Gene expression profiling of experimental asthma reveals a possible role of paraoxonase-1 in the disease

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Abstract

In this study, we aimed to identify novel genes involved in experimental and human asthma, importance of which has not yet been recognized. In an ovalbumin-induced murine model of asthma, we applied microarray gene expression analysis at different time points after allergen challenges. Advanced statistical methods were used to relate gene expression changes to cellular processes and to integrate our results into multiple levels of information available in public databases. At 4 h after the first allergen challenge, gene expression pattern reflected mainly an acute, but non-atopic, inflammatory response and strong chemotactic activity. At 24 h after the third allergen challenge, gene set enrichment analysis revealed significant over-representation of gene sets corresponding to T₄₂-type inflammation models. Among the top down-regulated transcripts, an anti-oxidant enzyme, paraoxonase-1 (PON1), was identified. In human asthmatic patients, we found that serum PON1 activity was reduced at exacerbation, but increased parallel with improving asthma symptoms. PON1 gene polymorphisms did not influence the susceptibility to the disease. Our observations suggest that an altered PON1 activity might be involved in the pathogenesis of asthma, and serum PON1 level might be used for following up the effect of therapy.

Introduction

Asthma is a complex chronic lung disease characterized by airway inflammation, hyper-responsiveness, remodeling and obstruction (1). In spite of the intense ongoing asthma research, there is currently an epidemic of allergic disorders including asthma in developed countries (2), and the prevalence of the disease is on the rise. A recent review of the literature revealed >120 genes correlating with asthma in humans in association studies and >150 genes in animal models (3). These results indicate that asthma is a polygenic disease and its complexity originates from interaction of an unknown number of genes and environmental factors. This interaction leads to a dramatic change in the airway microenvironment including the activation of inflammatory pathways and recruitment of immune cells that are not usually present in the airway, and the resulting pulmonary inflammation induces bronchoconstriction, airway hyper-responsiveness (AHR) and airway remodeling.

The advantage of high-throughput microarray technologies offers a new opportunity to gain insight into the global gene expression profiles in asthma, leading to the identification of new asthma-associated genes and pathways. Using animal models of allergic airway disease, researchers applied microarrays to identify potential regulators of asthmatic airway inflammation such as C5 (4), ARG1 (5), ADAM8 (6), SPRR2 (7) and CHIA (8). These studies also indicated that several additional, still unidentified, genes might play an
important role in the disease; all of them are potential therapeutic targets. It must be noted, however, that several studies reveal limitations of the animal models of asthma because there can be significant differences in the pathomechanisms (9); consequently, the results must be interpreted accordingly.

The aim of our current study was to identify novel genes involved in the pathogenesis of experimental asthma by applying microarray gene expression analysis at different time points of the asthmatic process. We also set out to discover gene groups which become highly over-represented in our microarray data and correlated them to characteristic cellular processes during the development of the disease. Furthermore, to be able to integrate our results into multiple levels of information available in public databases, gene set enrichment analyses (GSEAs) were applied. The possible role of a selected gene, paraoxonase-1 (PON1), was also studied in more detail at protein level and in human asthmatics.

Materials and methods

Animals

Six- to 8-week old, female, pathogen-free BALB/c mice were purchased from Charles River Laboratories (Isaszeg, Hungary). Mice were barrier housed in high-efficiency particulate-filtered, air-ventilated, climate-controlled (24°C) animal housing system with 12:12 h light–dark cycle. Animals were late-filtered, air-ventilated, climate-controlled (24°C). Mice were barrier housed in high-efficiency particulate-filtered, air-ventilated, climate-controlled (24°C) animal housing system with 12:12 h light–dark cycle. Animals were kept on ovalbumin (OVA)-free diet (SSniff, Soest, Germany) and had access to sterilized food and tap water ad libitum. The experimental protocol was approved by the Institutional Animal Ethical Committee of Semmelweis University.

Sensitization and airway challenge, bronchoalveolar lavage fluid (BALF) sampling, lung histology and measurement of airway responsiveness were carried out as described previously (10, 11) and are presented in this article’s Supporting Information (Supplementary Data are available at International Immunology Online).

Experimental design

Three groups of mice (six mice per group) were sensitized and challenged with allergen (OVA) and one group (control group) was sensitized and challenged with placebo (PBS) and served as a control. The outline of the experiment is shown in Fig. 1(A). On days 28 and 30, 4 h after the first and third allergen challenge, mice in groups 1 and 2 were anesthetized by an intra-peritoneal injection of ketamine and xylazine, BALF was isolated and the lungs were removed for further analysis. On day 31, 24 h after the third (last) allergen challenge, mice in group 3 and the controls were anesthetized, and AHR was assessed. After the AHR measurement, BALF sampling and lung tissue collection were performed the same way as it was carried out in groups 1 and 2. Cellular composition of BALF and lung histology were examined in all mice in all groups. AHR was measured in controls and group 3 on day 31.

RNA isolation from the lung tissues, quality determination, real-time PCR, immunohistochemical and western blot analyses for PON1 are described this article’s Supporting Information (Supplementary Data are available at International Immunology Online).

Microarray experiments

For microarray experiments analyzing the gene expression patterns in the lungs, Agilent Whole Mouse Genome Oligo Microarray 4 × 44 K chips were used. Details on sample preparation, microarray procedure, data normalization and analysis steps are provided in the Supporting Information (Supplementary Data are available at International Immunology Online).

Human studies

The −108 T/C and Q192R polymorphisms in the PON1 gene were genotyped in 302 children with physician-diagnosed asthma and in 188 healthy control subjects. The selection criteria for the asthmatic and control patients and the genotyping methods are presented in this article’s Supporting Information (Supplementary Data are available at International Immunology Online).

Paraoxonase and arylesterase activities were measured as described in the Online Repository (Supplementary Data are available at International Immunology Online). Enzyme activities were monitored in serum of eight adult asthmatic patients (three males and five females; age: 54.5 ± 14.3 years) at three time points—(1) day 0: the patient was taken to the hospital with serious asthma exacerbation; the blood was taken before the start of intravenous (i.v.) corticosteroid (80 mg methylprednisolone per day) treatment; (2) day 4: switch from i.v. to oral methylprednisolone (16 mg); (3) day 10: the exacerbation symptoms were relieved and the patient was released from the hospital. Informed consent was obtained for each patient and the study was approved by the Institutional Review Board of Semmelweis University. The study was undertaken with the understanding and written consent of each subject and the methodologies conformed to the standards set by the Declaration of Helsinki.

Statistical analysis and bioinformatics

Microarray data normalization, data analysis, Gene Ontology (GO) and GSEAs are presented in the Supporting Information (Supplementary Data are available at International Immunology Online). Unpaired t-test, one-way analysis of variance (ANOVA) and Tukey honestly significant difference (HSD) post hoc test were applied to analyze the statistical significance of our results. Allele frequencies were calculated by allele counting. Data were analyzed using MedCalc, Microsoft Excel or Statistica 7 programs. ‘Hardy–Weinberg’ equilibrium was tested by using $\chi^2$ goodness-of-fit test. Chi-square test was used to test for differences in allele distribution between the groups.

Results

Airway inflammation and BALF cellular composition

The outline of the experiment is shown in Fig. 1(A). Inflammatory influx of eosinophils into the peribronchial and perivascular tissues could be seen in the lung tissue from mice treated with OVA in group 2, and this lung inflammation
became more pronounced in group 3 (Fig. 1B). No marked lung inflammation and eosinophil influx were detectable either in group 1 or in controls.

The lung resistance measurements on day 31 revealed strong AHR to inhaled methacholine in the OVA-challenged mice compared with the placebo-challenged controls (t-test, \( P < 0.05 \), Fig. 1C), demonstrating the development of AHR in mice undergoing OVA sensitization and challenge.

Figure 2 shows the cellular composition of the BALF in each group of mice. In the first experimental group of animals (on day 28, 4 h after the first allergen challenge), a marked increase in BALF neutrophil cell count was observed. This early neutrophil inflammation showed a decreasing trend and was almost completely absent in the third experimental group (on day 31, 24 h after the last allergen challenge). In contrast, no eosinophil cell was found in group 1, but in groups 2 and 3, a marked increase in BALF eosinophil cell counts was observed. Total cell counts were elevated at all time points after allergen challenge with a peak in group 2. There were no neutrophil and eosinophil cells in the BALF of the placebo-challenged control mice. After completing one-way ANOVA, Tukey HSD post hoc test was applied to compare the experimental groups to the control group (\( *P < 0.05 \), **\( P < 0.001 \) (\( n = 6 \) per group)).

**Gene expression analysis**

Microarray analysis of the lung revealed a marked change in the gene expression profile during the asthmatic response. Five hundred and thirty three, 1554 and 1134 transcripts showed >2.0-fold statistically significant differential expression in groups 1, 2 and 3 relative to the control, respectively.

Eight hundred and sixty one transcripts were >2.0-fold differentially regulated between the experimental groups. These results are presented in Tables S3–S6 in the Supporting Information (Supplementary data available at International Immunology Online). Our microarray data have been submitted to the GEO database with the accession number GSE11911.

Self-organizing map clustering of our gene expression data revealed marked separation of group 1 (representing the allergen-evoked early response) from all the subsequent groups. Interestingly, these later time points (groups 2 and 3) showed a highly similar signature to each other (Figure S1, Supporting Information, Supplementary Data are available at International Immunology Online). Furthermore, in accordance with the marked neutrophil infiltration at the early time point after the first allergen challenge, the gene expression pattern of group 1 reflected mainly an acute, non-atopic inflammatory response and strong chemotactic activity. Among them, the expression of pro-inflammatory genes known to be involved in promoting neutrophilic inflammation (\( \text{TNF, CXCL1}, \text{CXCL2}, \text{IL6} \)) and macrophage chemotaxis (\( \text{CCL2}, \text{CCL7} \)) were highly up-regulated. In group 3, representing a developed OVA-induced experimental asthma response, a strong up-regulation of many recently described (e.g. \( \text{ARG1}, \text{CHIA}, \text{TFF2}, \text{and CLCA3} \)) genes was observed.

Next, we validated some microarray results by quantitative real-time PCR. As presented in Fig. 3(A), microarray and real-time PCR resulted in strikingly similar gene expression changes in the case of the up-regulated \( \text{CLCA3} \) and \( \text{CH1A} \) and the top down-regulated \( \text{PON1} \) confirming the reliability of our microarray results.
GO analysis

To be able to extract more biological information from our microarray data, GO analysis was performed on the significantly differentially regulated genes, revealing statistically over-represented gene families (\( P < 0.01 \)) in the respective experimental groups. Selected gene families which were most strongly enriched can be seen on Fig. 4. As expected, GO gene sets related to immune response, inflammatory response, cytokine and chemokine activities were induced in all experimental groups compared with the control. In contrast, gene sets related to acute inflammatory response and serine-type endopeptidase inhibitor activity were exclusively enriched in group 1, while sets corresponding to positive regulation of phagocytosis, humoral immune response, cell cycle and mitosis were highly over-represented in groups 2 and 3.

Gene set enrichment analysis

Traditionally, gene expression analyses focused on statistically differentially expressed genes, which may lead to overlooking many, perhaps important, disease or phenotype-related genes. Therefore, the comprehensive analysis of large number of data derived from microarray experiments often necessitates other alternative approaches, especially in
cases when the number of differentially regulated genes is too high or too low for an interpretable biological conclusion. The GSEA is able to overcome this problem by focusing on gene sets rather than individual genes. It tests the hypothesis whether the members of a given gene set (e.g. *a priori* defined pathways) are enriched in the rank-ordered list generated on the basis of degree of difference between two biological states.

Our GSEA focused on predefined gene sets of pathway databases (see in the Supporting Information, Supplementary Data are available at *International Immunology* Online) and six gene sets known to be associated with several models of lung diseases. These sets reflect the participation of genes involved in the pathophysiology of diseases, including bacterial infection, bleomycin-induced lung disease and T_{H}2 inflammation models. GSEA was used to rank these gene sets in terms of coupling them to the different progression states of the allergen-induced airway inflammation in our model. Therefore, we compared the early response, representing group 1 to the developed OVA-induced phenotype, modeling group 3. We drew conclusions from the top gene sets that had a false discovery rate <5% and *P*-value <0.05, both of which are acceptable cutoffs for the identification of biologically relevant gene sets (Fig. 5). Among these, pathways related to cytokine and IL-R1 signaling...

**Fig. 4.** GO analysis. The $-\log_{10} (P$-value) for individual GO terms signifies the relative importance of the GO term among the entities in the selected gene list compared with the entities in the whole data set. Darker color indicates a higher level of significant over-representation of the ontology set in the group. *P*-values <0.01 were considered to be significant (the method is detailed in the Supporting Information, Supplementary Data are available at *International Immunology Online*).

**Fig. 5.** GSEA. Positive normalized enrichment score (NES) indicates correlation with group 3 (dark yellow-pink), while negative values indicate correlation with group 1 (green). The nominal *P*-value estimates the statistical significance of the enrichment score for a single gene set. Significant false discovery rate (FDR) and nominal *P*-values were <5% and 0.05, respectively (the method is detailed in the Supporting Information, Supplementary Data are available at *International Immunology* Online).
showed the strongest correlation with group 1, while pathway sets related to cell cycle, TCR signaling, T<sub>h</sub> and T cytoxic response correlated with group 3. The gene set representing the gene expression changes related to bacterial infection models characterized generally by neutrophil inflammation and macrophage infiltration to the lung was strongly enriched in group 1. In contrast, the gene sets related to T<sub>h</sub>2-type inflammation models and related to T<sub>h</sub>2 cytokine production, eosinophilic inflammation and airway hyper-reactivity showed strong correlation with group 3. The data generated by GSEA confirmed our model system in large-scale gene expression level, and it was able to subtract clear biological meaning from the microarray results.

Reduced PON1 protein level in the lung of mice with allergic airway inflammation

Interestingly, among the top down-regulated transcripts, we identified PON1 which is a high-density lipoprotein-associated lactonase possessing anti-oxidative properties (12). Homeostasis of the reduction–oxidation (redox) state is critical for the protection against oxidative stress in the lungs. The number of inflammatory cells—particularly eosinophils—is increased in the airways of asthmatic patients, and the pulmonary inflammatory cells release large amounts of harmful reactive oxygen species (ROS) and nitrogen species. As the striking down-regulation of an enzyme being crucially involved in the protection against oxidative stress seems to be important in asthma, next we focused on the PON1 gene.

Immunohistochemistry showed a markedly reduced PON1 protein level in groups 2 and 3 compared with the controls (one-way ANOVA, Tukey HSD post hoc test, \( P < 0.0001 \)), validating our microarray data also at the protein level (Fig. 3B and Figure S2 in the Supporting Information, Supplementary Data are available at International Immunology Online). Interestingly, in lung tissues, PON1 protein was mainly localized in the non-ciliated bronchiolar epithelial cells (Clara cells) as well as in type I pneumocytes. The specificity of the antibody was confirmed by western blotting (Figure S3 in the Supporting Information, Supplementary Data are available at International Immunology Online).

Inverse relationship between serum PON1 activity and symptoms of asthma in humans

PON1 has not only a paraoxonase but also an arylesterase activity. To test whether PON1 activity has a correlation with the severity of asthmatic symptoms in humans, blood sample was taken from eight asthmatic patients at different time points of their medical treatment. Day 0 indicated the arrival of patients at the hospital with serious asthma exacerbation, but before the start of i.v. methylprednisolone treatment. Day 0 indicated the arrival of patients at the hospital with serious asthma exacerbation, but before the start of i.v. methylprednisolone treatment. Day 4 indicated the arrival of patients at the hospital with serious asthma exacerbation, but before the start of i.v. methylprednisolone treatment. Day 10 indicated the arrival of patients at the hospital with serious asthma exacerbation, but before the start of i.v. methylprednisolone treatment.

Compared with day 0, serum paraoxonase and arylesterase activities did not change on day 4 when patients were switched to oral methylprednisolone but increased significantly (main effects ANOVA, Tukey HSD post hoc test, \( P < 10^{-3} \)) on day 10 when the exacerbation symptoms were relieved and they were released from the hospital (Fig. 6).

Distribution of two polymorphisms of the PON1 gene in asthmatics and controls

We compared the distribution of the −108 T/C and Q192R polymorphisms of the PON1 gene in asthmatic and control children. All the genotypes were in Hardy–Weinberg equilibrium. As presented in Table S2 in the Supporting Information (Supplementary Data are available at International Immunology Online), neither the genotype distribution nor the allelic frequencies of the two polymorphisms differed between the two populations. Alletic frequencies for the −108C allele were 47.7 and 49.5%, while for the 192R were 30.2 and 27.4% in asthmatic and control subjects, respectively (\( \chi^2 \) test was used to test for differences in allele distribution between the groups).

Discussion

In this study, we investigated the development of allergic airway inflammation in a mouse model system. We demonstrated that the first airway inhalation of allergen led to a marked neutrophil and macrophage recruitment in the BALF of OVA-sensitized mice. This early neutrophil inflammation decreased during the progression and almost disappeared by the end of the protocol when the asthma-related T<sub>h</sub>2-type eosinophilic airway inflammation and hyper-reactivity developed. These data suggest that neutrophil recruitment may be an early event after allergen challenge. An early and transient infiltration of neutrophils into the airways, which precedes the infiltration of eosinophils, has also been described in other models of allergic airway inflammation (13, 14).
We carried out gene expression microarray profile analysis in the lung of mice undergoing allergen-induced experimental asthma at different time points during the experimental protocol. The study has shown extensive changes in gene expression in the lungs in response to allergen at all time points. Since the classical microarray data analysis generated a very high number of significantly differentially regulated genes, we applied GO and GSEA to be able to gain a comprehensive insight into the biological processes and functions of the asthmatic responses. GO analysis clearly revealed highly significant over-representation of gene set families related to immune response, inflammatory response, cytokine and chemokine activities in all groups compared with the controls. We identified the over-representation of ontology sets related to acute inflammatory response and serine-type endopeptidase inhibitor activity only in group 1, while sets corresponding to positive regulation of phagocytosis, humoral immune response, cell cycle and mitosis were strongly enriched in groups 2 and 3. Taking advantage of an unbiased approach offered by GSEA to rank gene expression pathways, as opposed to individual genes, we showed that pathways involved in cytokine and IL-R1 signaling had the strongest correlation with group 1. In contrast, pathway sets related to cell cycle, TCR signaling, \( T_h \) and T cytotoxic response correlated only with group 3. Besides the canonical pathway gene sets, the tested gene sets derived from other gene expression microarray studies (6, 15) using different lung disease models of mice were found to be coherent with our microarray data. According to the observed marked neutrophil infiltration at the early time point in group 1, we found highly significant enrichment of the gene set representing the gene expression changes related to bacterial infection models characterized by neutrophil inflammation and macrophage infiltration in the lung. This phenomenon has been completely replaced by the asthma-related \( T_h2 \)-type airway inflammation, indicated by the significant over-representation of gene sets related to \( T_h2 \)-type inflammation models in group 3. These results validated our observation about the kinetics of the leukocyte influx into the airways after allergen challenge. Our study differs significantly from previous studies, as we have monitored the development of allergic airway inflammation in a mouse model of asthma by applying GSEA to identify differentially expressed gene pathways and gene sets from other lung disease models as opposed to individual genes. As far as we know, we were the first who applied a comprehensive GSEA to analyze gene expression profile in the kinetics of asthma. Furthermore, GSEA also provided a rapid cross-platform comparison of the microarray data.

We could confirm not only many of the previous observations of differential gene expressions made by others but also identified \( PON1 \) as one of the genes that was strongly down-regulated in experimental asthma. Reduced \( PON1 \) expression in the lungs of groups 2 and 3 mice was also confirmed at the protein level by immunohistochemical analysis. \( PON1 \) is an extensively studied anti-oxidative enzyme, which is able to metabolize pro-inflammatory lipids formed during the oxidation of low-density lipoprotein and is therefore potentially anti-atherogenic (16). It was also shown that \( PON1 \) is able to attenuate diabetes development in mice through its anti-oxidative properties (17). Furthermore, there is increasing evidence that asthma is linked to the generation of oxidative stress. Cell-derived ROS are enzymatically produced by inflammatory (macrophages, eosinophils and neutrophils) and epithelial cells (18) within the lung as part of an inflammatory-immune response to a pathogen or allergen. Generation of ROS close to cell membranes can lead to lipid peroxidation, which can destabilize membrane function, inactivate membrane-bound receptors and enzymes and increase tissue permeability (19), and these events cause tissue dysfunction and destruction (19).

In clinical samples, we demonstrated that serum \( PON1 \) and arylesterase activities increased parallel with improving asthma symptoms. This finding confirms the results of Górnicka et al. (20) who found lower serum \( PON1 \) activity in children with exacerbated bronchial asthma. These data indicate that measurement of \( PON1 \) and/or arylesterase activities might be used to follow the effect of the asthma therapy. It must be noted, however, that owing to the high inter-individual variation in the serum \( PON1 \) and arylesterase activities, a single serum activity measurement in a person cannot have a particular diagnostic value and only the individual changes in the enzyme activity might give useful information.

According to these results, it is reasonable to hypothesize that development of acute asthma coincides with reduced expression of \( PON1 \) in humans. The mechanism of the reduction of serum \( PON1 \) activity in these patients is not clearly understood. This reduction could be related to increased lipid peroxidation caused by ROS produced by inflammatory and epithelial cells, since oxidized lipids are reported to inhibit \( PON1 \) activity (21). In addition, the activity of \( PON1 \) has been reported to be significantly reduced in some conditions accompanying oxidative stress and inflammatory conditions, including rheumatoid arthritis (22, 23), diabetes mellitus (24), cardiovascular disease (25) and lung cancer (26).

It must be noted, however, that the results of the animal experiments, in which the amount of the \( PON1 \) was reduced on the mRNA level, suggested another hypothesis. According to these results, the allergen-induced processes reduced the \( PON1 \) activity not by inhibiting the enzyme but by lowering the expression of the gene. Currently, it is hard to give a coherent explanation of these observations. Additional studies are necessary to elucidate the exact mechanisms how the activity and level of the \( PON1 \) are regulated.

The two most investigated \( PON1 \) polymorphisms are the Q192R, which was found to influence the activity of the enzyme (27), and the \(-108\) T/C promoter polymorphism associated with altered expression of the gene (28). Both polymorphisms have been found associated with several diseases like atherosclerosis (29), diabetes complications (30) and polycystic ovary syndrome (31). As oxidative stress plays an important role also in asthma (32) and polymorphisms influencing the anti-oxidant capacity of \( PON1 \) might change the susceptibility of the disease, we studied the distribution of these polymorphisms in asthmatic and control patients, but found no difference. This means that these polymorphisms do not play an important role in the susceptibility to the disease.

Our observations suggest the hypothesis that reduced \( PON1 \) activity may be involved in the pathogenesis of
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asthma. This is also supported by a study on adults in which apple consumption was negatively associated with asthma and moderate consumption of red wine was associated with less severe asthma attacks (33). These foods are rich in anti-oxidants (flavonoids) and in another study it was shown that consumption of wine flavonoids increased paraoxonase activity (34). This increased PON1 activity might contribute to the protective effect of these foods.

In conclusion, we applied comprehensive analyses including GO and GSEA to our microarray data to determine gene expression profile changes during the development of asthma at different time points. Furthermore, we identified PON1 which showed a correlation with the severity of the asthmatic symptoms and therefore it might be a potential new therapeutic target, as well as a potential diagnostic tool for following up the effect of therapy in asthma.

Supplementary data

Supplementary data are available at International Immunology Online.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AHR</td>
<td>airway hyper-responsiveness</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
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<td>GO</td>
<td>Gene Ontology</td>
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<td>GSEA</td>
<td>gene set enrichment analysis</td>
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<td>HSD</td>
<td>honestly significant difference</td>
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<td>i.v.</td>
<td>intravenous</td>
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<td>OVA</td>
<td>ovalbumin</td>
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<td>PON1</td>
<td>paraoxonase-1</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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References


