Changes in the elastase activity and colonization ability of *Aspergillus fumigatus* after successive inoculations in mice

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Summary

In a previous work we demonstrated a clear link between elastase activity and pathogenicity using what we have named the Elastase Activity Index (EAI). In the present study we have evaluated the possible variability of this index as a consequence of successive inoculations in mice. Two strains of *Aspergillus fumigatus* isolated from the environment without elastase activity were used. These strains were inoculated into successive batches of ten mice. Our results showed that with each inoculation there was an increase in the number of mice on each batch from which the strain could be isolated and an increase in the number of strains with an EAI>1. This study suggests that *A. fumigatus* could adapt to the environment in which it is developed, increasing its pathogenic capabilities from host to host.

Key words *Aspergillus fumigatus,* Adaptation, Elastase, Pathogenicity

Cambios en la actividad elastasa y la capacidad colonizadora de *Aspergillus fumigatus* tras inoculaciones sucesivas en ratones

Resumen

En estudios previos en nuestro laboratorio hemos demostrado la existencia de una clara relación entre la actividad elastasa y la patogenicidad mediante el cálculo de lo que hemos denominado Índice de Actividad Elastasa (IAE). En el presente trabajo hemos evaluado la posibilidad de variación de este índice como consecuencia de inoculaciones sucesivas en ratones. Hemos utilizado dos cepas de *Aspergillus fumigatus* aisladas del ambiente que no presentaban actividad elastasa. Estas cepas se inocularon a grupos de diez ratones en sucesivos lotes. Nuestros resultados muestran que con cada inoculación se producía un incremento en el número de ratones de cada lote de los que se podía aislar la cepa fúngica, así como un incremento del número de aislados con un IAE>1. Esto nos sugiere que se produce una adaptación del hongo al medio en que se desarrolla y un incremento de su patogenicidad en su paso de hospedador a hospedador.

Palabras clave *Aspergillus fumigatus,* Adaptación, Elastasa, Patogenicidad

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Different compounds have been incriminated in the pathogenicity of *Aspergillus fumigatus*, from adherence factors, such as fibrinogen or laminin [3,4] to toxins, such as gliotoxin [14,17] or restrictoxin [1,11]. The presence of elastase activity has been considered particularly relevant because elastin constitutes a significant proportion of total lung proteins [13] and it is an important virulence factor in other lung pathogens such as *Pseudomonas aeruginosa* [8].

In a previous study, the *in vitro* elastase activity of 198 strains of *A. fumigatus* obtained from clinical and environmental samples and the usefulness of this characteristic for predicting invasiveness with clinical isolates were evaluated [2]. A clear link between elastase activity and pathogenicity, using what we have named the Elastase Activity Index (EAI), was demonstrated. This index was calculated after the growth of the evaluated strains in solid medium with elastin, dividing the elastase diameter by the growth diameter. Higher EAIs are related to strains isolated from invasive aspergillosis, whereas if the EAI is < 1, the probability of suffering from invasive disease is very low.

In the present study the possible variability of this index as a consequence of successive inoculations in mice has been investigated, which suggested a possible adaptation of fungal growth in *vivo*.

Two strains of *A. fumigatus* isolated from the environment, which originally could not grow in the solid medium with elastin, and therefore lacked elastase activity were used.

To the inoculation of the mice (male CD-1 weighing between 18 and 20 g), 30 µl of a suspension of 7 x 10^5 conidia/ml was introduced in the nostrils of the animal, keeping the mouse anaesthetised using inhalatory anaesthesia with isoflurane, and in the vertical position. The use of a P-200 pipette and its corresponding tips was utilized for this purpose. Once the animal had inhaled the conidia, 5 µl of a saline solution was introduced into the nostrils with the objective of drawing out some of the spores which could possibly have been left in this area.

To immunosuppress the mice, 0.5 ml of cyclophosphamide at a concentration of 200 mg/ml was injected intraperitoneally three days before inoculation, and 100 mg/ml on the day of the inoculation. Dexamethasone, in a concentration of 2 mg/l was administered in the drinking water during the week before inoculation, and 4 mg/l from the day of the inoculation until the end of the experiment. Each strain was inoculated into a batch of 10 mice which were then euthanised at 14 days, although some died earlier. Mice were euthanised 14 days after the inoculation, by injecting sodium pentobarbital intraperitoneally at doses of 200 mg/kg, followed by cervical dislocation. Small pieces of lung tissues were placed onto Sabouraud Dextrose Agar plates. These plates were incubated at 37 °C until growth was developed. The strains were then cultured to study their elastase activity on the solid medium described by Kothary et al. [10]. Plates were inoculated in a central spot with an *Aspergillus* spore suspension (10^6/ml) and were incubated for 15 days at 37 °C. The diameter of colony and the diameter of the halo of elastin lysis (elastase activity) was measured on day 10 of incubation. We calculated the elastase activity index (EAI) by dividing the elastase diameter by the growth diameter [2].

Two different experiments were carried out:

Experiment A: From each batch, one of the isolated strains with no elastase activity was chosen and then inoculated into 10 mice.

Experiment B: Similarly, in a far location, a strain with EAI>1 was chosen to inoculate a batch of 10 mice.

The process was repeated until four inoculations in each experiment were achieved.

Non-parametric Friedman ANOVA test was used to statistically analyse differences in EAI of the strains isolated from the 10 mice between different inoculations (1st to 4th).

The results of this experiment are shown in tables 1-4, indicating the animals from each batch in whose lungs growth *A. fumigatus* was obtained and the EAI values of these strains.

In both strains and in both experiments (starting from strains with no elastase activity and with an EAI>1)

Table 1. Growth from the lung and EAI values of strain 1 after successive inoculations in mice taking the inoculated strain as a variant with EAI=0.

Inoculation	Animals with growth from lungs	strains with EAI>0	EAI mean	Range	strains with EAI>1
1st	5	2	0.25	0.1-0.4	0
2nd	6	2	0.65	0.6-0.7	0
3rd	8	5	0.7	0.3-1.5	1[a]
4th	8	7	0.75	0.3-1.5	2[b]

[a]EAI value: 1.5 (strain used to the 1st inoculation in Experiment B).
[b]EAI values: 1.5 / 1.1

Table 2. Growth from the lung and EAI values of strain 2 after successive inoculations in mice taking the inoculated strain as a variant with EAI=0.

Inoculation	Animals with growth from lungs	strains with EAI>0	EAI mean	Range	strains with EAI>1
1st	6	4	0.52	0.4-0.7	0
2nd	7	4	0.65	0.3-1.1	1[a]
3rd	7	5	0.74	0.4-1.1	1[b]
4th	8	5	0.8	0.5-1.3	1[c]

[a]EAI value: 1.1 (strain used to the 1st inoculation in Experiment B).
[b]EAI value: 1.1
[c]EAI value: 1.3

Table 3. Growth from the lung and EAI values of strain 1 after successive inoculations in mice taking the inoculated strain as a variant with EAI>1.

Inoculation	Animals with growth from lungs	strains with EAI>0	EAI mean	Range	strains with EAI>1
1st	8	5	0.72	0.1-1.5	2[a]
2nd	9	9	1.05	0.7-1.4	5[b]
3rd	10	10	1.02	0.5-1.8	5[c]
4th	10	10	1.20	0.5-1.9	8[d]

[a]EAI values: 1.5* / 1.2
[b]EAI values: 1.4* / 1.4 / 1.3 / 1.3 / 1.1
[c]EAI values: 1.8* / 1.4 / 1.4 / 1.1 / 1.1
[d]EAI values: 1.9 / 1.7 / 1.4 / 1.2 / 1.2 / 1.1 / 1.1 / 1.1
*Strains used to the following inoculation.

Table 4. Growth from the lung and EAI values of strain 2 after successive inoculations in mice taking the inoculated strain as a variant with EAI>1.

Inoculation	Animals with growth from lungs	strains with EAI>0	EAI mean	Range	strains with EAI>1
1st	7	5	0.84	0.5-1.1	2[a]
2nd	7	7	1.06	0.4-1.4	5[b]
3rd	10	10	1.34	0.4-2.3	9[c]
4th	10	10	1.52	1.1-2	10[d]

[a]EAI values: 1.1* / 1.1
[b]EAI values: 1.4* / 1.4 / 1.1 / 1.1 / 1.1
[c]EAI values: 2.3* / 1.8 / 1.6 / 1.3 / 1.1 / 1.1 / 1.1 / 1.1
[d]EAI values: 2 / 1.9 / 1.8 / 1.8 / 1.6 / 1.4 / 1.4 / 1.1 / 1.1 / 1.1
*Strains used to the following inoculation.

we observed: (i) an increase in the number of mice of each batch from which the strain could be isolated, and (ii) at the same time as the number of strains with an EAI>1 increase. This characteristic was more noticeable in the case of strains with an EAI>1. A statistically significant increase of EAI was detected with each inoculation ($p<0.01$, Friedman ANOVA test).

Some authors have suggested that elastase activity is an important, but not a determining factor in the pathogenicity of *A. fumigatus* [15]. Also, in a study of 38 isolates from patients with invasive aspergillosis in solid medium with elastin, Rhodes et al. [12] observed that all the isolates involved in cases of aspergillosis expressed elastase, but not all those that produced elastase were associated to clinical aspergillosis.

Other authors consider the involvement of elastase activity a factor in pathogenicity. Then, elastase activity is well known in *A. fumigatus* and other species and occurs in at least 36 to 94% of all strains [5-7,10,12]. Some studies showed a correlation between elastase activity and strains of *Aspergillus* isolated from invasive mycoses [6,9]. It was, however, only recently that a link between elastase activity and pathogenicity had been clearly demonstrated [2].

In the present study we have demonstrated that strains without elastase activity are capable of colonizing the lungs of experimentally infected mice. This colonization ability is noticeably increased with successive passes in the mouse. Also, the ability to colonize the lungs enhances the capacity to develop a higher elastase activity. Specifically we have shown how, in four successive inoculations, the colonization ability of a strain with zero elastase activity reached eight of the animals in both strains. When we used a strain with high elastase activity to inoculate, in the 4th inoculation it reached 10 mice colonized in both strains, in every case with elastase activity, presenting in one case 8 new isolates with an EAI greater than 1, and in another 10 (index of maximum pathogenicity).

Tomee et al. [16] believe that the pathogenicity of *A. fumigatus* involves a selection of spores with a natural phenotype able to grow on elastin-containing medium. This *in vitro* adaptation can also be observed *in vivo* as shown in the present study. Moreover, it is well known in other microbes that repeated passes through the same animal species tend to increase the virulence of the tested microorganism. According to the data in this study, this also appears to be the case with *A. fumigatus*.

In conclusion, and in light of the results obtained, it was found that after consecutive inoculations in mice, *A. fumigatus* strains undergo an increase in their elastase activity and their capacity to colonize the lungs of the inoculated mice. This finding suggests that there is an adaptation by the fungus to the medium in which it develops. These results may suggest an increase in pathogenicity from host to host, which might open the door to future studies orientated to elucidate whether this adaptation is due to physiological mechanisms of the fungus, or, on the other hand, to genetic changes (mutation-selection) which enables the fungus to evolve in a short time.

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