**INCO / International Scientific Cooperation Projects (1998-2002)**

**Contract number : ERB IC 15 CT 98 0322**

**FINAL REPORT**
Covering period from 1st October 1998 to 31 December 2002

# Title: "Automated Immunoassay System for continuous Air safety Control (AISAC) and its application to air pollution and aero-allergen monitoring".

Project Homepage: http://www2.pote.hu/bioanal/AISAC/WEB/AISAC.html

Keywords: Pollen, allergens, air pollution, immunodetections, health

Contract number: ERB IC 15 CT 98 0322

**TITLE:** "Automated Immunoassay System for continuous Air safety Control (AISAC) and its application to air pollution and aero-allergen monitoring".

**COORDINATOR**
Dr Gabriel PELTRE ESPCI, LECA, Allergie et Environnement, 10 rue Vauquelin, 75005 Paris (F)Tel 33 1 40 79 46 44Fax 33 1 40 79 46 54

**CONTRACTORS**
Prof. Ferenc KILAR, Scientific CoordinatorMedical University, Central Research Laboratory, University of Pécs, Pécs. (HU)Tel 36 72 32 41 22Fax 36 72 31 58 64E-mail : kilar@main.pote.huProf. Vaclav KASICKA, ContractorAcademy of Sciences of the Czeh Republic, Institute of Organic Chemistry and Biochemistry,Flemingovo 2, 16610 Prague 6 (CZ)Tel : 420-2 20 18 32 39Fax :420-2 24 31 00 90E-mail: kasicka@uochb.cas.czProf. Dietmar KNOPP, ContractorInstitute of Hydrochemistry, Technischen Universität, Munich (D) Tel : 49 89 70 95 79 94.Fax: 49 89 70 95 79 99E-Mail : Dietmar.knopp@ws.chemie.tu-muenchen.deProf. Vladimir VIKLICKY, Associated Contractor.EXBIO PRAHA Ltd. Videnska 1083, 142 20 Prague (CZ'). Tel: 420-2 47 52 151Fax : 420-2 47 52 151E-mail: Viklicky@biomed.cas.cz

# **Abstract of Final Report** on the activity of the project no. ERB 3512 PL97 3145, contract no. ERB IC15 CT98 0322 of the 4th Framework Program of the European Commission, INCO-Copernicus

## Automated Immunoassay System for continuous Air safety Control (AISAC) and its application to air pollution and aeroallergen monitoring

**Objectives:** Our aim was to contribute to build an automated air trap which should collect the air dust and, at regular intervals, should extract allergens and organic compounds and quantitate them rapidly by an ELISA type of immunoassay. The main core of our project was to identify and isolate a few allergens and organic compounds extracted from collected air dust, prepare antibodies to them to allow their identification and quantification by an ELISA. Such a method applied in a suitable automated air trap should allow the detection and quantitation of any pathogenic agent, besides aero-allergens and their adjuvants, present in the air, such as bacteria or viruses provided that specific antibodies could detect them.

**Activities and methodologies:** Our work consisted in extracting organic compounds and aero-allergens from urban aerosols highly polluted by air traffic, analysing their respective contents and producing either polyclonal or monoclonal antibodies to a few relevant well purified and characterised fractions or molecules. These antibodies were used to develop an ELISA type of immuno-assay to allow the automatised detection and quantitation of the selected antigens.

**Major results:** An optimised extraction procedure has been developed for both the organic compounds and the aero-allergen fractions from complex urban aerosol samples. In vivo and in vitro toxicity studies of these fractions have been made. The stimulation of the allergenicity (or **adjuvanticity)** of clean pollen by the organic compounds present in the urban aerosol has been tested in an original mouse model of allergy. We showed that car exhaust particles from Diesel engines mainly were able to stimulate the pollen allergenicity. Two sub-fractions representing only one tenth of the organic matter of the air dust are highly stimulating the induction of pollen allergy. Monoclonal antibodies were produced in mice against an important organic compound from the Diesel car exhaust particles, the Benzo-A-Pyrene (BAP) and to aero-allergens like latex and grass pollen. Polyclonal antibodies were also produced to epitopes common to many different kinds of pollen making possible the immunodetection of a wide spectrum of aero-pollens. All these antibodies were used in ELISA showing that the concept that we proposed to built an « Automated immuno-assay for Continuous Air Safety Control » (AISAC) was feasible.

**Problems encountered:** The first one was a very late financing of our project inducing the need for its prolongation and the modification of the money spending. The second was the moving from the Group of Partner F, the coordinator, first inside the Pasteur Institute and then to the ESPCI in Paris. This great loss of working time was mainly the reason for the very late final report delivery.

**Steps taken to ensure the application of the results:** The monoclonal antibodies to BAP are produced and sold by our Partner CZ'. The feasibility of an AISAC is studied further together by the Partner F and the French Company Bertin technologies.

**Summary of Final Report** on the activity
of the project no. ERB 3512 PL97 3145, contract no. ERB IC15 CT98 0322 of the 4th Framework Program of the European Commission, INCO-Copernicus

# Automated Immunoassay System for continuous Air safety Control (AISAC) and its application to air pollution and aeroallergen monitoring

## Objectives

Our aim was to contribute to build an automated air trap which should collect the air dust and, at regular intervals, should extract allergens and organic compounds and quantitate them rapidly by an ELISA type of immunoassay. The main core of our project was to identify and isolate a few allergens and organic compounds extracted from collected air dust, prepare antibodies to them to allow their identification and quantification by an ELISA. Such a method applied in a suitable automated air trap should allow the detection and quantitation of any pathogenic agent, besides aero-allergens and their adjuvants, present in the air, such as bacteria or viruses provided that specific antibodies could detect them.

## Activities

Our work program was:

- **Task 0**: the collection of air dust samples at different times and locations in Paris, Munich, Prague and Pecs (Partners F, D, CZ, HU)
- **Tasks 1 and 2**: the extraction of organic compounds and aero-allergens (F, D, CZ, HU) from the collected dust
- **Task 3**: the coulometric analysis of the insoluble organic residue of the extracted air dust
- **Tasks 4 and 5**: the physico-chemical characterisation and purification (Task 6) of some major organic compounds using HPLC and capillary electrophoretic methods (D, CZ, HU)
- **Tasks 7 and 8**: in vitro and in vivo testing of toxicity or adjuvanticity for aero-allergens of these compounds (F, CZ, HU)
- **Tasks 10, 11 and 12**: the physico-chemical characterisation and purification of major aero-allergens, mainly from pollen of grasses and trees and also from latex due to their presence in aerosols originating from the wear and tear of truck tires (F, HU, CZ)
- **Task 13**: the determination of the allergenicity of the aero-allergen fractions (F)
- **Tasks 9 and 14**: the preparation of polyclonal and monoclonal antibodies to the molecules, aero-allergens and organic compounds, that will be selected, analyzed and purified or synthesized (F, D, CZ, HU)
- **Task 15**: the production of immunoassays (F, D) as ELISA kits to the aero-allergen and organic compounds studied.

## Results achieved

This work program was carefully followed and our major results were:

Task 1, 4, 5 and 6: an **optimized extraction procedure** of the organic fraction, called "PAHs+", of the air dust collected in a Prague tunnel (Tunnel Particulate Matter or TPM) and its preparative fractionation into 7 sub-fractions. These sub-fractions were used for **toxicity studies** either in vitro by Partner H or in vivo in mice by Partner F. The **in vitro** studies used an EPR spectroscopic method on whole red blood cells and isolated cell membranes. The **in vivo** studies used mainly the Lactic Dehydrogenase (LDH) release in broncho alveolar lavages (BAL) from mice immunized and intra-trachealy challenged with the unfractionated TPM, the pollen suspensions respectively as positive and negative controls or the pollen suspensions to which the 7 sub-fractions of the organic compounds were added. Both methods showed that the clean pollen alone didn't induce any cytotoxicity whereas the air pollution fractions had some cytotoxic effects (ref F 6, 7 and H in progress).

These TPM organic sub-fractions were then used to test their **adjuvanticity** (Task 8) that is a stimulation of the allergenicity of the aero-allergen fractions in vivo in an **original mouse model of allergy and asthma**. Two sub-fractions ( 2 and 7) representing respectively only 3 and 7.4 % of the total organic matter of the TPM had a marked "adjuvant" effect on birch pollen allergenicity as expressed by the IgE antibody production and also by the degree of broncho-constriction observed. This very important and original result confirms what has already been shown by many authors that **car exhaust particles from Diesel engines mainly are able to stimulate the allergenicity of pollen**. What we are adding to that knowledge is one step further in the identification of the culprit of the allergy inducing components present in the urban air pollution. **Two sub-fractions representing only one tenth of the organic matter of the air dust are highly stimulating the induction of pollen allergy** in a mouse model. Preliminary analysis of these 2 sub-fractions suggests that they are highly heterogeneous at the molecular level. It would be very important to try to identify, at the molecular level, the true "adjuvants" of pollen allergenicity. Carmakers who know that Diesel car exhaust particles are responsible of the induction of an allergy to aerosolized pollens are able to modify, either by acting on the engine tuning or on the Diesel fuel the chemical and particulate composition of the car exhaust. They could then try to reduce the production of these pathogenic molecules if and when they will be fully identified.

The second main result of this programme lies in the development of **new instruments to detect, automaticaly, aero-allergens** (pollens, latex,…) **and some of their "adjuvants"** from the air pollution by a standard ELISA. Partner D with the contribution of Partners CZ, H and CZ' have successfully prepared an immunogenic preparation of an haptenic molecule isolated from the organic part of the TPM. Polyclonal antibodies have been produced to Benzo-A-Pyrene (BAP) with a specificity and avidity suitable to develop an immuno-assay able to detect and quantify this molecule in air dust by a standard ELISA. More importantly, **monoclonal antibodies were successfully produced** by Partners CZ' and D **against BAP**, allowing thus a constant supply of an identical immuno-reagent to detect BAP**. This monoclonal is now for the first time commercially available** through our Partner CZ' **and constitutes a reference** for further developments of such reagents. On the other hand, Partner F has, during this programme, characterized and identified more than 12 new allergens either from pollen or latex. Several polyclonal antibodies have been produced against pollen or latex allergens. Monoclonal antibodies to the Group 4 and 1 from grass pollen (Dactylis glomerata)

were obtained. With great difficulties, **two monoclonal antibodies to latex allergens were finally obtained** allowing the development of an ELISA to these molecules extracted from indoor as well as outdoor air dust. Very interestingly, Partner F produced several rabbit antisera directed to non-water soluble grass pollen. Some of these sera were able to recognize a great diversity of pollen, even some produced by plants phylogenetically very distant from grasses such as trees and other dicots. This type of immuno-reagent is able to detect with a great sensitivity the presence of **many different kinds of pollen grains in the air**. Another important feature is that **intact as well as parts of pollen grains can be detected**, which is often the case for pollen present in urban air dust. This work opens the **possibility to detect and quantify by ELISA the presence of many if not any type of pollen in the air by using a fully automated immuno-detection system**. This important result shows the feasibility of our initial "dream" as we proposed this project, to build an **"Automated Immuno-assay for Continuous Air Safety Control" (AISAC).**

## Problems encountered

The 1st problem we encountered was **the late financing of our project**. We could place the first orders only nine months after the official start of the programme. The money we had scheduled to be spent on equipment in order to start working was taken from another source and had to be devoted to consumables which were in fact more expensive than expected. This **late financing of the programme continued**: the support expected for February 2001 arrived only in December 2001.

The 2nd main problem that we experienced was due to **the move of the Partner F group** first **in April 2001** inside the Pasteur Institute **and in April 2002** from the Pasteur Institute to a new Institution, the Ecole Supérieure de Physique et Chimie Industrielles in Paris (ESPCI). It was due to the conjunction of 2 factors: the retirement of the former head of the "Immuno-Allergy Unit" to which the group belonged and the election of a new board of Directors at the head of the Pasteur Institute. The decision was made to dismantle the former Immuno-Allergy Unit. The Group F had to move a first time in inappropriate labs inside the Pasteur Institute. A severe accidental flooding of the lab happened on a week end in November 2001 resulting in the loss of precious experimental results, documents, pieces of equipment and of course a lot of time. The Group F decided then to leave the Institute. Among several offers, the "Allergy and Environment" group choose to move to the ESPCI, a prestigious engineer school in the centre of Paris. This moving was done in very bad conditions, as the new lab was not ready to receive it. Several precious months of work were again lost, justifying the extension of this programme and the very late report delivery. Finally, the Partner F is very happy of its new working conditions and is, more than ever before, effective at work with a group enriched and rejuvenated by 2 excellent PhD students and a post-doctoral fellow.

## Technology implementation plan

1- **Monoclonal antibody** production to Benzo-A-pyrene by partner D **produced and sold** by Partner CZ'. ELISA kit development available from Partner D.
2- **Monoclonal antibody** to Latex allergens and to grass pollen allergens 1, 3 and 4 produced by Partner F. ELISA kit development available from Partner F.
3- **Polyclonal antibodies** to non-water soluble antigens from grass pollen and cross reacting with many different pollens available as an ELISA kit from Partner F. Application to an automated detection of pollen in an air trap developed by the French Company Bertin technologies.

4- The results of the whole programme have shown that our initial proposal to contribute to build an Automated Immuno Assay System for Continuous Air Safety Control (AISAC) was feasible. Discussions with another SME as the one initially approached (SFRI from Bordeaux, France, which has disappeared since) namely the French Company Bertin Technologies are in progress to develop such equipment.

## Publications and papers

Main publications


1- Chardin H, Mayer C, Desvaux F-X, Sénéchal H, Peltre G. Latex allergy: characterization of major allergens and isotypic expression. Allergy, 1999, 54, 872-877.

2- Fernvik E., Peltre G., Sénéchal H., Vargaftig B. B. Effects of birch pollen and traffic particulate matter on Th 2-cytokines, immunoglobulin E levels and bronchial hyperresponsiveness in mice. Clin Exp Allergy 2002,32, 602-611.

3- Fernvik E., Scharnweber T., Knopp D., Niessner R., Vargaftig B. B., Peltre G. Effects of fractions of air polluted urban dust on Th 2 cytokines, IgE levels and bronchial hyperresponsiveness in mice. J Tox Envir Health Part A, 2002, 65, 101-121.

4- Chardin H, Raulf-Heimsoth M., Chen Z, Mayer C, Desvaux F-X, Sénéchal H, Peltre G. Interest of 2-D electrophoretic analysis for the characterization of the individual sensitization to latex allergens. Int Arch Allergy Immunol. 2002, 128,195-203.

5- Chardin H, Mayer C, Sénéchal H, Wal J.M, Poncet P, Desvaux F.X, Peltre G. Lipid Transfer Protein 1 is a possible allergen in *Arabidopsis thaliana.* Int Arch Allergy Immunol. 2003, 131, 85-90.

6- Kustos I, Toth V, Kocsis B, Kerepesi I, Emody L, Kilar F. Capillary electrophoretic analysis of wild type and mutant Proteus penneri outer membrane proteins. Electrophoresis. 2000 Aug; 21(14):3020-7.

7- Kustos I, Toth V, Kilar F, Kocsis B, Emody L. Effect of spontaneous and induced mutations on outer membrane proteins and lipopolysaccharides of Proteus penneri strain 357. Adv Exp Med Biol 2000; 485:177-81

8- Kilar F, Visegrady B. Mapping of stereoselective recognition sites on human serum transferrin by capillary electrophoresis and molecular modelling. Electrophoresis 2002 Mar; 23(6):964-71.

9- Kustos T, Kustos I, Gonda E, Kocsis B, Szabo G, Kilar F Capillary electrophoresis study of outer membrane proteins of Pseudomonas strains upon antibiotic treatment. J Chromatogr A. 2002 Dec 6; 979(1-2):277-84

10- Kasicka V, Prusik Z, Sazelova, Brynda E, Stejskal J. Capillary zone electrophoresis with electroosmotic flow controlled by external radial electric field. Electrophoresis. 1999 Sep; 20(12):2484-92.

11- Kasicka V, Prusik Z, Sazelova P, Chiari M, Miksik I, Deyl Z. External electric field control of electroosmotic flow in non-coated and coated fused-silica capillaries and its application for capillary electrophoretic separations of peptides. J Chromatogr B Biomed Sci Appl. 2000 Apr 28; 741(1):43-54.

12- Sazelova P, Kasicka V, Koval D, Prusik Z, Peltre G. Evaluation of the efficiency of extraction of ultraviolet-absorbing pollen allergens and organic pollutants from airborne dust samples by capillary electromigration methods. J Chromatogr B Analyt Technol Biomed Life Sci. 2002 Apr 25; 770(1-2):303-11.

13- Sazelova P, Kasicka V, Koval D, Kilar F, Knopp D, Peltre G. Analysis of water extracts from airborne dust samples by capillary isotachophoresis. J Chromatogr A. 2003 Mar 21; 990(1-2):303-9

14- Spitzer B, Cichna M, Markl P, Sontag G, Knopp D, Niessner R. Determination of 1-nitropyrene in herbs after selective enrichment by a sol-gel-generated immunoaffinity column. J Chromatogr A. 2000 Jun 2;880(1-2):113-20.

15- Suchanek M, Scharnweber T, Fisher M, Knopp D, Niessner R. Monoclonal antibodies specific to polynuclear aromatic hydrocarbons. Folia Biol (Praha). 2001; 47(3):106-7.

16- Scharnweber T, Fisher M, Suchanek M, Knopp D, Niessner R. Monoclonal antibody to polycyclic aromatic hydrocarbons based on a new benzo[a]pyrene immunogen. Fresenius J Anal Chem. 2001 Nov;371(5):578-85.

17- Schedl M, Wilharm G, Achatz S, Kettrup A, Niessner R, Knopp D.Monitoring polycyclic aromatic hydrocarbon metabolites in human urine: extraction and purification with a sol-gel glass immunosorbent. Anal Chem. 2001 Dec 1; 73(23):5669-76.

Ph.D. degrees resulting from work under the contract: Tim Scharnweber (Partner D), Dusan Koval (Partner CZ, since July 2000),

Post doctoral Fellow: Eva Fernvik (Partner F).

# Conclusions

### 1- Benefits arising directly or indirectly from the project:

**The scientific collaboration**, which was set up to propose first, then to accomplish our programme was truly needed: no single partner could have done alone the totality of the work achieved in common. Our project put together four complementary Partners mastering their own part of the work without too much overlapping of competencies producing a synergistic collaboration.

**Each Partner had his own benefit**: those who required the acquisition of dearly needed equipment (CZ, H) could obtain it, Partner D invested his whole budget into the salary of a Ph.D. student responsible of the work and Partner F shared his budget into expensive needed consumables and a post-doctoral fellow salary. Sub-contractor CZ' has contributed to produce monoclonal antibodies and is selling them successfully.

**The common benefit** is the successful collaboration and its rich production in terms of publications, contributions to meetings and broadening of the scientific interest of each of the Partners.

### 2- Recommendations for future actions:

1- **Careful preparation** of the project is a fundamental condition to warrant its success: all the Partners were already knowing each other by attendance to meetings and through the literature. Some of them had bilateral collaborations in the past. We have never worked together before this programme but we knew, by mutual esteem and confidence, that our collaboration will be a success. A first meeting was organized to prepare together the proposal. This constituted a firm commitment of all the partners into the proposed programme and was certainly the best insurance for a successful and enjoyable collaboration.

2- An adequate number of **meetings** among the 5 partners was important in order to have a good exchange and a good control of the evolution of the work. Some of us have underestimated the cost of these meetings but have managed to meet with other funding.

3- To have a **young scientist responsible** of the work in the lab, meaning a personnel interest in the fulfillment of it (Ph.D. thesis, post doc work,…) was an important incentive for a successful project. These young scientists are a very strong driving force to achieve the project, to publish the results, to bind links between the participating labs and especially with the other young scientists involved in the project. We witnessed the organization of a very fruitful network of exchange at that level.

### 3- This project was a true "success story".

We consider this Programme really as a success story on many points of view:

- **Scientific achievement**: we believe that the results of our project are very important and open the way to the production of a fully automated air trap needed for the air safety control. No better period than now can be expected to show the urgent need for such equipment: political fanatism and terrorism may be a threat for the air safety of all major urban areas. Furthermore the actual rapidly spreading of bacterial and viral epidemics or epizootics in the world are one more reason to develop a performing network of air safety control automates.

- **European dimension of the Scientific Community**: the success of our project is mainly based on the true complementarity of the 5 Partners and the excellent synergy that was created at the European level.

- **Human experience**: our collaboration was very friendly and enjoyable. We are ready to continue to work together and some collaborative projects are already prepared among the Partners and submitted for financing. We are frequently in contact by mail or by visits or attendance to common congresses.

**Consolidated scientific Report** on the activity
of the project no. ERB 3512 PL97 3145, contract no. ERB IC15 CT98 0322 of the 4th Framework Program of the European Commission, INCO-Copernicus

# Automated Immunoassay System for continuous Air safety Control (AISAC) and its application to air pollution and aeroallergen monitoring

## Objectives

Our aim was to contribute to build an automated air trap which should collect the air dust and, at regular intervals, should extract allergens and organic compounds and quantify them rapidly by an ELISA type of immunoassay. The main core of our project was to identify and isolate a few allergens and organic compounds extracted from collected air dust, prepare antibodies to them to allow their identification and quantification by an ELISA. Such a method applied in a suitable automated air trap should allow the detection and quantitation of any pathogenic agent, besides aero-allergens and their adjuvants, present in the air, such as bacteria or viruses provided that specific antibodies could detect them.

## Activities

Our work program was:

- **Task 0**: the collection of air dust samples at different times and locations in Paris, Munich, Prague and Pecs (Partners F, D, CZ, HU)
- **Tasks 1 and 2**: the extraction of organic compounds and aero-allergens (F, D, CZ, HU) from the collected dust
- **Task 3**: the coulometric analysis of the insoluble organic residue of the extracted air dust
- **Tasks 4 and 5**: the physico-chemical characterization and purification (**Task 6**) of some major organic compounds using HPLC and capillary electrophoretic methods (D, CZ, HU)
- **Tasks 7 and 8**: in vitro and in vivo testing of toxicity or adjuvanticity for aero-allergens of these compounds (F, CZ, HU)
- **Tasks 10, 11 and 12**: the physico-chemical characterization and purification of major aero-allergens, mainly from pollen of grasses and trees and also from latex due to their presence in aerosols originating from the wear and tear of truck tires (F, HU, CZ)
- **Task 13**: the determination of the allergenicity of the aero-allergen fractions (F)
- **Tasks 9 and 14**: the preparation of polyclonal and monoclonal antibodies to the molecules, aero-allergens and organic compounds, that will be selected, analyzed and purified or synthesized (F, D, CZ, HU)
- **Task 15**: the production of immunoassays (F, D) as ELISA kits to the aero-allergen and organic compounds studied.

# Results achieved

## Partner F

**Task 0.** Air dust samples have been collected in Paris on different types of filters and at different time periods, mainly in relation to the pollinic seasons of birch (March-April) and grasses (May-June). As big amounts of air dust samples were needed for our planned research **we decided to design and built our own air trap able to filter several cubic meters of air per minute** on a polyester mesh of 20 micro meters. The air dust collected with this machine located in our lab in Paris was compared to the big amounts of dust collected by the CZ partner in Prague and used by all the partners of this project.

**Task 2. Water extraction** of the air dust was optimized first at the begining of our project with the aim of extracting mainly the pollen water soluble allergens. As our work progressed, we became convinced that the allergen rich water-soluble fraction was not the only fraction we should be looking at. Some air dust fractions collected in rainy periods were particularly poor in the well known and characterised water-soluble allergens. We then decided to further extract the solid residue of the water extraction of the air dust by a mixture of detergents and chaotropes (Thiourea, Urea and CHAPS), a solution compatible also with our usual analysis of allergen extracts by 2D gel electrophoresis. **This extraction was highly successful and produced a new set of proteins, some being recognized as allergens for allergic patients and so far not yet characterized. This very important aspect of our work is still under development and not yet published**. These new „non-water soluble allergens and antigens“ required to prepare specific antibodies to recognise and quantify them by immuno-assays (tasks 14-15).

**Task 7. Toxicity** of the „PAHs+“ fractions was studied in vivo as a logical consequence of our comitment to immunise mice (and rabbits) with these fractions, together with allergenic preparations, in order to evaluate the adjuvanticity of the former fractions. This work was done on the BP 2 selection of mice from Biozzi, known as good immune responders. The test we used is the LDH (Lactic Dehydrogenase) release in the Broncho-Alveolar Lavage (BAL) fluid. No toxicity was found in mice immunised with pollen alone. The highest toxicity was found in mice immunised and challenged with a mixture of pollen and Tunnel Particulate Matter (TPM). Intermediate toxicity were found in mice receiving either pollen or TPM, **suggesting strongly that pollen and TPM may act synergistically on the inflammatory and the immune systems, the two major systems involved in allergy** (ref 6,7).

**Task 8. We developed an adjuvanticity test** in the BP 2 mice in order to detect, from the air dust, the presence of “adjuvant” molecules stimulating the allergenicity of known allergens (ref 6,7). We choose clean pollen as allergen source, Prague car tunnel dust (or TPM from Partner CZ) samples as source of urban air pollutants. BP 2 mice were immunized with clean pollen alone, or pollen together with TPM, or with pollen together with an IgE inducing adjuvant, Al (OH)3. The **bronchial hyperresponsiveness** (BHR) was measured in a whole body plethysmograph after a methacholine challenge and an intra-nasal allergen exposure. Th2 cytokines (IL-4 and IL-5) as markers of an induced allergic state and both fibronectin and lactate dehydrogenase (LDH) were determined as **inflammatory markers** in the Broncho-Alveolar Lavage (BAL) fluid. Antibody titres to pollen extract and cytokine levels were

quantified in the sera. Specific IgE titres, BHR, the number of recruited eosinophils and levels of fibronectin and LDH in BAL were increased in mice immunized and challenged with a mixture of pollen and TPM. However, mice immunized with pollen alone and challenged intranasally with pollen or a mixture of pollen and TPM showed higher levels of IL-4 and IL-5. **In conclusion, we have demonstrated that polluted urban dust has an effect on both the inflammatory and immunological components of experimental allergy.**

**Our search for adjuvant activity stimulating the pollen allergenicity was then straightforward: the organic phase of the TPM (from Partner CZ) was separated into 7 sub-fractions obtained by liquid extraction with organic solvents of increasing polarity (from Partner D)** (figure 1, see next page).

The BP 2 mice were immunised and intranasally exposed either with clean pollen alone, as above described, or pollen to which the different TPM sub-fractions (1-7) were added. They were then provoked intranasally with a mixture of birch pollen and TPM. The same tests (BHR, cytokines and fibronectin in BAL and IgE production in sera) were performed as above described.

The highest titers of IgE and highest BHR were found in the positive control mice (immunized and provoked with a mixture of pollen and unfractionated TPM), followed by mice immunized with pollen and fractions 2 (which contains organic acids) and 7. Fraction 2 also induced the highest number of eosinophils and increased levels of interleukin 5 (IL-5) in the BAL fluid. With exception of control mice, and those immunized with fractions 2 and 7, the levels of IgE were overall very low or completely absent with the other fractions.

**In conclusion, we have demonstrated that fractions 2 and 7, which are in mass minor sub-fractions of the total TPM, respectively organic acids and highly polar compounds, do contain potential adjuvant molecules having a clear effect on both the inflammatory and immunological components of our experimental allergy model.**

**Task 11.** Characterisation and identification of aero-allergens constituted a great part of the work done by our group.

Grass pollen. We have studied mainly the allergens from Dactylis glomerata. Most of the water-soluble allergens are well known and characterised at the molecular level: pI; Mr and their location on 2D gel maps. However some minor allergens (pI 4.2-4.6, Mr 42-48 kDa) were detected by a few patient sera and will be further analysed, described and worth to be published. The effects of pollen exposure to urban air pollution at different time periods around the year modify greatly the allergen spectrum. These results should be also published. **Among the non-water soluble proteins a few allergens were recognized by patient sera. This very important result need more work for a complete molecular identification (amino acid sequencing) as a preliminary screening in the existing protein and nucleic acid data banks didn't allow their identification**.

Tree pollen. We have studied mainly the birch pollen allergens recognised by mice in our new animal model of allergy (ref 6, 7), the only ethical way to study the effects of air pollution on allergy. The allergens recognised by the BP 2 mice were also recognised by some of our allergic patients. Balb/c mice were also used to set up this animal model. These mice had another spectrum of allergen recognition that was similar to a small fraction of our birch pollen allergic population. These results are stimulating us to extend in the future a set of mice strains that should be more representative of the actual human immune response to allergens. Furthermore, the non-water soluble allergens from birch pollen should be looked for as we did with the grass pollen.

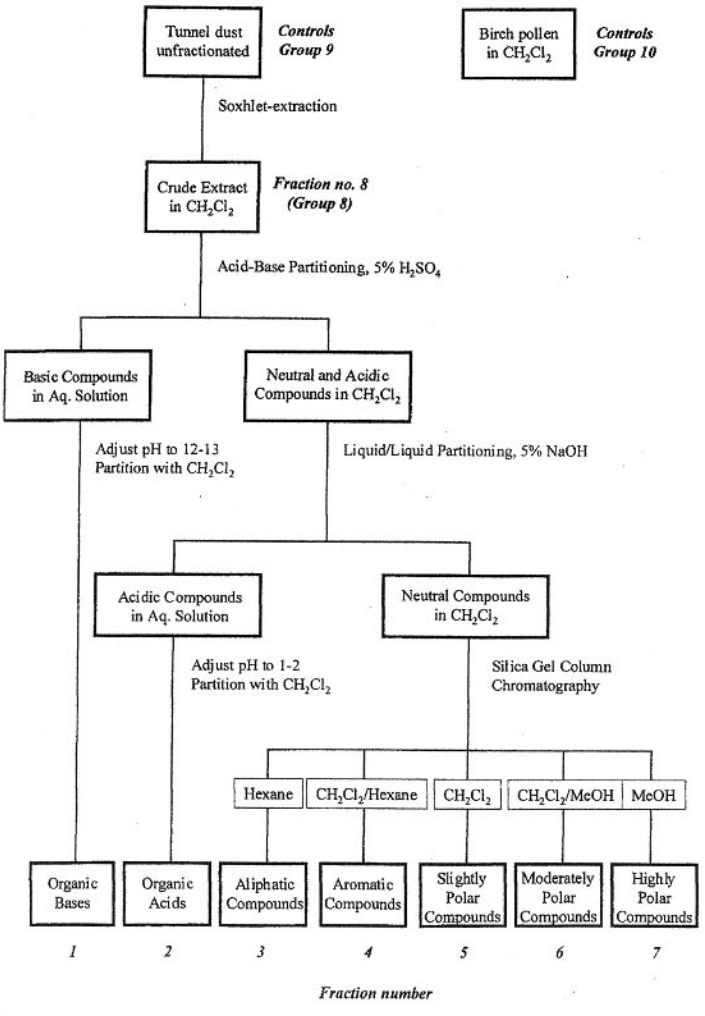



**FIGURE 1.** Extraction scheme for the traffic particulate matter (TPM). Mice were immunized with a mixture of birch pollen and fraction 1–8. As controls, one group of mice (group 9) was immunized with unfractionated TPM and pollen, and another group of mice (group 10) was immunized with dichloromethane-treated pollen.

Rape seed. Up to **7 allergens have been detected, characterised and identified**. One of them, a polygalacturonase or pectinase (pI 6.5-8.5, Mr 43 kDa) was described for the first time (ref 5,11).

Arabidopsis thaliana. In this plant, genetically closely related to rape seed we found, described and published **the first allergen identified** (ref 12). As the genome of this plant is known we were able to locate, most likely, on chromosome 2 the gene coding for this allergen, an LTP 1 (Lipid Transfer Protein).

Hevea brasiliensis or latex. (ref 1, 2, 4, 8). This allergen has been rather recently introduced in our indoor environment, since the AIDS outbreak in the mid 80s, mainly in hospitals and research centers as a consequence of an extensive use of latex gloves. Some reports have also shown that the wear and tear of truck tyres could induce latex aerosols as an allergenic contribution to the already polluted urban air. Our contribution, within this EU programme was to analyse the different latex allergens in the indoor (gloves, mattresses,…) as well as the outdoor environments. With the skilled help of Partner CZ' we have tried to produce monoclonal antibodies to some different latex preparations. **Four papers were published** on the detection, characterisation and identification of these allergens (ref 1, 2, 4, 8). The human antibody response to these allergens was also carefully described. The Task 2, the optimisation of the allergen extraction, was very fruitful as we introduced new methods of extracting **non-water soluble allergens** from the different latex allergen sources. The Task 11, devoted to the immunodetection of the allergens was particularly well studied: large numbers of patients were screened for their sensitivity to latex. Besides their IgE antibodies to latex allergens we have looked for some other Ig classes and subclasses to the latex extracts and found some original results concerning the **IgG3 response** (ref 2). The Task 14 took us much effort but induced a lot of frustration as concerns the monoclonal antibody production. Our Partner CZ', a recognised specialist in monoclonal antibody production made 3 successive cell fusions to try to induce monoclonals without success. Due to our strong investment into this subject we tried, helped by Dr Pascal Poncet from the Pasteur Institute who joined our group in 2001, again to induce such antibodies. With difficulties we succeeded finally in producing 2 good IgG producing clones against 2 latex allergens. This point should need further research to understand why an initial successful immunisation of mice with latex preparations led, reproducibly to the death of the cell clones producing actively antibodies to these particular molecules that are the latex allergens.

**Tasks 13 and 14.** The **allergenicity of pollen extracts**, grasses and birch, were extensively studied during this Programme. In this project we decided to develop **new mouse models of allergy to intact clean and air-polluted pollen grains**. Our new immunisation schedule allowed us to test rather small amounts of air pollutants, the TPM collected in Prague, or fractions of it. We were also very surprised to observe that the method used to induce IgE antibodies to grass pollen was not exactly the same as for inducing IgE antibodies to birch pollen. Grass pollen was more immunogenic in BP2 than birch pollen at equal amounts, an observation which is also true for some human beings (ref 6 and 7). These facts are very valuable observations and could led to further investigations.

As already mentioned above in Task 11, latex extracts were very immunogenic in rabbits and mice. However the monoclonal antibody production was very difficult. Our main competitors in that field, from Finland, told us the same. They finally succeeded also to produce monoclonal antibodies to most of the major latex allergens.

**Task 15.** Our group has produced an **ELISA for detecting and quantifying the grass and birch pollen antigens in air dust samples**. This test is more sensitive for grass pollen but is

also able to detect, besides birch pollen, many other pollen grains. This test is able to detect far below one single pollen grain, in the order of 2 nano grams of total pollen per ml of air dust extract. **This sensitivity is far better than the usual detection level** obtained up to now by the conventional volumetric pollen traps that are in use in Europe and world wide to detect pollen in the air. **This achievement is a proof that our initial project to build an "Automated (Immuno-Assay) System for Continuous Aeroallergen Monitoring" was feasible.**

## Partner HU

**Task 0**. Dust collection was performed in Pécs by using the common equipment of a National Environmental Control Station established in the city or a simple filter, respectively. Generally monthly periods were chosen to follow the changes in the air pollution in urban area with high traffic and industrialization. The glass filter was prepared for a minimum 6 hour collection with heat treatment, and measured in weight for later use. The amount of air sucked through the filter was similar to other dust collection procedures used by other Contractors in the *Project*.

**Tasks 1 and 2**. Extraction procedures were tried to collect substances from the glass filter cuts. Different organic solvents and methodologies were tested and the recovery of substances was controlled. The experiments resulted in a standard procedure to obtain an acetonitrile solution of PAHs for HPLC studies.

**Task 7**. It is known that the effect of pollutants on biological objects is a complex process. According to the literature the first possible target of the pollutants is the biological membrane. In order to obtain information about the structural and dynamic changes induced by pollutants, studies were performed by spectroscopic methods in two biological model systems: effects of chromium VI on yeast cell membranes and effects of drugs on human erythrocyte membranes. After the model studies the biological effects of several polycyclic aromatic hydrocarbons were investigated by EPR spectroscopy using three different membrane systems. Whole red blood cells, ghost membranes and liposomes were applied and 6 PAH compounds were tested. Although the effect of the PAHs were observed only at higher concentrations, ie higher than the concentration in the air, but the long exposure of membranes might have similar effect. It was found that the PAH molecules with different chemical structure have different impact on the membranes. The rigidity of the membranes in the presence of these aromatic organic substances changes by temperature, but a more pronounced effect can be visualised in the case of naphtalene compared to pyrene. The incorporation of the different structures in the membrane is resulting in altered mobility of the spin labels. Although the PAHs are not water soluble molecules the exposition of the membranes to such compounds might influence the chemical structure and biological activity of cells.

**Tasks 4 and 10**. Two different sets of extracts were investigated by capillary electrophoresis and HPLC methods. ln order to have a standardized comparison of the different methods used by the network partners, a larger amount of dust was extracted in Munich. The aliquots of the extracted materials were then distributed to the other partners. These aliquots were analysed in Pécs. Water based capillary electrophoresis experiments and HPLC runs were performed. Since the extraction method resulted in organic solutions in dichloromethane, the samples were transferred into solvents that are compatible with water-based capillary electrophoresis. Therefore, highly acidic buffers, methanol and ethanol, as well as acetone mixed with buffers

solutions were applied. The 7 fractions (see details in the report of Partner 4, TUM) after re-dissolving the extracts in the different solvents did not show any detectable amount of components except Fraction 1, the basic extract. However, the amounts of the recovered components were low, and therefore the analysis was not possible with UV detection. The HPLC analysis of the same fractions applying a fluorescent detector showed, however, numerous components, and with an extensive calibration study all the PAH components in the extracts were quantified. The sensitivity of the HPLC experiments is higher due to a bigger injection volume and the fluorescent detection technique.

A part of the glass filters obtained from dust collection was investigated by HPLC. The chromatograms allowed a precise quantification of PAH components from the air and their changes during the seasonal variations.

## Partner D

**Tasks 0, 1, 4, 7. Determination of PAHs in air particulate extracts (air particulates, tunnel dust)** Real samples of particulate matter were collected on glass-fiber filters with a low-volume sampler with PM-10 sampling head at a high traffic crossing in Munich. The residues were re-dissolved in acetonitrile and PAHs determined directly by HPLC or, after a dilution with water, by ELISA.

Six traffic tunnel dust samples were collected at different times from ventilation shafts of two tunnels in Prague (CR) and made available (to all project participants) by the Czech partner (Dr. Kasicka). All dust samples were extracted with toluene/dichloromethane/methanol (1:1:1, v/v) by ultrasonication. PAHs were determined by HPLC and ELISA as described above. Additionally, one dust sample extract was fractionated according to a scheme developed by LEWTAS et al. (Int. J. Environ. Anal. Chem. 39, 1990, pp 245-256) and modified by TOPINKA et al. (Mutat. Res. 19, 1998, pp 91-105). Seven different fractions (organic bases, organic acids, aliphatic compounds, aromatic compounds, slightly polar compounds, moderately polar compounds and high polar compounds) were obtained and measured by the ELISA. Further, these fractions were delivered to our French partner (Dr. Peltre) and used for in vivo toxicological experiments with mice.

Three tunnel dust samples were selected for SEM/EDX analysis: the ground and sterilized sample 1/98 from LETNA, and non-homogenized samples from LETNA (4/99) and STRAHOV (1/01). As could be seen in the microphotographs, particle sizes were smaller (lower than 10 μm) and distribution more homogeneous in the treated sample (Fig. 2) than in the other ones (for example sample 1/01, Fig. 3).

**Fig. 2: SEM of Prague tunnel dust sample 1/98**

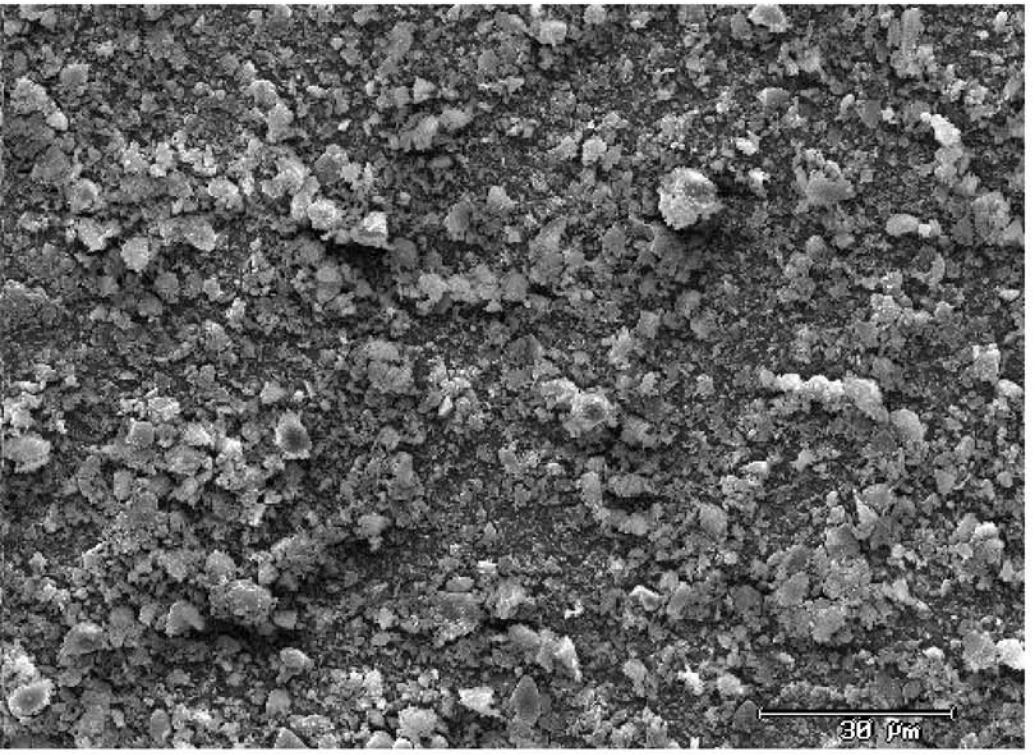
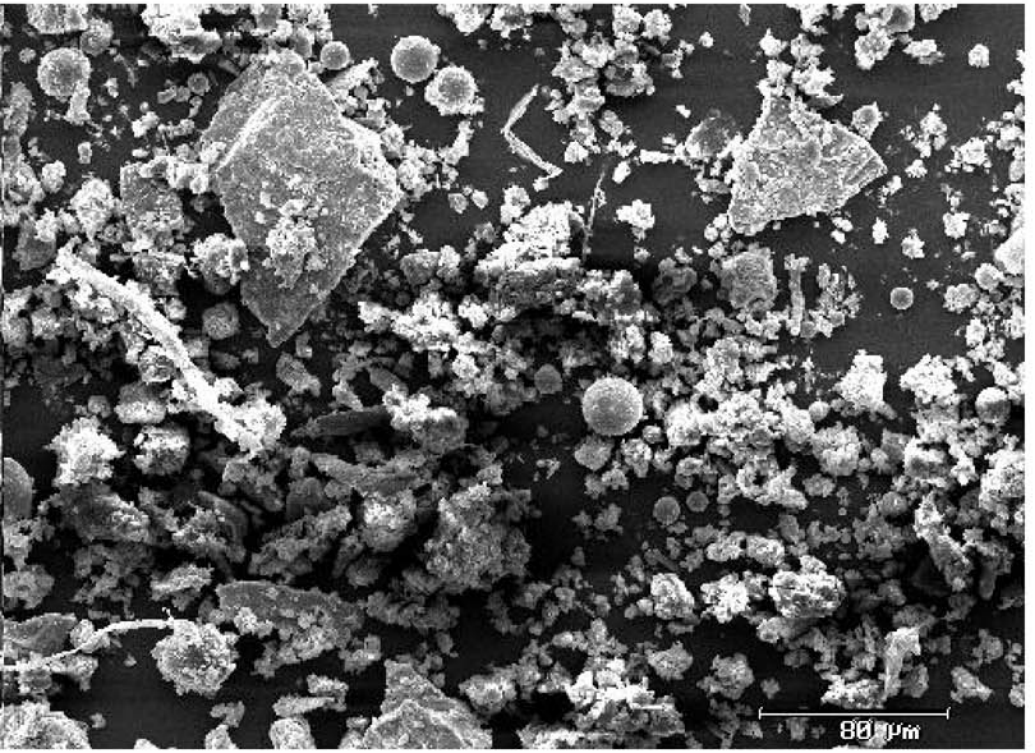



**Fig. 3: SEM of Prague tunnel dust sample 1/01**



**Task 3. Characterization of tunnel dust samples.**

Total carbon content (TC), elemental carbon content (EC) and organically bound carbon content (OC) were determined. In brief, TC was determined by burning the sample followed by the coulometric detection of the $CO_2$ formed. EC was also measured coulometrically after extraction of dust sample with toluene/2-propanol (1:1, v/v) and thermal desorption of non-extractable organic compounds under nitrogen. The OC (also designated as extractable organic matter-EOM) can be calculated as the difference between TC and EC.
Additionally (not contained in the proposal), three selected samples were analyzed by Scanning Electron Microscopy/Energy Dispersive X-Ray Analysis (SEM/EDX) to obtain a semi-quantitative estimation of the content of 13 selected elements (Si, C, O, Al, Ca, Na, Fe, K, Ti, Cu, Mg, Cl, Zn).

**Task 9. Antibody production**

A new benzo(a)pyrene derivative (1-benzo(a)pyrenebutyric acid) was synthesized and characterized by GC/MS and NMR. Then it was covalently coupled to various proteins using either the mixed anhydride method or carbodiimides. The conjugation ratio (degree of substitution) was determined using the TNBS method, i.e. the number of free (uncoupled) amino groups was measured. These conjugates have been used to immunize rabbits (for production of polyclonal antibodies) and/or mice (monoclonal antibody production) (Table 1).

*Table 1: Synthesized protein conjugates*

| Protein carrier | Used for immunization of rabbits | Used for immunization of mice |
|---|---|---|
| Bovine serum albumin (BSA) | X (three animals) | X (two animals) |
| Cationized bovine serum albumin (cBSA) | | X (eight animals) |
| Keyhole limpet hemocyanin (KLH) | X (two animals) | X (three animals) |
| Thyroglobulin (TG) | | X (three animals) |
| Ovalbumin (OA) | | |

**Task 15. Characterization of antibodies and ELISA development.**

*Polyclonal antibodies:*
Antibodies against benzo[a]pyrene were detectable in all sera and could be used to construct calibration curves. A lower sensitivity was found with antisera from rabbits which were immunized with the KLH-conjugate ($IC_{50}$ about 50 µg/L). An about tenfold higher sensitivity could be obtained with antisera dilutions of rabbits which were immunized with the BSA-conjugates ($IC_{50}$ about 3-4 µg/L). The polyclonal sera were not used for airborne aerosol measurements.

*Monoclonal antibodies (mab):*

Monoclonals have been prepared in collaboration with the Czech partner EXBIO, Prague (Drs. Viklicky and Suchànek). The clone designated B[a]P-13 was used in the following investigations because it had the highest affinity for the target analyte. However, affinity was not higher ($IC_{50}$ about 4 µg/L) than compared to the polyclonal antisera which were obtained with the BSA-conjugates.

The monoclonal antibody (ascites) produced by EXBIO was delivered to us and then purified by the company EUROGENTEC (Belgium). The isotype was $IgG_1$, kappa. It was further characterized using an indirect competitive ELISA technique.

The relative sensitivity (cross-reactivity) of the antibodies towards chemicals structurally similar to benzo[a]pyrene was determined by assaying a dilution series of each compound in water with 10% methanol. To take into account the weak affinity of the antibody towards some compounds, which leads to inhibition only at high concentration, the dilutions were prepared from 0.01 µg/L to 10.000 µg/L. The $IC_{50}$ values (concentration of inhibitor that produces a 50% decrease in the maximum normalized response), expressed as molar concentrations, were compared and expressed as a percentage $IC_{50}$ based on 100% response of benzo[a]pyrene. The $IC_{50}$ can be considered a measure (inverse) of the affinity of an antibody for a given analyte.

It was our intention to generate antibodies of broad specificity to bind as many different PAHs as possible and to use the obtained ELISA signal as a sum parameter of the PAH fraction (class-specific ELISA). Therefore, a detailed characterization of the affinity of the antibody to as many related compounds as possible, was absolutely essential in immunoassay development. The cross-reactivity of 21 parent PAH, 16 EPA PAH standards, several hydroxylated and methylated derivatives was determined. Investigations revealed that the antibody had broad specificity, especially for four- to six-ring compounds (Table 2). The latter are considered to be very toxic.

With obtained sensitivity (limit of detection) of the ELISA of 0.3 µg/L for benzo[a]pyrene the assay is the most sensitive ELISA for this analyte that was reported so far. Robustness is acceptable (intra- and interassay precision was below 10%).

*Table 2: Cross-reactivity of antibody B[a]P-13*

| Compound | **CR [%]** |
|---|---|
| *a) Parent PAHs* | |
| Acenaphthene* | 1 |
| Acenaphthylene* | < 1 |
| Anthracene* | 17 |
| Benzo[a]anthracene* | 40 |
| Benzo[a]pyrene* | 100 |
| Benzo[b]fluoranthene* | 26 |
| Benzo[e]pyrene* | 55 |
| Benzo[g,h,i]perylene* | 17 |
| Benzo[j]fluoranthene** | 84 |
| Benzo[k]fluoranthene* | 15 |
| Chrysene* | 38 |
| Dibenzo[a,h]anthracene* | 10 |
| Fluoranthene* | 48 |
| Fluorene* | 3 |
| Indeno[1,2,3-c,d]pyrene* | 69 |
| Naphthalene** | < 1 |
| Triphenylene** | 32 |
| p-Quaterphenyl** | < 1 |
| Perylene* | 8 |
| Phenanthrene* | 16 |
| Pyrene* | 37 |
| *b) Methylated PAHs* | |
| 10-Methylbenzo[a]pyrene# | 21 |
| 1-Methylanthracene# | 64 |
| 1-Methylchrysene# | 77 |
| 1-Methylfluoranthene# | 83 |
| 1-Methylpyrene# | 90 |
| 7, 10-Dimethylbenzo[a]pyrene# | 43 |
| 7-Methylbenzo[a]pyrene# | 37 |
| *c) Other PAHs* | |
| 1-Hydroxypyrene# | 56 |
| 1-Pyrenebutyric acid** | 197 |
| 1-Benzo[a]pyrenebutyric acid# | 1572 |

## Partner CZ

**Task 0**. Six different dust samples were collected in the air-filtration devices in the different locations, heavy-traffic car tunnels in Prague, Letna tunnel and Strahov tunnel, in the different periods, in the pollen-free period (December – January) and in the tree and grass pollen-rich periods (March – July), see Table 1.

**Tasks 1 and 2**. Different extraction procedures have been tested for isolation of "Aero-allergen+" fraction from the above mentioned dust samples:

i) water
ii) acid water solution: 0.5 mol/l acetic acid, pH 2.5 (HAc)
iii) alkaline water buffer solution with anionic detergent: 0.02 mol/l tris, 0.005 mol/l $H_3PO_4$, 0.05 mol/l sodium dodecylsulfate (SDS), pH 8.7 (TP-SDS)

**Tasks 4 and 10**. The water extracts and the acid water solution extracts were analyzed by capillary zone electrophoresis (CZE) in acid background electrolyte (0.5 mol/l acetic acid). Water extracts and alkaline water-TP-SDS-buffer extracts were analyzed by capillary micellar electrokinetic chromatography (MEKC) using 0.02 mol/l tris, 0.005 mol/l $H_3PO_4$, pH 8.7, as background electrolyte with 0.05 mol/l SDS forming a micellar pseudophase.

UV absorption detection in low UV region (206 nm) was used both in CZE and MEKC analyses. Generally more material was extracted and more components of the dust were found in the water-buffer extracts than in water extracts and a better resolution of the components of the dust extracts was achieved by MEKC than by CZE.

Significant differences have been found in the analyses of dust extracts of different origin. Much more material and more components have been found in the extracts of the dust from the pollen-rich period (March-July) than from the pollen-free period (December-January) as can be seen from the data presented in Table 3.

Some similarities have been found in the analyses of extracts from the dusts samples originating from the corresponding pollen-free (January-December) and pollen-rich periods (March –July) in the individual years, 1998-2001.

Examples of CZE and MEKC analyses demonstrating the above results are shown on the enclosed copies of the posters presented on the international symposia and conferences, and in the copy of the paper published in J. Chromatogr. B 770 (2002) 303-311, see below the list of publications and posters and the annexes of this report.

*Table 3: Evaluation of the efficiency of extraction of Prague tunnel dust samples by capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC).*
Extracted amount (in relative arbitrary units, AU) of UV-absorbing components, including pollen allergens and organic pollutants, and number of found components in the dust sample extracts obtained by different extraction agents and analyzed by CZE and MEKC methods using HAc and TP-SDS as background electrolytes (BGEs), respectively.

| Extracting agent | CE-mode | BGE | Extracted amount [AU] | | Number of found components | |
|---|---|---|---|---|---|---|
| | | | LT 1/98 | LT 4/99 | LT 1/98 | LT 4/99 |
| Water | CZE | HAc | 41 | 69 | 8 | 11 |
| Hac | CZE | HAc | 234 | 302 | 7 | 12 |
| Water | MEKC | TP-SDS | 231 | 459 | 14 | 24 |
| TP-SDS | MEKC | TP-SDS | 500 | 975 | 17 | 19 |

*HAc = 0.5M acetic acid, TP-SDS = 20mM Tris, 5mM $H_3PO_4$, 50mM SDS, pH 8.7*
*LT 1/98 – dust from the pollen-free season, LT 4/99 – dust from the pollen-rich season*

In addition to CZE and MEKC analyses, water extracts of all dust samples have been analyzed by capillary isotachophoresis (CITP) in anionic and cationic modes using the electrolyte systems the composition of which is given in Table 4.

*Table 4. Composition of CITP electrolyte systems*

| | Leading electrolyte (LE) | | | Terminating electrolyte (TE) | | |
|---|---|---|---|---|---|---|
| CITP mode | Leading ion | Counterion | pH | Terminating ion | Counterion | pH |
| Anionic | 10 mM $Cl^-$ | 20mM $His^+$ | 5.8 | 10 mM $MES^-$ | - | 3.7 |
| Cationic | 10 mM $K^+$ | 25 mM $Ac^-$ | 4.4 | 10 mM $BALA^+$ | $Ac^-$ | 4.4 |

Extracted amounts of UV-absorbing substances, including pollen allergens and organic pollutants, number of the found components and concentrations of some inorganic ions (e.g. $Cl^-$, $K^+$, $Na^+$, $Ca^{2+}$) in the dust samples were determined. It was found that the extracted amounts of anionic components and their number were much higher than those of cationic components. Significant differences have been found in the analyses of the extracts of different origin. Similarly as in CZE and MEKC analyses of the dust extracts much more material and more components were present in the extracts of dust samples from the pollen-rich period than from the pollen-free period, especially in anionic CITP mode (see Table 5).

*Table 5: Extracted amounts (in relative arbitrary units, AU) of UV-absorbing components*, including pollen allergens and organic pollutants, and number of components found in the water extracts of the dust samples from Prague traffic tunnels and from Parisian metro station, determined by capillary isotachophoresis.

| | | **Anionic mode** | | **Cationic mode** | |
|---|---|---|---|---|---|
| Season | Dust sample | Extracted amount [AU]* | Number of components found | Extracted amount [AU]* | Number of components found |
| Pollen- free | LT 1/98 | 73.0 | 19 | 0.9 | 3 |
| | LT 1/00 | 53.7 | 19 | 1.3 | 4 |
| | ST 1/01 | 118.2 | 20 | 0.9 | 5 |
| Pollen- rich | LT 4/99 | 133.0 | 28 | 1.4 | 3 |
| | LT 5/01 | 118.1 | 25 | 1.0 | 5 |
| | ST 7/00 | 200.5 | 30 | 1.3 | 5 |
| | PM 1 | 352.9 | 28 | 84.0 | 14 |
| | PM 2 | 371.4 | 30 | 61.0 | 13 |

***** Averaged values from two ITP analyses, the values of which differed less than 3-5 %.
LT m/yr – Letná tunnel dust samples, month/year
*ST m/yr – Strahov tunnel dust samples, month/year*
*PM 1 – Paris Metro sample, fine dust*
*PM 2 – Paris Metro sample, fibre-rich dust*

The dust sample collected in Letna tunnel in January 1998 was additionally characterized by the content of more than 40 elements, determined by the methods of neutron activation analysis, particle induced X-ray excitation and Röntgen fluorescence analysis.

A new methodology has been developed for conversion of analytical capillary electro-separations into the preparative separation process realized by continuous free-flow electrophoresis. This procedure has been utilized for integrated approach to analysis and preparation of biologically active peptides and their fragments and derivatives (insulins, dalargins, gonadotropin releasing hormones). The synthetic preparations of these peptides are first analyzed by capillary electromigration methods and suitable conditions are developed for separation of admixtures (impurities) of these peptides. Then, based on the early developed model of the correlation between capillary and free-flow electrophoresis, the optimized experimental conditions of capillary electrophoresis are utilized for separation of the admixtures of the biologically active peptides in preparative scale by free-flow electrophoresis.

The early constructed laboratory-made experimental device for capillary electrophoresis has been adapted for control of electroosmotic flow by external radial electric field which allows optimization of analysis time and separation resolution and efficiency according to the mobility differences of the analytes to be separated. This device was utilized for more efficient separation of dust extracts by capillary zone electrophoresis.

To summarize, the achieved results represent a contribution to the fulfillment of one of the project goals, to develop a procedure for efficient extraction of soluble components from the dust samples collected in the air dust traps and for the detection of organic pollutants and aero-allergens in these extracts by high-performance capillary electromigration methods.

## Problems encountered.

The 1st problem we encountered was the late financing of our project. We could place the first orders only nine months after the official start of the programme. The money we had scheduled to be spent on equipment in order to start working was taken from another source and had to be devoted to consumables which were in fact more expensive than expected. This late financing of the programme continued: the support expected for February 2001 arrived only in December 2001.

The 2nd main problem that we experienced was due to the move of the Partner F group first in April 2001 inside the Pasteur Institute and in April 2002 from the Pasteur Institute to a new Institution, the École Supérieure de Physique et Chimie Industrielles in Paris (ESPCI). It was due to the conjunction of 2 factors: the retirement of the former head of the "Immuno-Allergy Unit" to which the group belonged and the election of a new board of Directors at the head of the Pasteur Institute. The decision was made to dismantle the former Immuno-Allergy Unit. The Group F had to move a first time in inappropriate labs inside the Pasteur Institute. A severe accidental flooding of the lab happened on a week end in November 2001 resulting in the loss of precious experimental results, documents, pieces of equipment and of course a lot of time. The Group F decided then to leave the Institute. Among several offers, the "Allergy and Environment" group choose to move to the ESPCI, a prestigious engineer school in the centre of Paris. This moving was done in very bad conditions, as the new lab was not ready to receive it. Several precious months of work were again lost, justifying the extension of this programme and the very late report delivery. Finally, the Partner F is very happy of its new working conditions and is, more than ever before, effective at work with a group enriched and rejuvenated by 2 excellent PhD students and a post-doctoral fellow.

## Technology implementation plan

18- **Monoclonal antibody** production to Benzo-A-pyrene by partner D **produced and sold** by Partner CZ'. ELISA kit development available from Partner D.

19- **Monoclonal antibody** to Latex allergens and to grass pollen allergens 1, 3 and 4 produced by Partner F. ELISA kit development available from Partner F.

20- **Polyclonal antibodies** to non-water soluble antigens from grass pollen and cross reacting with many different pollens available as an ELISA kit from Partner F. Application to an automated detection of pollen in an air trap developed by the French Company Bertin technologies.

21- The results of the whole programme have shown that our initial proposal to contribute to build an Automated Immuno Assay System for Continuous Air Safety Control (AISAC) was feasible. Discussions with another SME as the one initially approached (SFRI from Bordeaux, France, which has disappeared since) namely the French Company Bertin Technologies are in progress to develop such equipment.

## Publications and papers

*Main publications*

1. Chardin H, Mayer C, Desvaux F-X, Sénéchal H, Peltre G. Latex allergy: characterization of major allergens and isotypic expression. Allergy, 1999, 54, 872-877.
2. Fernvik E., Peltre G., Sénéchal H., Vargaftig B. B. Effects of birch pollen and traffic particulate matter on Th 2-cytokines, immunoglobulin E levels and bronchial hyperresponsiveness in mice. Clin Exp Allergy 2002,32, 602-611.
3. Fernvik E., Scharnweber T., Knopp D., Niessner R., Vargaftig B. B., Peltre G. Effects of fractions of air polluted urban dust on Th 2 cytokines, IgE levels and bronchial hyperresponsiveness in mice. J Tox Envir Health Part A, 2002, 65, 101-121.
4. Chardin H, Raulf-Heimsoth M., Chen Z, Mayer C, Desvaux F-X, Sénéchal H, Peltre G. Interest of 2-D electrophoretic analysis for the characterization of the individual sensitization to latex allergens. Int Arch Allergy Immunol. 2002, 128,195-203.
5. Chardin H, Mayer C, Sénéchal H, Wal J.M, Poncet P, Desvaux F.X, Peltre G. Lipid Transfer Protein 1 is a possible allergen in *Arabidopsis thaliana.* Int Arch Allergy Immunol. 2003, 131, 85-90.
6. Kustos I, Toth V, Kocsis B, Kerepesi I, Emody L, Kilar F. Capillary electrophoretic analysis of wild type and mutant Proteus penneri outer membrane proteins. Electrophoresis. 2000 Aug; 21(14):3020-7.
7. Kustos I, Toth V, Kilar F, Kocsis B, Emody L. Effect of spontaneous and induced mutations on outer membrane proteins and lipopolysaccharides of Proteus penneri strain 357. Adv Exp Med Biol 2000; 485:177-81
8. Kilar F, Visegrady B. Mapping of stereoselective recognition sites on human serum transferrin by capillary electrophoresis and molecular modelling. Electrophoresis 2002 Mar; 23(6):964-71.
9. Kustos T, Kustos I, Gonda E, Kocsis B, Szabo G, Kilar F Capillary electrophoresis study of outer membrane proteins of Pseudomonas strains upon antibiotic treatment. J Chromatogr A. 2002 Dec 6; 979(1-2):277-84
10. Kasicka V, Prusik Z, Sazelova, Brynda E, Stejskal J. Capillary zone electrophoresis with electroosmotic flow controlled by external radial electric field. Electrophoresis. 1999 Sep; 20(12):2484-92.
11. Kasicka V, Prusik Z, Sazelova P, Chiari M, Miksik I, Deyl Z. External electric field control of electroosmotic flow in non-coated and coated fused-silica capillaries and its application for capillary electrophoretic separations of peptides. J Chromatogr B Biomed Sci Appl. 2000 Apr 28; 741(1):43-54.
12. Sazelova P, Kasicka V, Koval D, Prusik Z, Peltre G. Evaluation of the efficiency of extraction of ultraviolet-absorbing pollen allergens and organic pollutants from airborne dust samples by capillary electromigration methods. J Chromatogr B Analyt Technol Biomed Life Sci. 2002 Apr 25; 770(1-2):303-11.
13. Sazelova P, Kasicka V, Koval D, Kilar F, Knopp D, Peltre G. Analysis of water extracts from airborne dust samples by capillary isotachophoresis. J Chromatogr A. 2003 Mar 21; 990(1-2):303-9
14. Spitzer B, Cichna M, Markl P, Sontag G, Knopp D, Niessner R. Determination of 1-nitropyrene in herbs after selective enrichment by a sol-gel-generated immunoaffinity column. J Chromatogr A. 2000 Jun 2;880(1-2):113-20.
15. Suchanek M, Scharnweber T, Fisher M, Knopp D, Niessner R. Monoclonal antibodies specific to polynuclear aromatic hydrocarbons. Folia Biol (Praha). 2001; 47(3):106-7.
16. Scharnweber T, Fisher M, Suchanek M, Knopp D, Niessner R. Monoclonal antibody to polycyclic aromatic hydrocarbons based on a new benzo[a]pyrene immunogen. Fresenius J Anal Chem. 2001 Nov;371(5):578-85.
17. Schedl M, Wilharm G, Achatz S, Kettrup A, Niessner R, Knopp D.Monitoring polycyclic aromatic hydrocarbon metabolites in human urine: extraction and purification with a sol-gel glass immunosorbent. Anal Chem. 2001 Dec 1; 73(23):5669-76.

*Two Ph.D. degrees resulting from work under the contract:*
Tim Scharnweber (Partner D), Dusan Koval (Partner CZ, since July 2000),
Post doctoral Fellow: Eva Fernvik (Partner F).

*Conferences :*

1. Euroconference, Institut Pasteur, Paris, 25-27 January 2001, G. Peltre : Hygiène et Santé,
2. Euroconference, Institut Pasteur, Paris, 27-29 June 2001. G. Peltre : Poumon et Pollution

3. Farkas, N., Gombkötö, Zs., Belágyi, J.: Effects of drugs on plasma membrane of the human erythrocytes. An EPR study. *III Int. Conference on Molecular Recognition,* Pécs, 2000. Abstracts p. 63

4. Kašička V., Prusík Z., Sázelová P., Peltre G.: Analysis of liquid extracts from airborne dust samples by capillary electromigration methods, Electrophoresis Forum '99, Rouen, Francie, 24-26.11.1999, Program and Abstracts, Workshop "Application of electrophoresis in environment and taxonomy, p. L1

## *Posters :*

1. Congrès de la société Française d'électrophorèse, Rouen, Novembre 1999, *Effects of air pollution on pollen allergens*. Lhuissier F, Sénéchal H, Mayer C, Desvaux FX, Ripoll C, Peltre G.

2. Journées Parisiennes de l'Allergie, Paris, 12-15 janvier 2000: *La reconnaissance des allergènes moléculaires du pollen des graminées ne changent pas avec les années.* Pires AP, Correia S, Peltre G, Desvaux FX , Inacio F (A).

3. XIX th congress European Academy of Allergy and Clinical Immunology, Lisbonne, 1-5 juillet 2000: *Detection and 2D gel characterization of two major latex allergens in various latex extracts* Allergy, 2000, 55, sup 63, 127. Chardin H, Chen Z, Raulf-Heimsoth M, Mayer C, Sénéchal H, Desvaux F-X, Peltre G (A).

4. XIX th congress European Academy of Allergy and Clinical Immunology, Lisbonne, 1-5 juillet 2000: *Gaseous air pollution on allergenic pollen: effects of NO and CO exposures.* Allergy, 2000, 55, sup 63, 56. Lhuissier F, Sénéchal H, Mayer C, Desvaux F. X, Ripoll C, Peltre G (A).

5. XX th congress European Academy of Allergy and Clinical Immunology, Berlin, 9-13 mai 2001. *Bronchial hyperresponsiveness, cytokine levels and antibody-production pollen after latex provocation in two different mice-strains.* Allergy, 2001, 56, sup 68, 241. Fernvik E, Chardin H, Sénéchal H, Vargaftig BB, Peltre G (A).

6. XX th congress European Academy of Allergy and Clinical Immunology, Berlin, 9-13 mai 2001, *Effects of air pollution in birch pollen sensitised mice*. Allergy, 2001, 56, sup 68, 10. Fernvik E, Peltre G, Sénéchal H, Vargaftig B B (O).

7. XX th congress European Academy of Allergy and Clinical Immunology, Berlin, 9-13 mai 2001. *Pollen grains and urban air pollution: three years study*. Allergy, 2001, 56, sup 68, 133. Sénéchal H, Mislin-Bocquel C, Desvaux F-X, Verhille A, Peltre G (A et O).

8. 58th congress of the American Academy of Allergy, Asthma, and Immunology, New York, 1-6 mars 2002. *Two dimensional electrophoretic analysis of the specificity of the various antibody isotypes produced by latex-allergic patients*. Chardin H, Mayer C, Sénéchal H, Desvaux F-X, Peltre G (A).

9. XXII th congress European Academy of Allergy and Clinical Immunology, Paris, 8-11 juin 2003. *Two dimensional immunoblot analysis of the carbohydrate involvement in pollen polysensitization.* Chardin H, Mayer C, Sénéchal H, Wal JM, Desvaux F-X, Poncet P, Peltre G (A).

10. XXII th congress European Academy of Allergy and Clinical Immunology, Paris, 8-11 juin 2003. *Two dimensional immunoblot analysis of non water soluble grass pollen allergens.* Sénéchal H, Mayer C, Le Mao J, Desvaux F-X, Wal JM, Clément G, Peltre G (A).

11. Kašička V., Prusík Z., Sázelová P., Peltre G.: Analysis of liquid extracts from airborne dust samples by capillary electromigration methods, Electrophoresis Forum '99, Rouen, France, 24-26.11.1999, Program and Abstracts, p. P30.

12. Kašička V., Prusík Z., Sázelová P., Peltre G.: Analýza extraktů vzdušného prachu kapilárními elektromigračními metodami, Konference Pokroky v chromatografii a elektroforéze 2000, 5-6.9.2000, Pardubice, ČR, Sborník abstraktů, P12, p. 49.

13. Kašička V., Sázelová P., Prusík Z., Peltre G.: Analysis of water and water-buffer extracts from airborne dust samples by capillary electromigration methods, ITP 2000, 12th Int. Symp. on Capillary Electroseparation Methods, Bratislava, 10-13.9.2002, SR, Program and Abstracts Volume, p. A23.

14. Sázelová P., Koval D., Kašička V., Prusík Z., Peltre G.: Analysis of liquid extracts from air-borne dust samples by capillary electroseparation methods, ICES 2001, meeting of the International Council of Electrophoretic Societies, Verona, Italy, June 10-13, 2001, Abstracts Volume, CE22, p. 79.

15. Sázelová P., Kašička V., Koval D., Prusík Z., Peltre G.: Evaluation of the efficiency of extraction of pollen allergens and organic pollutants from airborne dust samples by capillary electromigration methods, 2nd Int. Symp. Separations in the BioSciences, SBS 2001, Prague, CR, 17-20.9.2001, Book of Abstracts, P75, p. 135.

16. Sázelová P., Kašička V., Koval D., Prusík Z., Peltre G.: Analýza vodných extraktů vzdušného prachu kapilární izotachoforézou, Konference Pokroky v chromatografii a elektroforéze 2002 a Chiranal 2002, Olomouc, 24-27.6,2002, Sborník Abstrakt, A. Gavenda a J. Ševčik (Eds.), Alga Press 2002, Olomouc, str. 88.

17. Sázelová P., Koval D., Kašička V., Kilár F., Knopp D., Peltre G.: Analysis of water extracts from the airborne dust samples by capillary isotachophoresis, 13th Int. Symp. on Capillary Electroseparation Techniques, ITP 2002, Helsinki, Finland, 1-4.9.2002, Book of Abstracts, P58, p. 105.

18. 2nd International Conference "Field Screening Europe: Strategies and Techniques for On-Site Investigation and Monitoring of Contaminated Soil, Water, Air and Waste", Karlsruhe 14.-16. May 2001. Oral Contribution: Scharnweber, T., Fisher, M., Suchànek, M., Knopp, D., Niessner, R. "Immunological detection of polycyclic aromatic hydrocarbons (PAHs) in airborne aerosols".

19. 2nd International Conference on Instrumental Methods and Analysis, Modern Trends and Applications, Ioannina, Greece, 5.-8. September, 2001. Oral Contribution: Knopp, D. "Determination of polycyclic aromatic hydrocarbons (PAHs) in water, soil, air and human body fluids".

20. Jahrestagung 2001 der Wasserchemischen Gesellschaft in der GDCh, Bad Wildungen, 21.-23. May 2001. Poster: Scharnweber, T., Knopp, D., Niessner, R., „Charakterisierung eines monoklonalen Antikörpers gegen Benzo(a)pyren und Entwicklung eines Enzym-Immunoassays (ELISA)".

# Conclusions

*4- Benefits arising directly or indirectly from the project:*

The scientific collaboration, which was set up to propose first, then to accomplish our programme was truly needed: no single partner could have done alone the totality of the work achieved in common. Our project put together four complementary Partners mastering their own part of the work without too much overlapping of competencies producing a synergistic collaboration.

Each Partner had his own benefit: those who required the acquisition of dearly needed equipment (CZ, H) could obtain it, Partner D invested his whole budget into the salary of a Ph.D. student responsible of the work and Partner F shared his budget into expensive needed consumables and a post-doctoral fellow salary. Sub-contractor CZ' has contributed to produce monoclonal antibodies and is selling them successfully.

The common benefit is the successful collaboration and its rich production in terms of publications, contributions to meetings and broadening of the scientific interest of each of the Partners.

*5- Recommendations for future actions:*

1- Careful preparation of the project is a fundamental condition to warrant its success: all the Partners already knew each other by attendance to meetings and through the literature. Some of them had bilateral collaborations in the past. We have never worked together before this programme but we knew, by mutual esteem and confidence, that our collaboration will be a success. A first meeting was organized to prepare together the proposal. This constituted a firm commitment of all the partners into the proposed programme and was certainly the best insurance for a successful and enjoyable collaboration.

2- An adequate number of meetings among the 5 partners was important in order to have a good exchange and a good control of the evolution of the work. Some of us have underestimated the cost of these meetings but have managed to meet with other funding.

3- To have a young scientist responsible of the work in the lab, meaning a personnel interest in the fulfillment of it (Ph.D. thesis, post doc work…) was an important incentive for a successful project. These young scientists are a very strong driving force to achieve the project, to publish the results, to bind links between the participating labs and especially with the other young scientists involved in the project. We witnessed the organization of a very fruitful network of exchanges at that level.

*6- This project was a true "success story".*

We consider this Programme really as a success story on many points of view:

- **Scientific achievement**: we believe that the results of our project are very important and open the way to the production of a fully automated air trap needed for the air safety control. No better period than now can be expected to show the urgent need for such equipment: political fanatism and terrorism may be a threat for the air safety of

all major urban areas. Furthermore the actual rapidly spreading of bacterial and viral epidemics or epizootics in the world are one more reason to develop a performing network of air safety control automates.

- **European dimension of the Scientific Community**: the success of our project is mainly based on the true complementarity of the 5 Partners and the excellent synergy that was created at the European level.
- **Human experience**: our collaboration was very friendly and enjoyable. We are ready to continue to work together and some collaborative projects are already prepared among the Partners and submitted for financing. We are frequently in contact by mail or by visits or attendance to common congresses.

Contract number : ICA.-CT Year 2004

**Data Sheet**
**for final report**
to be completed by the co-ordinator for the whole project

| ***1. Dissemination activities*** | Published | Submitted |
|---|---|---|
| Number of communications in conferences | 22 | |
| Number of publications in refereed journals | 36 | |
| Number of articles/Books | 1 | |

| ***2. Training*** | |
|---|---|
| Number of PhDs | 2 |
| Number of MScs | 1 |
| Number of exchanges of scientists (stay longer than 3 months) | 1 |

| ***3. Achieved results*** | |
|---|---|
| Number of new prototypes | 1 |
| Number of products developed | 3 |
| Number of new tests/method developed | 3 |

| ***4.Industrial aspects*** | | | |
|---|---|---|---|
| Industrial contacts | | yes | |
| Financial contribution by industry | | yes | |
| Industrial partners | Large | | no |
| | SME | yes | |

## Completed catalogue page

of the project no. ERB 3512 PL97 3145, contract no. ERB IC15 CT98 0322 of the 4th Framework Program of the European Commission, INCO-Copernicus

# Automated Immunoassay System for continuous Air safety Control (AISAC) and its application to air pollution and aeroallergen monitoring

## Summary (résumé):

The air quality in urban areas is of great importance. Many sources of pollution are threatening the quality of life and the public health.

**Present state of knowledge:** Gaseous pollutants are now well detected and quantified by automated equipments but aerosols of dust or pollen particles are not easily analysed currently. Allergenic pollens are responsible of a great part of the respiratory tract diseases. Their effect is frequently amplified by the air pollution mainly its particulate load. Pollen traps are available throughout Europe but they need a well trained expert to count under the microsope intact captured pollen grains. Their data are available only one or two weeks after the pollen capture. Diesel car exhaust gas particles are the major source of small particles (less than 2.5 μm) in the air of our cities. Their detection and quantification is not yet easy to perform. However their level in the air should be known accurately and rapidly in order first to confirm their direct responsibility on pulmonary functions in conjunction with aero-allergens and second to allow documented regulatory measures of the car traffic in polluted cities if needed.

**Joint research project partners:** Our project involves four truly complementary partners specialized respectively in immunoallergy (F), in chemistry of air pollutants (D), in analytical and preparative electroseparation techniques (CZ), in analytical biochemistry (HU), in collaboration with one french SME, expert in integrated automated immunoassays, and one czech SME (CZ'), as associated contractor, very successful in producing monoclonal antibodies and biological tests.

**Objectives:** Our aim is to contribute to build an automated air trap which will collect the air dust and at regular intervals, once or twice daily, will extract allergens and organic compounds representing mainly the Diesel car soot and quantitate them rapidly by an ELISA type of immunoassay. Such a versatile device should be able to detect any compound that could induce the production of antibodies, as an antigen or as a small haptenic molecule. For example it could also allow, if needed, to detect quickly and automatically any patho-genic agent present in the air, such as bacteria or viruses, provided that specific antibodies can detect them.

**Summary:** Our work program includes:

- the collection of dust samples at different times and locations in Paris,Munich,Prague and Pecs (F,D,CZ,HU)
- the extraction of organic compounds and aero-allergens (F, D, CZ, HU)
- the physico-chemical characterisation and purification of some major organic compounds using HPLC and capillary electrophoretic methods (D, CZ, HU)
- in vitro and in vivo testing of toxicity or adjuvanticity for aero-allergens of these compounds (F, CZ, HU)

- purification of organic compounds by preparative free-flow electrophoresis and HPLC (CZ, D)
- the physico-chemical characterisation and purification of major aero-allergens, mainly from pollen of grasses and trees and also from latex due to their presence in aerosols originating from the wear and tear of truck tyres (F, HU, CZ)
- the preparation of polyclonal and monoclonal antibodies to the molecules that will be selected, analyzed and purified or synthesized (F, D, CZ, HU)
- the integration of these developments into a new air trap to be built in close collaboration with the french SME "SFRI" from Bordeaux, a company specialised in the development and production of automated immunoassays (F).

**Organization and management:** A long lasting and friendly collaboration binds already 3 partners (F, CZ,HU) recently confirmed by bilateral exchange programs (Balaton 96-97, Barrande 97). The Coordinator favours enthousiastically european collaboration. He was or still is involved in FLAIR and TEMPUS programmes, in a European Science Foundation (ESF) network and in an ESF programme.

**Expected results:** Automatic measurements of aero-allergens and organic air pollutants will make possible precise preventive actions and treatments for a better air quality and safety in urban areas.

## Results achieved :

We have collected urban air dust samples and extracted from them the organic compounds and aero-allergens fractions. From one important sample from the city of Prague we have shown that the organic compound fraction was cytotoxic in vitro whereas the aero-allergens fraction alone was not. On an original in vivo mouse model af allergenicity we have shown that the organic compound fraction was stimulating the allergenicity of the aero-allergen fraction. By separating the organic compound fraction in increasingly polar solvants into seven sub-fractions we found that 2 rather minor sub-fractions were particularly stimulating the allergenicity of the aero-allergen fraction. Polyclonal were prepared against the organic compound fraction and monoclonal antibodies to one of its component, Benzo A Pyrene. Monoclonal antibodies were prepared against pollen and latex allergens. ELISA methods were developed and allowed the specific immunodetection of these antigens in urban air dust samples demonstrating the feasibility of the whole AISAC aim.