cereal is frequently contaminated with aflatoxins (AF) which are severe mutagens that pose a constant threat to the genome of an organism. Aflatoxins are secondary metabolites of the fungi Aspergillus flavus Link [10], A. parasiticus Speare [3]

Introduction

The Mexican population has maize as staple food, and this cereal is frequently contaminated with aflatoxins (AF) which are severe mutagens that pose a constant threat to the genome of an organism. Aflatoxins are secondary metabolites of the fungi Aspergillus flavus Link [10], A. parasiticus Speare [3]
and A. nomius [15] of well known chemical structure [2], biosynthesis [26], production conditions [6, 29] and toxic effects [23] such as carcinogenicity [20, 28], teratogenicity [17] and mutagenicity [17] among many other.

AF activate the proto-oncogene H ras to oncogene producing punctual mutations that cause substitutions G-C to T-A or G-C to A-T [30], most of the mutations are eliminated, but the carcinogen AFB, is accumulated in the DNA during lifetime and sometimes DNA cannot be repaired properly and these AF mutations can initiate a malignant neoplasia or cancer.

Aflatoxins are linked to DNA producing adducts (AFB-DNA) that are good biomarkers, there is a correlation of DNA adduct levels with tumor incidence [24].

Lee et al. [16] explained the molecular basis for the participation of mutation at codon 249 of the p53 gene (p53m249) induced by the potent mutagen AF, in the genesis of hepatocellular carcinoma (HCC).

AFB, induced mutation p53m249 is critical during the formation of HCC following hepatitis B virus infection. P53m249 markedly increases insulin-like growth factor II transcription from promoter 4, accumulating the fetal form of IGF-II. The blocking of apoptosis through enhanced production of IGF-II should provide a favorable opportunity for the selection of transformed hepatocytes.

In the ras gene superfamily, codon 12 (TGGTG-) of the K-ras gene is the most frequently mutated codon in human cancers. AFB1 targeted carcinogen-DNA adduct formation is a major reason for the observed high mutation frequency at codon 12 of the K-ras gene in human cancers [12].

One of the highest incidence rates of HCC is found in China where chronic infection with hepatitis B virus (HBV) and exposure to aflatoxins in foodstuffs are the main risk factors [25]. HCC is the most common type of liver cancer, the major risk factors being hepatitis B and C viruses and AF; other factors such as alcohol are also of importance in some populations. Aflatoxins exposure biomarkers include urinary aflatoxin metabolites and aflatoxin-albumin adducts in peripheral blood [21].

AFB, causes chromosomal aberrations and DNA ruptures in animal and plant cells [22], as well as mutations in bacterial genes when they are activated with rat microsomal fraction [31]. The Ames Test is a reliable model to check mutagenicity of AF. The mutated TA98 strain of *Salmonella typhimurium* lacks the enzymatic machinery to synthesize its own histidine and can not grow in minimal culture medium. The presence of a mutagen allows revertant (mutated) colonies to rise in proportion to the mutagenic strength of the sample tested.

It was interesting to note that caffeic acid and glutathione [14] and the plant *Maytenus ilicifolia* [11] had an effective antimutagenic effect against AFB in the Ames Test.

Commercial treaties between Mexico and United States have developed similar legislations in both countries with a maximum tolerance level of 20 µg/kg of total AF, in maize for human consumption, to be practical and keep these commercial transactions easy. This 20 µg/kg level is based on studies about the amount of AF that produced proliferation of biliary channels in ducts of one day of age, that was 21 µg/kg, so 20 µg/kg was considered “safe”.

The purpose of this work is to know the minimal amount of aflatoxin B, (AFB), necessary to produce a mutation, in order to understand if the AF contamination accepted by governmental tolerance limits in maize represents a health risk, and therefore to determine if the present legislations of Mexico and USA protect the human health.

### Materials and methods

#### Test strains for the Ames Test

*Salmonella typhimurium* TA98 strain [hisD3052, gpt, a (chl, uvrB, his, rfa, pKM101(MucA/A/B Amp)] were donated by Dr. Ames, in filter paper disks in agar. Disks were reactivated in 5 mL of nutritive broth OxoN2 (nutritive broth 1.25 g dissolved in 50 mL of distilled water and sterilized at 121 °C for 15 minutes, and disposed in tubes with 5 mL each added with 5 L of ampicillin trihydrated from Sigma-Aldrich, Cat. A 6140), incubated 16 hours at 37°C in a soft shaker (Lab.Line). The presence of the genetic markers, the frequency of spontaneous reversion and their sensitivity to known mutagens were tested. Extra stock cultures were obtained by adding 0.8 mL of bacterial suspension, incubated 16 hours, in dimethyl sulphoxide (DMSO) from J.T.Baker, frozen quickly with dry ice and stored at 80 °C (Forma Scientific Freezer). Disposable Petri dishes 10 x 15 mm of high transparency polystyrene sterilized with gamma radiation (Vection Dickinson) with Vogel-Bonner minimum media, complemented with an excess of L-histidine (Sigma-Aldrich, Cat.H 6034), were prepared. A sterile solution of 0.1 mM of ampicillin trihydrated (Sigma-Aldrich, Cat. A6140), at a concentration of 8 mg/mL, was added to the media with the bacterial strain TA98.

Bacterial cultures for mutagenicity tests were obtained taking a sample of the bacterial strains stock in Petri dishes, and sowing it in 5 mL of nutritive broth, incubating at 37°C with overnight agitation. For each experiment we used fresh bacterial cultures. All samples were tested in 3 replicated plates.

New bacterial cultures were prepared for each new experiment, they were taken from the stock of bacterial TA98 strain and never from the disposed Petri dishes, because these last could have lost the plasmid uvr Bt marker. All samples were tested in 3 replications.

#### Control treatments for Ames test

The following 4 negative control treatments were included:

a) Without microsomal S9 fraction, without phosphate buffer solution (PBS) nor AFB,

b) With 500 µL of microsomal S9 fraction (from Molecular Toxicology Incorporated Moltox induced with Arocrol 1254), without PBS nor AFB,

c) Without microsomal S9 fraction, with 500 µL PBS, without AFB,

d) Without microsomal S9 fraction, with no PBS, and with either 100 ng or 50 ng AFB. The stock AFB, (Sigma-Aldrich, Cat. A6363) concentration of 1µg/mL was dried at 45°C and reconstituted in 500 µL of DMSO for Ames test.

#### Agar medium preparation

1. **Top-Agar**

   Contained 0.6% agar (Merck Co.) and 0.5 % NaCl (Merck Co.) heated at boiling temperature until it looked translucent, when this mixture was at room temperature, 10 mL of a 0.5 mM solution of histidine-biotin that had 0.077 g of L-histidine and 0.0122 g of biotin (Sigma-Aldrich) in 100 mL of sterile distilled water was added. The histidine-biotin solution was stored at 4°C in a dark glass bottle. Later, all the components were mixed and aliquots of 2.5 mL were done in sterile tubes and stored in the freezer. When the tubes with top agar were needed, they were previously boiled in boiling water.

2. **Agar-plates**

   Petri dishes (Vection Dickinson) contained 30 mL of minimal agar (2% glucose, 1.5% of agar) (Merck Co) in Vogel-Bonner E medium.

   The minimal medium E of Vogel-Bonner included 7.5 g of Bacto Agar (Difco) or purified Agar-Agar (Merck Co.) without inhibitors, diluted in 300 mL of distilled water and sterilized at 121°C during 15 minutes. Ten grams of dry dextrose (Merck Co.) diluted in 100 mL distilled water were sterilized at 121°C for 15 minutes.

   The concentrated Vogel-Bonner solution was done with 10 grams of magnesium sulphate heptahydrated, 100 g of citric acid monohydrated, 500 g of dibasic anhydrous...
pH. The following formula was applied:

\[
\text{Highest absorbance} \times \text{AFB1 molecular weight (at 355-365 nm)} (\text{mw} = 312) = \text{AFB1 concentration} \text{ Extinction coefficient } (\lambda = 21800)
\]

To prepare a AFB1 standard solution (stock) of 1 µg/mL ( = 1000 ng/mL) we divided one between the AFB1 concentration obtained, and the result was subtracted from 1000 (because one mL = 1000 µL) in order to know how many mL we had to use from the standard and from methanol to make the stock dilution.

Ames Test

To establish the mutagenicity assay an aliquote of surface agar, melted at 45°C, was the substrate of 0.1 mL of TA98 strain of Salmonella typhimurium incubated overnight in nutrition broth, the AFB1 dilution to test, and 0.5 mL of S microsomal rat liver concentrate to facilitate the metabolic activation of the treatments. Bacteria can survive some minutes at 45°C, but S. fraction cannot, so it was added when the media was just warm, shaking the mixture and putting it in Petri dishes that already had minimum agar with glucose (Merck Chemical Co.), spreading in a uniform way in less than 20 seconds and letting it to solidify.

Results interpretation

A positive result is defined as an increase in the number of revertant (mutated) colonies that should be at least twice the frequency of spontaneous reversion shown when the colonies of the negative controls are counted. This fact shows an increment of the number of mutants in relation to the concentration of the tested compound. It is recommended to test concentrations in an ample range (2, 20 and 500 µg) per plate in the presence and absence of S, microsomal fraction. A positive result must have a clear dose-response using a narrow range of concentrations that must have a linear response [19].

Results and discussion

Calibration curve

The area results of the HPLC calibration curve (Table 1) of AFB1 standard are presented The coefficient of correlation of AFB1, (r = 0.99210447) shows the direct relation between the standard concentration and the chromatographic area. With the calibration curve and the area presented in the HPLC the concentration and purity of AFB1 standard were determined.

Ames Test

The Ames experiments with a range of concentrations from 0.39 to 100 ng of AFB1, are presented The coefficient of correlation of AFB1, (r = 0.99210447) shows the direct relation between the standard concentration and the chromatographic area. With the calibration curve and the area presented in the HPLC the concentration and purity of AFB1 standard were determined.

Ames Test with S. typhimurium strain TA98 negative control alone, gave a spontaneous reversion average of 20 revertant colonies and 10.0 ng of AFB1, as treatment, gave 68 colonies that were 3 times higher than the controls, and can be taken as a positive result. The number of reverted colonies is directly proportional to the mutagen effect. The results are compared...
Table 1: AFB<sub>1</sub> calibration curve.

<table>
<thead>
<tr>
<th>N° of dilutions</th>
<th>Concentrations of AFB&lt;sub&gt;1&lt;/sub&gt; standard µg/mL</th>
<th>Chromatographic Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25</td>
<td>450059</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>549085</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>4142473</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>9371459</td>
</tr>
</tbody>
</table>

Regression line: \(y = -1415569 + 53798272x\)
Coefficient of Correlation: \(r = 0.992210447\)

Table 2. Higher quantities of AFB<sub>1</sub> tested by Ames test with the strain Salmonella typhimurium TA-98 (100 µL) to know minimal amount to produce a mutation.

<table>
<thead>
<tr>
<th>N° of Dish</th>
<th>S&lt;sub&gt;9&lt;/sub&gt; Arochlor induced (µL)</th>
<th>PBS Control (µL)</th>
<th>AFB&lt;sub&gt;1&lt;/sub&gt; (ng)</th>
<th>Ames test</th>
<th>Number of revertant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Treatments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>S&lt;sub&gt;9&lt;/sub&gt;</td>
<td>Control</td>
<td>1</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>-</td>
<td>Control</td>
<td>2</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>500</td>
<td>Control</td>
<td>3</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>Control</td>
<td>4</td>
<td>500</td>
</tr>
</tbody>
</table>

with the control treatments and the Ames test is considered positive if the number of the reverted (mutated) colonies triplicates the number of the control treatments.

The IARC determines the carcinogenicity of a toxin not its minimal quantity to produce a mutation, this agency reports studies done in different animals and the way the ingested mycotoxin causes cancer in each species. They do not measure the minimal amount to cause a mutation, but the carcinogenic effect itself. There is a distance between causing the carcinogenic effect itself. There is a distance between causing a mutation and the Ames test is considered being produced frequently by different factors, among which aflatoxins are common, until DNA cannot repair itself and begins a malignant process, if promoters and an inheritance factor are present. The advantage of Ames Test is that it can measure mathematically the reverted (mutated) bacterial colonies.

In fact there is a relation between the amount of maize ingested by a person and the risk to develop a liver disease such as chronic hepatitis B and C, and viral cirrhosis, this was concluded from the correlation of the amount of AF contamination in the urine of 210 patients with 40,000 data of different kinds of “risk foods” ingested during the last week, month and year, established in a questionnaire that was applied to the patients [1].

Adducts (AFB<sub>1</sub> link to DNA) are considered good biomarkers of a high risk of disease, they are in direct relation with the malignity of the tumor. This fact is well accepted by the international scientific community, there are studies [7, 8, 9] that testify that adducts are the authentic activated carcinogens that produce punctual mutations, that eventually can initiate the malignant tumor.

AF are present in maize of Mexico, there is a good survey with 12,000 samples and 60,000 analysis in 5 years [13]. Also milk is contaminated with AFM<sub>1</sub> [4] and aflatoxicol [5], so this study becomes relevant because Mexico imports maize and milk and the maximum tolerance level of these foods in the legislation is the only protection for the Mexican population at this point.

If the maximum legal tolerance level of AFB contamination for human foods in U.S.A., Canada and Mexico (NAFTA) is 20 µg/kg, this amount means that the addition of the four most common AF (AFB<sub>1</sub>, AFB<sub>3</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>) should be 20 ng at the most, but in maize that has mainly A. flavus that produces only AFB<sub>1</sub> and AFB<sub>3</sub>, 20 ng would be divided in the 2 AF that are 10 ng for AF<sub>1</sub> and another 10 ng for AFB<sub>3</sub>, that is certainly producing a mutation. If the contaminated food is peanuts, then A. parasiticus is the common fungi producing the 4 mentioned AF (AFB<sub>1</sub>, AFB<sub>3</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>), if AF are present in equivalent amounts, the legal protection is “adequate” because the accepted contamination for AFB<sub>1</sub>, should be 5 ng and legal maximum tolerance level of 20 µg/kg do protect the population, although peanuts and maize are frequently with higher amounts of AF contamination.

Codex Alimentarius has a maximum tolerance limit of 10 µg/kg that is more strict than NAFTA (Canada, U.S.A. and Mexico) 20 µg/kg level, and can “protect” the health of the human beings, but the frequent ingestion of these amounts can trigger the cancer mechanism in susceptible persons.

The problem is that the present experiment represents a single dose per triplicate and the question is what happens with the ingestion of several of these doses that a person ingests in maize, dairy products, wheat, rice, species, raisins, figs, etc. daily during years and that accumulate in DNA of all the cells.

The population is accumulating these carcinogens and no legal limit can prevent this fact. So these legal limits are helpful for commercial transactions only but do not represent a real protection to the health of the human population. We have to remember that we cannot know the AF contamination of all the food that we eat, because the analyzed samples are few and if they are chemically extracted with organic solvents (methanol, acetonitrile, etc.), they cannot be eaten.

Prokaryotic or eukaryotic organisms are exposed to a multitude of DNA damaging agents such as AFB<sub>1</sub>, as a result, organisms have evolved important mechanisms to repair DNA damage and systems (cell cycle checkpoints) that delay the resumption of the cell cycle after DNA damage, to allow more time for these accurate processes to occur. The difficulty to repair DNA damage can let a mutagenic event to occur. Most bacteria, including Escherichia coli, have evolved a coordinated response to these challenges to the integrity of their genomes and this inducible system is named SOS response and it controls both accurate and potentially mutagenic DNA repair functions [27].

The problem is starvation, because most of the cereals and maize [13], dairy products [4, 5], oils, seeds, spices, etc. have AF contamination and if legal standards are very strict, there will be no enough amount of foods for humans. The problem has no easy solution, is either to eat risky food or starve. Only governmental or industrial check-ups made by each food company, of statistically useful number of samples of food for humans and feed for animals, can prevent the ingestion of AF that are very frequent and dangerous contaminants, and can give some security to protect human and animal health. But again, the price of food will increase with the additional cost of these extra analyses, and economically low income people will suffer the consequences.

Conclusions

The minimal quantity of AF, that can produce a mutation was 10 ng which gave 68 colony revertants, 3 times higher than the controls had an average of 20 revertant colonies. Maximum tolerance legal limits applied in Canada, Mexico and United States (NAFTA) are useful for commercial transactions, but do not protect human health against these potent mutagens and carcinogens.

Acknowledgements

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References