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Measurement of air-borne pyrogens by the *in vitro* pyrogen test (IPT) based on human whole blood cytokine response

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The impact of environmental micro-organisms as well as their fragments and components, especially endotoxins, on human health is increasingly recognised. Different syndromes have been described in connection with inhaled air-borne microbiological contamination, e.g. sick building syndrome, humidifier lung, organic dust toxic syndrome (ODTS) and "Monday illness". Air-conditioning systems intensify this problem, as does the collection of biological waste in households, which represents a substantial source of air-borne pollution.

In 1995 we described a new method for the detection of pyrogenic contamination[1,2]. This sensitive test (*in vitro* pyrogen test: IPT) uses the natural reaction of the immune system to detect a broad spectrum of pyrogens in human blood. Safety tests in injectable drugs represent the main application and the test has been successfully validated for inclusion into the European Pharmacopoeia.

Here, the test was adapted to the detection of environmental air-borne pyrogens. Air was drawn through a filter which was then incubated directly with diluted human whole blood. The release of the inflammatory cytokine, interleukin-1β (IL-1β), was measured by ELISA. In animal stables, up to 3 x 10^6 endotoxin equivalent units (EEU) were found per cubic meter of air.

The problem

Both our well-being and our health depend substantially on the quality of the air in our environment. The quality of the room climate is mainly determined by the components: humidity, air temperature and pollutant content, including dust, environmental micro-organisms and their decay products.

In particular, fungal spores and bacteria, as well as their toxins, are the main microbiological components of the air. The pollution of interior air with bacteria and moulds represents the causal factor for "sick building syndrome"[3]. This syndrome is characterised typically by lack of well-being, faintness, lack of concentration, headache, affection of mucous membranes, and increased incidence of allergy and infection. According to a study by the World Health Organisation (WHO), 30% of the 3 million people employed in air-conditioned rooms suffer these symptoms[4]. Investigations at the University of Kiel demonstrated a connection between pulmonary disease, i.e. the frequency of infections and allergies, and the presence of environmental micro-organisms (bacteria and moulds) in households[5].

The main cause of microbiological contamination of living areas with bacteria and fungi is continuous dampness in or on building materials. This problem is exacerbated by the continuous trend towards more efficient sealing of buildings to save energy. A continuous exchange of air is obstructed by these sealing measures. The result is moisture in the building materials and subsequent increasing loads of microbiological pollutants in the air. Due to this connection, it can be assumed that the microbiological burden in living and work areas will increasingly cause health problems in the next few years. Therefore, research methods and evaluation criteria must be improved for the characterisation of environmental micro-organism loads in living and work areas.

So far, microbiological contamination of households is normally, if at all, evaluated only by determination of the concentration of fungal spores in room air, which is insufficient for a global evaluation of the microbial load as:

- Moulds represent only one form of microbiological pollutant; an important causal relevance is attributed to Gram-negative bacteria and their endotoxins in "sick building syndrome".

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- The measurement of the load of air-borne moulds is based on classical microbiological methods, i.e. only micro-organisms which can be cultured are represented. Dead fungi and bacteria, which are not detected in cultures, pose an additional health risk to humans.
- The determination of the concentration of micro-organisms, i.e. colony-forming units per air volume, does not reflect the pathogenicity or inflammatory potential of different fungi or bacteria for humans.

A comprehensive estimate of environmental micro-organism load should include a variety of relevant micro-organisms and their toxic metabolic products and reflect their pathogenicity for humans.

Beside the micro-organisms themselves, their cell wall constituents, i.e. what remains after the micro-organisms are dead, represent a substantial risk factor for the well-being and health of humans. These structures (eg. endotoxins and glucans) are recognised by human immune cells, e.g. after inhalation, leading to an inflammatory reaction, the cause of the above mentioned health problems. The load with such environmental micro-organisms is approximately three times higher in households that collect biological waste within the household; the fungal load is about eight-fold higher. If carpets are used as floor covering, these values rise dramatically up to 800-fold[6].

The working environment represents another relevant area where workers are increasingly exposed to air-conditioning systems; for example, in 1993 three million people in the Federal Republic of Germany worked in offices with air-conditioning[3]. Today, the following health problems are described in connection with air-conditioning systems: sick building syndrome, building-related illness, infections and allergies.

The detection of endotoxin as a marker for contamination with pathogenic micro-organisms is considered a very important factor; six-fold increased endotoxin quantities, i.e. 36-800 ng/m^3 air are considered a significant load[7]. Gram-positive micro-organisms are also found to a substantial extent; however they do not seem to correlate with the pathogenic importance suggested by micro-biological investigations, i.e. based on the count of living organisms that can be cultured. However, it should be noted that there is no specific *in vitro* test system to detect dead material from Gram-positive bacteria as there is for Gram-negative endotoxin, i.e. the Limulus Amoebocyte Lysate assay (LAL). Therefore, a more detailed evaluation is still awaited; similar considerations apply to fungal constituents.

The WHO estimated more than 10 years ago that about one third of all new or reconstructed buildings are so-called potentially "sick" buildings[7]. Since the health problems apply less to naturally ventilated than to fully air-conditioned buildings, the numbers are increasing in parallel with the increasing use of air conditioning. Systems which operate with air humidifiers appear to represent a special risk factor since high humidity favours contamination and micro-organism growth. Due to energy saving measures, the regulation of the humidity is often omitted and humidity increases. Both Gram-negative and Gram-positive bacteria, as well as fungi and amoebae, are causally related to "humidifier lung", which is particularly common in the printing industry[3]. The so-called "humidifier fever" is directly correlated with endotoxins[8].

It is well known in a variety of working environments that illnesses frequently occur on Mondays, when the body is abruptly confronted with a multiplicity of air-borne contaminations after a recuperative period over the weekend. This "Monday syndrome", characterised by general malaise combined with shortage of breath, coughing and thoracic tightness, improves during the course of the working week. Inhalation of organic dust, endotoxins and other factors are thought to be causes of this illness, known in the cotton industry by the name "byssinosis"[9].

People employed in pet shops, animal stables, zoos, poultry farms or refuse sorting facilities are also affected. In addition, workers in the steel industry are particularly exposed, because the recirculating cooling emulsions (oil/water mixtures) spread pyrogenic pollution after microbiological overgrowth especially with *Pseudomonas* species. This is exacerbated by the high temperatures and the damp environment.

Detection possibilities

Essentially, the following procedures are available:

1. Microbiological testing methods, where sample material is incubated together with a nutrient substrate. Depending on the substrate, specific micro-organisms can grow, which can then be identified. Incubation takes up to 14 days.
2. 'Rapid tests' for live microbiological contamination are bioluminescence and impedance monitoring, which also require previous growth of the organism. The optical system of direct epifluorescence is very time-consuming, insensitive and unsuitable for routine analysis of large sample numbers. With methods of direct fluorescence marking (e.g. ChemScan system, based on measurement with an ultrasensitive laser scanner), living micro-organisms are detected very rapidly. It is intended for raw materials or finished products, e.g. in pharmaceutical manufacture, and unsuitable for air control, because the naturally occurring micro-organism spectrum would disturb this test substantially.
3. Test of endotoxin content. After separation of air-borne endotoxins from filters, these structures can be detected specifically and very sensitively in the Limulus Amoebocyte Lysate test (LAL) (see also procedures for the determination of the endotoxin concentration in the working environment, BIA working folder "Measurement of dangerous materials", Index 9450).

These microbiological differentiation methods are useful for analysing the living micro-organism spectrum while dead ones escape detection. Notably, these methods are very labour and material intensive. This is hardly useful for routine purposes, and the direct effect on humans is not reflected. In addition, putatively infectious material is

produced and has to be processed and disposed of under appropriate safety conditions.

Endotoxins can be used as markers for contamination with Gram-negative bacteria. The LAL test is a well established and sensitive method. However, only a rinsing solution of the contaminated filter can be examined. Tightly bound and non-extractable endotoxins escape detection.

New method: IPT

Pyrogens are inflammation-inducing substances which, depending upon the route of uptake, can lead to general malaise, breathing problems, fever, circulatory and organ failure up to fatal shock in humans. These disease symptoms can also manifest after uptake of endotoxin via the lung and are nowadays regarded as a unique syndrome in environmental medicine[10].

The human immune system reacts very sensitively to environmental micro-organisms or their products and initiates appropriate defence mechanisms. It is unimportant for the host defence whether microbiological material (living micro-organisms, endotoxins or other pyrogens) threatens the organism directly via the blood, skin or the respiratory system. After contact with relevant contaminations, white blood cells (monocytes/ macrophages) release pro-inflammatory signal molecules such as interleukin-1β (IL-1β). The pro-inflammatory cytokines change the set-point for thermo-regulation in the brain and cause a fever reaction in the organism. We used this reaction to develop a test for pyrogenic contaminations in drugs based on whole blood incubation[1,2,11-13]. Blood from a healthy donor is diluted in clinical saline and incubated with the liquid sample at 37°C for 6–24 hours. IL-1β release is quantified by ELISA measurement. Recovery of an endotoxin spike in the sample rules out interference.

For adaptation of the test to the detection of air-borne pyrogens, air was drawn onto 8mm nitrocellulose filters (Sartorius, Germany) using a PGP dust sampling system (Ströhlein GmbH, Germany) or bubbled through pyrogen-free water using AGI-30 impingers. The blood can be incubated directly with the filter in a Petri dish or with a sample of the collector liquid.

Results of the new whole blood method (IPT)

First, we tested whether the nitrocellulose filters interfere with the whole blood test. Different concentrations of lipopolysaccharide (LPS) from *Salmonella abortus equi* were applied to the filters in a volume of 10µl and left to dry. The IL-1β response to these filters placed in 24-well cell culture plates and covered with 1ml whole blood diluted 1:5 in saline was compared with the response of the blood to LPS without filters or to untreated filters plus LPS added directly to the incubation. As can be seen in **Figure 1**, the filter alone does not induce significant cytokine release. However, it appears to improve the presentation of LPS to the blood, resulting in a more sensitive IL-1β response (12.5pg were detected) and in higher levels of cytokine release compared to the samples

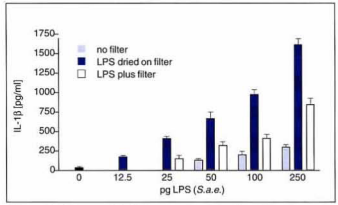

Figure 1: Release of IL-1β by human whole blood in response to nitrocellulose filters coated with different concentrations of LPS in comparison to LPS without filters and incubation of LPS with untreated filters.

without the filter or to the co-incubation of LPS with untreated filters.

Air samples from animal stables were then examined. The contamination of air in animal stables is particularly problematic for humans working there[14] and was used here to check the applicability of the test.

Air was drawn onto 8µm nitrocellulose filters (Sartorius, Germany) with a PGP dust sampling system (Ströhlein GmbH, Germany) at a rate of 3.5 l/min for 60 min. The exposed filters were incubated with 8ml saline and 2ml blood overnight in a Petri dish. IL-1β release was measured in the supernatant. Control endotoxins employed in increasing concentrations on clean filter material induced an increasing interleukin-1β release, i.e. there was a concentration/response-relationship (**Figure 2**, left part). Contaminated samples from a sheep stable showed clear signal responses (**Figure 2**, right part) dependent on their load.

Air samples from two pig stables (K and V) and samples from outside air were gained by impingement[15]. The air was bubbled through 50ml pyrogen-free water (Aqua ad iniectabile, Braun Melsungen AG, Melsungen, Germany) in order to dissolve the pollution by AGI-30 impingers (AGI = All Glass Impinger) operated for 20

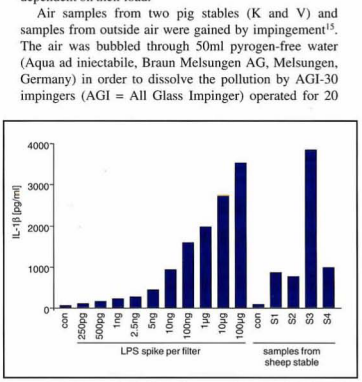

Figure 2: Release of IL-1β in human whole blood initiated by nitrocellulose filters contaminated with defined LPS-concentrations or filters contaminated in a sheep stable by collecting 3.5 l/min of air for 1 hour (S1 to S4).

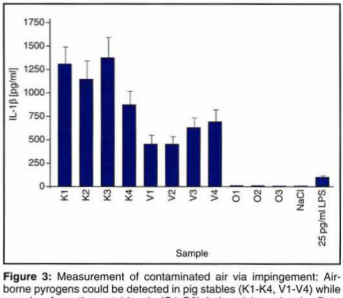

Figure 3: Measurement of contaminated air via impingement: Airborne pyrogens could be detected in pig stables (K1-K4, V1-V4) while samples from the outside air (O1-O3) induced low signals. Data represents mean ± SEM of five blood donors.

minutes at an air flow rate of 12.5l/min. Samples of this washing liquid contaminated with air-borne materials were employed in the whole blood test as liquid samples (100µl sample, 900µl saline and 100µl blood) (**Figure 3**).

The microbiological contamination in the examined animal stables was also characterised in comparison to the outside air with standard culture procedures and the LAL test in parallel with the whole blood test (**Table 1**). The PGP dust-sampling system (Ströhlein GmbH, Germany) was used to collect the inhalable dust fraction. The PGP system was operated at an airflow rate of 3.5l/min for 60 min. For measurement of endotoxin, the exposed nitrocellulose filters were rocked (22rpm; WS 5 shaker, Edmund Bühler GmbH, Tübingen, Germany) for 2h in 50 ml pyrogen-free water. After centrifugation of the washing fluid with 2000g, an aliquot was measured in the LAL assay QCL 1000 (BioWhittaker, Walkersville, MD, USA) as recommended by the manufacturer. The amount of endotoxin in the supernatant of the sampler fluids was converted from EU/ml to EU/m^3 using the airflow rate of the sampler and the sampling time.

Andersen samplers were operated for 1 min at an air flow rate of 28.3l/min to obtain the total number of airborne aerobic bacteria (on 5% sheep blood agar) and for 20 min to obtain the number of airborne Gram-negative aerobic bacteria (on MacConkey 3 agar; Oxoid, Wesel, Germany). The sheep blood agar plates were incubated at 37°C for 24h; the MacConkey agar plates were incubated at 37°C or 22°C for 24h. All bacteria grown on MacConkey agar were tested for their Gram-reaction by staining to give the real number of Gram-negative bacteria in the samples. For the airborne moulds the samplers were equipped with DG-18 agar plates and operated for 1 min at an air flow rate of 28.3 l/min. The exposed agar plates were incubated at 37°C for 48h and at 22°C for 72h.

The data gained with the whole blood method with filters or by impingement were related to the contamination in 1m^3 for comparison with the other measured parameters.

The signals measured in the whole blood test correlate roughly with the levels of microbial contamination found in the different stables and the outside air. Further experiments will be necessary to examine in more detail how well this method reflects the risk of exposure of farm workers or highly sensitive individuals and to optimise the methodology, i.e. optimisation of filter material, pore size, further simplification of incubation procedure, etc.

Conclusion

We showed that in the problematic field of animal stables air contamination with living environmental microorganisms and dead material can be assessed with the *in vitro* pyrogen test. The test method was adapted for these specific conditions (filtration and impingement for the collection of air-borne contamination). Air-borne pyrogens are not only of importance in animal stables, but for the entire human living and working area. The special advantages of the test lie in its species-specificity, the medical relevance, the broad detection spectrum and the possibility of checking the blood of the affected person directly. There is no comparable test system that can cover both dead and living material in an integral manner.

We intend to evaluate this innovative method further with partners from environmental and occupational medicine work safety and industry to optimise the methodological developments described here and to adapt them to the different environmental conditions relevant to humans. The crucial advantage of the new test is that the reaction mimics the reaction that air contamination would cause in humans, thereby reflecting potential dangers for humans. First results promise that a simple, standardised method will be available shortly for the measurement of air-borne pyrogens, especially since a standardised kit version of the IPT is now available (Charles River

Table 1: Overview of the microbial air contamination in the investigated stables

air hygenic parameters	sheep stable	pig stable K	pig stable V	outside air
inhalable dust [mg/m^3]	0.5	3.1	2.8	0.1
inhalable endotoxin [EU/m^3]	512	3 994	3 841	15
Gram-negative bacteria [CFU/m^3]	10	115	100	3
total bacteria [CFU/m^3]	40 000	175 000	132 000	2 055
moulds [CFU/m^3]	94 007	247	230	n.d.
IPT [mg IL-1β/m^3]	0.4*	25.8†	12.3†	0.1†

Arithmetic average of at least 5 measurements
n.d. = not done
CFU = colony forming units
EU = endotoxin units
*measured on filters
†measured by impingement

Endosafe). This is an important prerequisite for the development of international guidelines and thresholds and respective preventive measures in the long term. The test may even be adapted to gauge the inflammatory reaction of animals living in the stables and to compare their sensitivity to that of humans.

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