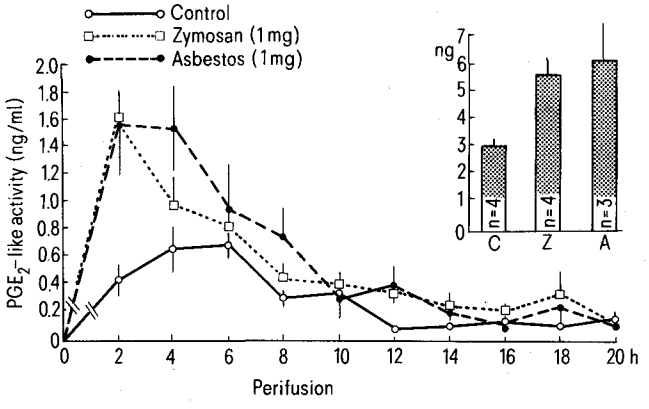

Figure 2. Release of prostaglandin E_2-like activity by 1×10^7 macrophages perifused for 20 h in a Nuclepore chamber in the absence (C; open circle) or the presence of zymosan (Z; open square) or asbestos (A; closed circle). The histogram on the right hand side of the figure represent the cumulated release of prostaglandins following a total of 20 h of perifusion.

population perifused under control conditions gave a relatively stable basal release with values decreasing slowly and progressively during the experiment.

The addition of a suspension of chrysotile (Canadian asbestos B; 1 mg) to cells in the chamber produced a large stimulation of the synthetic activity of the macrophages. However, the stimulation of prostaglandin release by this particulate pollutant was transient; the peak was reached at the beginning of the incubation, and maintained during the first 4 h, but activity decreased during the following 2 h and returned to control values after 6 h. This initial period of active release, similar to the one produced by zymosan, may also be related to an active phase of phagocytosis. Comparing the cumulative results, the same amount of either particulate compound approximately doubled the output of mediators. When phorbol myristate acetate (100 ng/ml) or the endotoxin from *Salmonella minnesota* (30 µg/ml) were added to the perifusion fluid, a marked increase in prostaglandin release was also noted. On a cumulative basis there was a 2-fold increase of PGE_2 release with the endotoxin and a 3-fold increase with phorbol myristate acetate when compared to control cells. On the other hand, when the non-steroid anti-inflammatory drug indomethacin (20 µg/ml) was added to the perifusion fluid, a complete inhibition of the release of PGE_2-like activity was seen throughout the experiment. The amount of PGE_2 released during 2 h of perifusion was undetectable with our bioassay, which can detect 0.025 ng PGE_2.

The viability of the cells in the control chamber was high but the asbestos fibers produced a toxic effect on the macrophages with decrease in viability (down to 20% in some experiments). Lactate dehydrogenase (LDH) activity in cell effluents showed an increase during the first h and remained stable for the reminder of the experiments (data not shown).

Discussion. Bronchoalveolar lavage in the guinea-pig lung is a simple way to obtain large amounts of lung cells[16]. Because very few manipulations are required to liberate and purify them, FAC can be maintained alive for relatively long periods[18]. Under our experimental conditions, the cell viability was not significantly lowered following 20 h of perifusion with appropriate media. As suggested by many authors, the study of FAC offers enormous potential in the evaluation of lung defense mechanisms since these cells are located on the first line of defense when intruders find their way into the bronchial tree and the alveoli[3,5,18,19]. Furthermore, functional alterations of these cells measured in vitro after exposure to inhaled pollutants in vivo could be helpful diagnostic aids. In our model, FAC were obtained from untreated lungs, incubated in the presence of pharmacological or particulate substances and the release of mediators used as indices of acute toxicity.

In the present study, we have shown that perifusion of FAC brings a new dimension to cell culture. With this easy technique, it is possible to maintain the cells in a constantly renewed medium and flush the chamber of by-products which could, if they reached certain concentrations, alter the normal metabolism via positive or negative feedback. Furthermore, this technique allows the study of biochemical events which take place at various times during the incubation. In the present experiments, we have measured prostaglandins in the effluent as an index of inflammatory reaction but obviously many other mediators could also be determined.

Among the various substances released into the medium during incubation of macrophages, prostaglandins are believed to be very important indices of the inflammatory reaction[9-13]. Our results showed clearly that they are released in large amounts by macrophages exposed to toxic agents, thus confirming previous observations[10,11,13,20].

Some reports suggested that the release of these fatty acids does not take place[12] or is very weak[11] during phagocytosis of inert material such as latex beads. On the contrary, phagocytosis of inflammatory particles parallels the synthesis and release of prostaglandins. In a recent publication, Hsueh et al.[21] showed that the phagocytosis of red blood cells induces the release of prostaglandins from rabbit alveolar macrophages. Further experiments are necessary to throw light on this process and determine whether the action of asbestos on prostaglandin release is related to phagocytosis or simply a toxic effect on the membrane.

Our results with endotoxin and indomethacin confirmed previous reports on the synthetic potential of macrophages for fatty acids[11,13,20,22,23] and showed that our perifusion system is adequate for short term culture of the cells. Cumulated amounts of prostaglandins released are comparable to those reported using classic techniques[11,20,24,25]. In our system, unstimulated cells release detectable levels of the mediators and endogenous substrate appears to be sufficient for maintenance of a very satisfactory level of synthesis.

As described before[10,25,26], zymosan and asbestos are powerful stimulants of phagocytosis and prostaglandin release. These particulate substances produced only a transient stimulation which could probably correlate with active phagocytosis. However with asbestos fibers the increase in the liberation of LDH corresponds the decrease in viability of the cells observed at the end of the experiments and confirms the cytotoxic effect of this particulate pollutant.

In conclusion, the results presented extend previous observations on the action of various pharmacological and particulate substances on the release of prostaglandins by free airway cells (mainly macrophages). Our technique represents an application of perifusion to the study of the progression of inflammatory reactions in the macrophage. The utility of this technique for the evaluation of the toxicity of various types of asbestos and other inhaled particles is warranted by the clear advantages which it offers over the standard culture methods: continuously renewed medium and sequential event analysis.

Because of the ever increasing feasibility of bronchoalveolar lavages in animals and man we believe that this approach will contribute to the clarification of the early biochemical events in the host response to various environmental toxic substances.

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The preparation of colloidal gold particles using tannic acid as an additional reducing agent

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Summary. A description of a simple procedure using a tannic acid/citrate reducing agent for the preparation of gold particles having an average diameter of 5.7 nm is given.

Colloidal gold particles, being electron dense and non-cytotoxic markers, are increasingly used in cytochemistry[2]. Various reducing agents have been employed in the preparation of colloidal gold[3]. Up until now, the smallest particles have been obtained with white phosphorus[4]. However, after having investigated the reduction of gold solutions with the aid of a combination of tannic acid and sodium citrate, we now describe a simpler alternative to the white phosphorus method of preparing stable gold particles.

A stock solution of 0.1% (w/v) $HAuCl_4$ (Riedel de Haen) is prepared in water distilled twice in glass. This solution can be stored for months in well-stoppered brown glass bottles. This stock solution is diluted with distilled water to provide a fresh working solution of 0.01% (w/v). 100 ml of a 0.01% (w/v) freshly prepared chloroauric acid solution is trans-