In 1981, the Spanish toxic oil syndrome (TOS) affected more than 20,000 people, and over 300 deaths were registered. Assessment of genetic polymorphisms on xenobiotic metabolism would indicate the potential metabolic capacity of the victims at the time of the disaster. Thus, impaired metabolic pathways may have contributed to the clearance of the toxicant(s) leading to a low detoxification or accumulation of toxic metabolites contributing to the disease. We conducted a matched case-control study using 72 cases (54 females, 18 males) registered in the Official Census of Affected Patients maintained by the Spanish government. Controls were nonaffected siblings (n = 72) living in the same household in 1981 and nonaffected nonrelatives (n = 70) living in the neighborhood at that time, with no ties to TOS. Genotype analyses were performed to assess the metabolic capacity of phase I (cytochrome P450 1A1 [CYP1A1], CYP2D6) and phase II (arylamine-N-acetyltransferase 2 [NAT2], GSTM1 [glutathione S-transferase M1] and GSTT1) enzyme polymorphisms. The degree of association of the five metabolic pathways was estimated by calculating their odds ratios (ORs) using conditional logistic regression analysis. In the final model, cases compared with siblings (72 pairs) showed no differences either in CYP2D6 or CYP1A1 polymorphisms, or in conjugation enzyme polymorphisms, whereas cases compared with the unrelated controls (70 pairs) showed an increase in NAT2 defective alleles [OR = 6.96, 95% confidence interval (CI), 1.46–33.20] adjusted by age and sex. Glutathione transferase genetic polymorphisms (GSTM1, GSTT1) showed no association with cases compared with their siblings or unrelated controls. These findings suggest a possible role of impaired acetylation mediating susceptibility in TOS. Key words: CYP1A1, CYP2D6, enzyme genetic polymorphisms, GSTM1, GSTT1, molecular epidemiology, NAT2, Spanish toxic oil syndrome, xenobiotic metabolism. Environ Health Perspect 109:369–375 (2001). [Online 16 March 2001] http://ehpnet1.niehs.nih.gov/docs/2001/109p369-375ladona/abstract.html

Among food-related toxic outbreaks that have occurred in the world, the Spanish toxic oil syndrome (TOS) emerges as a significant disaster because of the degree of severity and the huge population involved (1,2). In May 1981 the TOS appeared in Madrid and northwestern areas of Spain as a unique disease caused by the ingestion of adulterated rapeseed oil denatured with aniline (3–7). More than 20,000 people were affected; of these, over 11,000 required hospitalization and over 300 deaths were registered in the first 2 years (1,8). Although the majority of patients recovered after a long period, 30–40% continue to suffer mild symptoms or severe sequelae (9–12). TOS was characterized as a multisystemic disease with three consecutive phases. In the acute phase (1–2 months), patients presented fever, rash, eosinophilia, pulmonary edema, and myalgia. Many patients (59%) progressed to an intermediate phase with pulmonary hypertension, thromboembolism, persistent myalgia and eosinophilia, skin edema, alopecia, and sicca syndrome. The clinical signs of the chronic phase were principally pulmonary hypertension, scleroderma, peripheral neuropathy, and liver disease. A summary of clinical and epidemiological findings has been compiled in recent reviews (9–11,13,14).

Rapeseed oil, denatured with 2% aniline, was imported for industrial purposes and illegally refined and delivered for human consumption. A strong association of TOS with ingestion of this oil was proven (3–5,15); thus, the syndrome was caused by toxicants in the oil (1,6,7,13,15,16). Despite the analytical efforts seeking toxic substances in these oils, only aniline derivatives such as fatty acid anilides (1,3,17,18) and fatty acid esters of 3-phenylamino-1,2-propanediol (PAP esters) (19–21) have been identified in toxic oil batches. The content of oleanilides and PAP esters in the oil has been strongly associated with the morbidity caused by these oil batches in the corresponding households (16,22,23). In particular, the di-oleyl-PAP ester (OOPAP) is considered the putative toxic substance generated during the refining process (23,24); however, its toxicity mechanism in biological systems has not yet been fully clarified. Extensive experiments in diverse animal species fed with toxic oil or administered aniline derivatives have failed to reproduce the full spectrum of the disease (1,25,26). This may suggest a species-specific toxicity for humans; in this respect, species differences in aniline toxicity have been recognized for decades and attributed to metabolic differences (27–30).

The disease tended to cluster in families, and the exposure factor was shown to be closely related to household life (4–7). Nevertheless, members of the same family seemed to differ in their risk of becoming ill (4,5), which suggested consumption of different amounts of the oil (a dose factor) and/or a susceptibility trait. With respect to the latter, an immunological mechanism was initially suggested as a toxicity target and was extensively investigated (31–33) because the disease resembled an allergic-toxic syndrome in the acute phase and an autoimmune condition in the chronic phase. However, with regard to a dose factor in toxicity, the patients’ detoxification mechanisms have not yet been investigated. The real toxic dose ingested by patients before the oil was officially recalled was unknown. Epidemiological studies on dietary habits in 1981 failed to conclusively establish a correlation between oil consumption and severity of the disease (4,5). However, these studies did not provide analytical data on aniline-derivative content in the household-distributed oil batches; to date, it is known that oleanilide and OOPAP content varied several folds in oil batches (18,22–24). This would suggest that some families might have suffered a poisoning dose due to a high toxicant(s) content in their edible oil batch, whereas other families may have reflected a susceptible trait.

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even with low toxicant content in their oil batch (Figure 1).

TOS, as the result of a toxic chemical ingestion, would invite investigation on the subject’s capacity to biotransform and eliminate the toxic agent(s). Thus, differences in xenobiotic metabolism and inherited genes among exposed subjects may have contributed to the overall clearance and elimination of the toxicant(s), resulting in an accumulation of toxic metabolites, or a low detoxification, contributing to the disease. In this context, polymorphic genes that encode drug-metabolizing enzymes are attractive candidates for unraveling mechanisms of genetic susceptibility in adverse drug reactions or in xenobiotic exposure toxicity (34,35). Phase I enzymes may metabolically activate xenobiotics and procarcinogens, yielding toxic or carcinogenic electrophiles, respectively; phase II enzymes may be implicated in detoxifying such products. In this study we attempt to identify host-metabolism differences (i.e., genetic susceptibility factors) that may have played a role in the pathogenesis of TOS. In other words, our goal was to determine whether the TOS population inherited a particular genetic profile with regard to xenobiotic enzyme metabolism, which would imply impaired or increased metabolic capacity toward chemical exposure.

Methods

Population selection and study variables. The study was designed as a matched case-control study with two different controls—nonaffected siblings and unrelated nonaffected subjects—hereinafter referred to as siblings and friends, respectively. Cases were recruited from five areas where the Associations of TOS-Affected Patients cooperated.

Figure 1. Factors that contribute to TOS differential morbidity observed in households: toxic dose and metabolism.
nomenclature of Taïoli et al. (45) to name the CYP1A1 genotypes: C, wild-type allele; M, the allele with the M sp1 site at the 3′-flanking region (T5315C); D, the allele with M sp1 site plus valine mutation; and A, the allele with the M sp1 site at 3315 nucleotide (T5315C).

Gene deletions causing impairment of GSTM1 (46) and GSTT1 (47) were analyzed by well-described allele-specific PCR methods (47, 48). These methods permitted identification of the homozygous deleted gene, the so-called null allele (GSTM1− or GSTT1−), from the heterozygous and homozygous wild type haplotype, the wt allele (GSTM1+ or GSTT1+). Finally, we assessed the NAT2 genetic polymorphism to identify m1 (T341C), C481T, m2 (G590A), and m3 (G657A) point mutations in the coding region (49). These mutations account for 90–95% of the enzyme’s capacity variability described for NAT2 polymorphism (41, 50, 51).

All chemical reagents, of molecular and analytical grade, were purchased from Sigma Chemical (St. Louis, MO, USA) and Merck (Darmstadt, Germany). We obtained synthetic primers and 2′-deoxynucleosides-5′-triphosphate from Pharmacia Biotech (Upp sala, Sweden), restriction enzymes from Gibco-BRL (Gaithersburg, MD, USA), and Taq polymerase from Perkin Elmer (Norwalk, CT, USA).

**Statistical analysis.** Each genetic variable was tested separately for its distribution in each group (cases, siblings, and friends) and given as allele frequencies.

We defined three metabolic categories on the basis of their functional and nonfunctional derived haplotypes: wt/wt, wt/mutant (wt/m), and m/m. We then designed dummy variables to evaluate the independence of each genetic category. The absence of mutations (wt/wt) was considered the metabolic basal risk, which was compared with the heterozygous (wt/m) or homozygous (m/m) mutations. We used univariate conditional logistic regression to measure the relative risk of cases versus siblings and cases versus friends. Data are shown as odds ratios (OR) with 95% confidence intervals (CIs).

We performed multivariate analysis by applying multivariate conditional logistic regression following a backward method, including all metabolic pathways of phase I and phase II to test their interactions and adjusting by age and sex. Variables were retained if they achieved statistical significance (p ≤ 0.05) or if, in order to control confounders, their absence changed the remaining estimated coefficients by at least 15%. To give adjusted estimators, sex and age were also retained despite their significance. We used the change in the -2 log likelihood to compare different models. Analyses were performed with the Epidemiological Graphics Estimation and Testing software (EGRET, analysis module version 0.26.6, EPIXACT version 0.03 1985–1991; Cytel, Cambridge, MA, USA).

**Results.**

We collected and analyzed samples from 236 subjects (80 cases, 80 siblings, and 76 friends) with their corresponding questionnaires. We excluded 21 subjects from the statistical analyses. Of these, 7 cases were excluded because 2 were not officially registered as cases and 5 did not fulfill inclusion criteria; their corresponding 7 siblings were also excluded. In addition, 1 sibling was excluded because there was no age match (born after the outbreak). In the friends group, 4 were excluded because they had no case for comparison and 2 were excluded because they did not live in same geographical area as their case at the time of the disaster. As a result, we identified 215 subjects as cases, siblings, and friends (73 cases, 72 siblings, and 70 friends, thus yielding 72 and 70 matched-pair series for comparison. Women were overrepresented among cases (75% vs. 54% in siblings and 48.6% in friends). On average, cases were younger than their friends but not younger than their siblings (median: 27 years for cases, 26.5 years for siblings, and 29 years for friends). The clinical symptom profile of the included cases at the time of the outbreak is shown in Table 1.

Descriptive results of phase I metabolic pathways, CYP1A1 and CYP2D6 enzyme genetic polymorphisms are shown in Table 2 and Figure 2. Allele frequencies of CYP1A1 polymorphism in the seventh exon (isoleucine/valine amino acid exchange) and at the 3′-gene-flanking region (Msp1 restriction sites) are listed in Table 2. Cases presented a higher frequency of mutated alleles with M sp1 and valine point mutations (M, A, and D alleles, respectively); however, they did not reach statistical significance compared to controls. M utation at the seventh exon was always linked to the presence of the M sp1 site (T5315C), i.e., the D allele. For two subjects in the friends group, the M sp1 restriction site reported for African Americans (44) was confirmed by sequencing analyses. The functional metabolic categories established included the haplotype combinations of wt (C allele) and the mutant alleles (M or D) (Figure 2A) and reflected a higher proportion of C/D haplotype in the case group compared to friends (OR = 2.8; 95% CI, 0.9–9.0).

CYP2D6 metabolic capacity was assessed by determining point mutations and the genotypic locus, as described in “M methods.” Genotype categories were defined according to established nomenclature (39). XbaI and EcoRI restriction endonucleases permitted detection of deletions and clear differentiation of 44 and 42 kb fragments. No subject
presented homozygous gene deletions (i.e., the 11.5 kb fragment). The Xbal 16+9 fragments were linked to a G1934A mutation and accounted for 3% in the entire sample collection. The Xbal-derived and EcoRI-confirmed 44 kb fragment was also linked to the presence of the splicing defect G1934A, as determined by PCR analyses. In contrast, the duplication fragment i.e., the 42 kb allele was always associated with wt PCR alleles and was clearly differentiated from 44 kb fragments by EcoRI restriction endonuclease. A novel Xbal RFLP of 33+9 kb was found in three subjects as a heterozygous 29 kb haplotype with an EcoRI pattern indistinguishable from the 42 kb allele. Thus, this may suggest that a new Xbal mutation had appeared in a 42 kb allele, giving the 9 kb fragment (52). This 33+9 RFLP fragment was also linked to mutation G1934A and considered as a CYP2D6*4 nonfunctional allele. Prevalence of CYP2D6 defective alleles among cases was higher compared to their relative controls (Figure 2B), which could imply that an impaired metabolism may have operated in some patients.

Genotype distributions of conjugation pathways (glutathione S-transferase and arylamine-N-acetyltransferase major detoxification pathways) are shown in Table 3 and Figure 3. NAT2 point mutations (m1: C341T, m2: A590G, m3: A857G) causing enzyme-impaired function by posttranscriptional mechanisms were distributed in eight functional mechanisms were distributed in eight

| Allele1 | wt | m1 | m2 | m3 | m | GSTM1 | GSTT1 |
|---------|----|----|----|----|---|-------|-------|
| wt      | 0.27 | 0.38 | 0.32 | 0.03 | 0.73 | 0.52 | 0.71 |
| m1      | 0.30 | 0.43 | 0.25 | 0.02 | 0.70 | 0.54 | 0.74 |
| m2      | 0.30 | 0.43 | 0.25 | 0.02 | 0.70 | 0.54 | 0.74 |
| m3      | 0.30 | 0.43 | 0.25 | 0.02 | 0.70 | 0.54 | 0.74 |
| m       | 0.30 | 0.43 | 0.25 | 0.02 | 0.70 | 0.54 | 0.74 |

Table 3. Phase II metabolism: allele frequencies of NAT2 and GST genetic polymorphisms.

Nomenclature is as reported for NAT2 (49), GSTM1 (48), and GSTT1 (47).

**Figure 3.** Phase II metabolism: conjugation pathways of NAT2 and GST genetic polymorphisms. Functional categories of (A) NAT2, (B) GSTM1, and (C) GSTT1 expressed as a percentage in the population.
homozgyous mutant haplotypes would be the first at risk; heterozygous and homozygous wild types consecutively would be involved because accumulative doses would exhaust each subject’s metabolic capacity. Some factors should be highlighted in connection with the TOS problem: a) a large proportion of the population was exposed compared with the number of cases; b) patients presented a great diversity of clinical manifestations of the disease; and c) a different degree of morbidity was observed among members of the same household who shared meals. These aspects suggest that a metabolic factor is the basis of these differences. Genetic, immunologic, or metabolic factors are frequently involved in the pathogenesis of this type of disease (53).

Alternatively, the present results may point to a metabolic characteristic of TOS survivors with regard to TOS deceased patients. Thus, a particular metabolic profile of the survivors of TOS may have acted as a prognostic factor more than as a risk factor. It was impossible to confirm this hypothesis, which would have substantially reinforced our results, because of the absence of frozen tissue samples at the beginning of the study. We attempted to extract DNA from tissue blocks fixed in formalin and were unsuccessful due to the quality of the available samples at the time of the study.

One of the best designs for investigating potential risk factors in an outbreak is a case-control study (54). With regard to the control group, the selection of “the best friends of the case” has been used successfully (55). Moreover, we selected two different control groups, siblings and friends, to test the hypothesis under different scenarios. Both control groups were used only on the basis of the absence of TOS disease, but not as representative of a population (no sampling was performed). Nevertheless, CYP2D6 and NAT2 allele frequencies found in friends were similar to those reported in other studies with larger Spanish populations (56–58).

To avoid confounding factors, we decided to choose matched controls to adjust for exposure to oil consumption and other habits. However, one of the drawbacks of this kind of design is the possibility of overmatching (55,59). In our opinion, this may help to explain the absence of significant NAT2 results in cases/siblings comparison. We assumed that siblings and cases had experienced the same exposure, although slight differences in the amount of oil ingested could have influenced results. In contrast, we were not able to check whether the group of friends actually were nonexposed. Chemical analysis of the oils collected from the epidemic showed that more toxic oil was sold than was consumed by the families with cases (60). Thus, we can assume that unaffected subjects and cases from the same population area had the same probability of being exposed.

It is impossible to recognize any individual biomarker before analyses are performed unless the marker and the case selection methods are associated. Being related to a specific group of victims or being a friend of a case cannot justify a selection bias for this specific metabolic profile. Thus, the pharmacogenetic differences observed among these groups could only be explained by a true risk factor. It was not feasible to sex-match the control group of siblings. Similarly, we also decided not to pair the friends group by sex; in fact, none of the metabolic factors under study are sex-linked. Thus, the presence of defective alleles in NAT2 and female cases in the final logistic regression models cannot be justified by intergroup sex differences. The overrepresentation of females among cases was a feature of the TOS epidemic (1,4,5). One explanation may be a chance exposure related to household life or an unidentified factor, perhaps epigenetic modulation, associated with females.

An interesting point regarding xenobiotic metabolism being a susceptibility factor is that some pathways are tissue-specific markers. It is feasible that the toxic agent(s) in the oil would have followed two possible absorption routes: directly to the lung through the thoracic duct or through the liver (3). Ultimate toxic derivatives in the blood stream may therefore be the result of these metabolic circuits. Moreover, experiments in rabbits and mice known to have NAT polymorphisms, revealed toxification symptoms depending on the administration route (25,61). Anilides and PAP esters identified in oil batches should be considered arylamides and arylamines, respectively. These two types of chemical species differ in their basicity, nucleophilicity, and ionization potential; as a consequence, their chemical reactivity and biotransformation may follow different pathways, resulting in several nucleophiles (62).

In addition, the fatty acid moiety in either anilides or PAP esters confers a lipophilic characteristic for their distribution. These compounds share a chemical characteristic at the aromatic moiety of being oxidized; this is followed by complex conjugations, reductions, and/or hydrolysis (62), plausibly by some of these enzyme pathways reported in the present study. In particular, the contribution of CYPIA1 and NAT2 enzymes to the metabolism of aniline-derived xenobiotics such as acetanilide and phenacetin is well known (63). In this respect, it is worth noting

Table 4. M etabolic study variables among matching pairs of cases/siblings (n = 72) and cases/friends (n = 70).

| Variable | Case/sibling pairs | Case/friend pairs |
|----------|-------------------|------------------|
|          | No. (%)           | No. (%)          | No. (%) |
| Sex      |                   |                  |         |
| Male     | 18 (25)           | 33 (45.8)        | 18 (25.7) |
| Female   | 54 (75)           | 39 (54.1)        | 52 (74.3) |
| CYP1A1   |                   |                  |         |
| (C/D)    | 11 (15.3)         | 7 (9.7)          | 11 (15.7) |
| (C/D + C/M, A) | 20 (27.8)        | 16 (22.2)        | 20 (28.6) |
| CYP2D6   |                   |                  |         |
| (wt/m + m/m) | 34 (47.2)        | 29 (40.3)        | 33 (47.1) |
| NAT2     |                   |                  |         |
| (wt/m + m/m) | 68 (94.4)        | 66 (91.6)        | 66 (94.3) |
| GSTM1    |                   |                  |         |
| GSTT1    |                   |                  |         |

Distribution of functional metabolic categories derived from Figure 2 and Figure 3 were calculated in cases to compare with the corresponding siblings or friends for statistical analyses.

* Nomenclature from Taioli et al. (45).
* Heterozygous and homozgyous nonfunctional haplotypes.
* Homozygous deleted gene.
that the molecular structure of fatty acid anilides is similar to that of acetalanilide and that fatty acid anilides exert a specific inhibitory effect on benzphetamine 3-hydroxylation, a CYP1A1 marker (64). The fact that patients ingested both anilides and PAP esters adds more complexity to their biotransformation and/or mutual interaction.

Although a theory of free radicals that might involve glutathione conjugation was initially postulated (1,3), the present study clearly shows no involvement of glutathione transferase polymorphisms, M enzymatic imbalance or tissue lesions such as those described by a typical aniline-acetanilide intoxication (3) have not been observed in TOS patients. Thus, if aniline had been released from the toxicant (i.e., by aniline/amide hydrolysis), it would have been converted by conjugation or other biotransformations. The potential co-biotransformability of anilides is intriguing. For example, Berking et al. (65) recently reported a lethal wasting disease in A/J mice treated with oleylanilide. The disease observed in A/J mice, a slow acylator strain, paralleled some of the human TOS disease features, whereas their homologous C57BL/6J strain (a fast acylator) did not have symptoms. The immunoreactivity observed in these murine strains shows a profile similar to that described in TOS cases (33). The study of Berking et al. (65) suggests that acetylation may afford protection from reactive metabolites derived from oleylanilide, leading to toxicity. Using the same mouse strains, ongoing studies in our laboratory have shown that PAP oxidized metabolites at the aniloy moiety (66) and oxidized acetalanilide metabolites (67) were present in the animals’ urine after intraperitoneal administration of 14C-labeled PAP or oleylanilide.

Genetic polymorphisms and epidemiological tools such as those used in this study might be useful in the examination of susceptibility factors in other diseases caused by toxicants. As far as TOS is concerned, the consideration of altered phase II metabolic pathways could be an important issue in obtaining an animal model that permits more in-depth analysis of the causal hypothesis. We believe that the present results strongly suggest the presence of a metabolic factor in the presentation of the disease. Further studies should be designed to confirm these findings. One of our greatest future interests is to clarify whether NAT 2 mutations in TOS cases are associated with particular symptoms or sequelae, such as autoimmune disorders, because impaired acetylation has been associated with skin disorders produced after chemical exposure (68–71). Our laboratory is currently investigating metabolic and distribution pathways of TOS-implicated aniline derivatives in an animal model. We hope to determine how other unexpected enzymes may contribute to TOS pathogenesis and how genetic polymorphisms may explain why people living in the same household had different degrees of morbidity.

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