The Spanish Toxic Oil Syndrome 20 Years after Its Onset: A Multidisciplinary Review of Scientific Knowledge

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In 1981, in Spain, the ingestion of an oil fraudulently sold as olive oil caused an outbreak of a previously unrecorded condition, later known as toxic oil syndrome (TOS), clinically characterized by intense incapacitating myalgias, marked peripheral eosinophilia, and pulmonary infiltrates. Of the 20,000 persons affected, approximately 300 died shortly after the onset of the disease and a larger number developed chronic disease. For more than 15 years, a scientific committee supported by the World Health Organization's Regional Office for Europe and by the Institute of Health Carlos III in Madrid has guided investigation intended to identify the causal agent(s), to assess toxicity and mode of action, to establish the pathogenesis of the disease, and to detect late consequences. This report summarizes advances in research on this front. No late mortality excess has been detected. Among survivors, the prevalence of some chronic conditions (e.g., sclerodermia, neurologic changes) is high. Attempts to reproduce the condition in laboratory animals have been unsuccessful, and no condition similar to TOS has been reported in the scientific literature. Laboratory findings suggest an autoimmune mechanism for TOS, such as high levels of seric soluble interleukin-2 receptor. Epidemiologic studies integrated with chemical analyses of case-related oils have shown that the disease is strongly associated with the consumption of oils containing fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol (PAP). These chemicals have also been found in oils synthesized under conditions simulating those hypothesized to have occurred when the toxic oil was produced in 1981. Whether PAP esters are simply markers of toxicity of oils or have the capability to induce the disease remains to be elucidated. *Key words*: autoimmunity, foodborne intoxication, oleyl anilide, 3-phenylamino-1,2-propanediol esters, rapeseed oil, toxic oil syndrome. *Environ Health Perspect* 110:457–464 (2002). [Online 29 March 2002] *http://ehpnet1.niehs.nih.gov/docs/2002/110p457-464gelpi/abstract.html*

Since its identification in 1981, several publications have described the condition currently known as toxic oil syndrome (TOS) and the outbreak of TOS that affected some 20,000 people (and killed over 300 in the first 20 months) in Madrid and in the northwest provinces of Spain. The novelty of the condition (which did not correspond to any known nosologic category), the complexity of its etiology and pathogenesis, and the uncertainties of its evolution led the Spanish government to request the collaboration of the World Health Organization (WHO) Regional Office for Europe, which has now lasted for almost 20 years.

In 1987, the WHO Regional Office for Europe and the Spanish government formalized the collaboration and laid the foundation for a jointly supervised, intensive international research program. In 1996, the Spanish government created a center for investigating the disease at the Institute of Health Carlos III in Madrid (Centro de Investigación para el Síndrome del Aceite Tóxico, or CISAT). The collaboration is being carried out under the guidance of the joint WHO/CISAT Scientific Committee for the Toxic Oil Syndrome. The joint committee had held 29 meetings by mid-2001.

Case definition and identification of TOS patients have not been major problems. Four major and a number of minor criteria for case definition were established early after the outbreak of the condition (*1*), and their application has met with very few difficulties. The same criteria were applied in the retrospective creation in the mid-1980s of an exhaustive registry of affected people [the "census," which is kept at CISAT and was retrospectively and carefully reviewed in the late 1980s; see World Health Organization (*2*)]. The census has been the basis for follow-up studies of the TOS cohort.

In the early phases of the outbreak of TOS, because the clinical picture was similar to that of acute infectious pneumonitis, the possibility of an infectious etiology, such as *Legionella* and/or *Mycoplasma*, was seriously considered. Many patients were treated with erythromycin and/or tetracycline antibiotics, with no benefit. At the same time, rumors or reports surfaced regarding an association of the illness with a particular vector or vehicle, frequently with an unclear notion of the precise nature of the causal agent. Onions, strawberries, asparagus, and chicken were variously implicated by different observers. Reports that the disease was being carried by cats, dogs, and birds led to the slaughter of many pets.

After what most considered the definitive identification of oil as the vehicle of the TOS agent, on 10 June 1981, only a few alternative theories maintained any support. The most prominent and persistent of these was that the epidemic was the result of contamination of tomatoes and perhaps other produce grown in Andalusia with organophosphates, particularly isofenphos and fenamiphos. Although the theory generated much media publicity, it was to be discarded because of the clear-cut differences of the syndrome from classical organophosphate toxity, the lack of any report on the presence of organophosphorus compounds in case-related oils (*2*), and the absence of any other supporting evidence.

In 1987, the epidemiologic evidence was strong that TOS was causally associated with

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the ingestion of a food-grade rapeseed oil containing aniline derivatives and sold for human consumption by itinerant street vendors (*2*). Such evidence satisfied some of Bradford Hill's (*3*) traditional causality criteria: the strength of the association, the temporal sequence, and the evidence for dose–response relationship. The latter had been shown in a study [so-called "toxi-epi" study; see Kilbourne et al. (*4*)] that compared the concentration of fatty acid anilides [particularly oleylanilide (OA) given that oleic acid is the most abundant fatty acid in edible rapeseed oil] between case-related and control oils. Findings of the toxi-epi study (*4*) were subsequently reproduced on a different subset of oils (*5*), thus satisfying Bradford Hill's criterion of consistency of results among independent studies. Posada de la Paz et al. (*6*) also found that rapeseed oils originating from France had been imported to Spain (allegedly for industrial use) after denaturation with 2% aniline, illegally refined to remove aniline, and finally mixed with other edible oils at a clear profit for the people involved in this operation.

Reports on the etiology, epidemiology, clinics, immunology, and pathology of TOS collected up to 1992 have been published by the WHO (*2*). The condition reflects a multisystemic disease of unknown pathogenesis, initiated by a non-necrotizing endothelial injury, with a likely autoimmune component in its inception and/or evolution. Among TOS survivors, chronic conditions affecting the skin, lungs, neurologic system, muscles, liver, and other organs have been reported. The present report reviews estimates of the prevalence of these changes. No excesses of complicating conditions (such as cancer) had been detected during the first decade of the disease (*2*). Rapeseed oils refined with processes thought to mimic those leading to the production of the toxic agent in 1981 had failed to produce relevant toxic effects in laboratory animals (*2*). As substantiated by a systematic literature search (*7*), no animal model for TOS (i.e., either the spontaneous occurrence in laboratory or domestic animals of a complex condition comparable with TOS or its experimental induction by any treatment) can be found in the scientific literature. The toxic agent(s) in the rapeseed toxic oil had not been identified: aniline itself could be ruled out, given that its toxic effects are totally unrelated to changes typical of TOS.

Thus, in the early 1990s, further investigation focused on the natural history of TOS, the composition of case-related oils and their effects on laboratory animals, and the simulation of the refining process (*8*). The present report describes advances along these lines made during the last decade. The scientific strategy that has been developed may be a model for investigating foodborne chemical disasters caused by previously unknown hazards and producing novel pathologic conditions.

Mortality of the TOS Cohort in the Second Decade after the Disaster

Early and late death rates among censused TOS victims have been compared with both the Spanish population and the population living in the 14 provinces hit by the outbreak (*9,10*). Since the late 1980s, intervening deaths are identified at yearly intervals through contact (via mail or telephone) with the patient or the patient's family (*9*). A number of quality controls have provided confidence on the exhaustiveness of the identification of deaths (*10*). Certified causes of death have been collected from other sources (registrars of the town where death occurred, files of the Spanish National Institute of Statistics).

During the first 20 months of the outbreak, 262 excess deaths occurred (75 men and 187 women) among individuals who had been affected by TOS, a figure similar to early estimates (*11*). A higher mortality rate among women, particularly young women, has been recognized since 1981 (*11*), which may reflect a higher consumption of toxic oils and/or a greater sensitivity of women to the toxic compound.

The excess deaths observed through the end of 1982 were followed by a persisting deficit. A total of 832 men and 858 women died between 1983 and 1997: during the triennia 1983–1985, 1986–1988, 1989–1991, 1992–1994, and 1995–1997, standardized mortality ratios for all deaths were, respectively, 0.68, 0.73, 0.64, 0.87, and 0.82 in men and 0.52, 0.78, 0.75, 0.91, and 0.81 in women (*10,12*). Reasons for these low estimates have not been clarified (*10*).

The only sex/age group in which an excess of deaths persisted after 1983 comprised women under 40 years old, in which observed-to-expected ratios were 38/16.4 during 1983–1988 and 24/15.6 during 1989–1994, both statistically significant at the $p < 0.05$ level. The ratio decreased to 7/8.43 during 1995–1997 (*13*). In 31 of 62 women who died before 1994, the persistence over the years of a severe condition typical of the intermediate or early chronic phases of the condition (e.g., cachexia, lung hypertension) could be traced in the clinical records (*13*).

Prevalence of Chronic Conditions in the 1990s

A systematic and consistent pattern of late features of TOS has been identified from three large studies (*14–16*). All provide point prevalence estimates (persons showing signs among persons affected by TOS) several years after the outbreak. Findings are consistent among the three studies: Neuromuscular and skin changes are common, as well as subjective complaints. Prevalence of both contractures and obvious motor neuropathy was around 10%; cramps and paresthesias were reported, respectively, by 70–80% and 60–70% patients, whereas patients complaining from musculoskeletal pain ranged between 30% and 80%. Clear sclerodermia was present in 9–13% patients. Pulmonary hypertension and chronic liver impairment were diagnosed in 1–3% and 1–7% patients, respectively. Other conditions noticed in long-term TOS survivors were hyperlipidemia (total cholesterol > 240 mg/dL) in 44%, obesity (body mass index > 30) in 40%, hypothyroidism in 7%, and diabetes mellitus in 7–9%. The latter finding is of interest because another study estimated a 2-fold risk for diabetes among TOS patients and, in addition, reported vascular pancreatic lesions in some dying patients (*17*).

Although these prevalence estimates are somewhat imprecise because of selection bias (insufficient participation in otherwise well-designed studies and lack of an adequate comparison group), consistency among different studies is remarkable.

TOS patients have frequently reported peripheral neurologic impairment that could not be detected by the traditional neurologic examination. A recent investigation using quantitative neurologic tests has shown a good correlation between subjective complaints and objective findings. In women of all ages and in men more than 55 years old, distal strength has the highest predictive value for the identification of objective findings (*18*).

An overall poor self-assessment of health status has been estimated in studies using tools such as the Health Assessment Questionnaire (*19*) and the Nottingham Health Profile Questionnaire (*20*). Compared with previous studies on samples of the Spanish general population, impairment scores of TOS patients obtained with the latter questionnaire were three times higher (*21*).

In an ongoing activity related to the process held in the Spanish court in 1997, TOS-induced damage over 15 years is being medically and legally evaluated in 17,000 individuals. Those recognized as suffering from "great incapacity" (who require the help of others to carry out daily life activities) and "absolute permanent incapacity" (inability to carry out any type of work) number, respectively, 101 and 209 (*22*). Those recognized by the Spanish National Institute of Social Security to suffer from "total permanent disability" (corresponding

to an inability to carry out common activities) number 3,477, two thirds of whom are women, with a median age of 37 years (*23*).

Searching for the Causal Agent: Characterizing Case-Related Oils

The two toxi-epi studies (*4,5*) could be carried out thanks to the creation of a permanent collection of oils and their containers that consumers returned in exchange for safe olive oil provided by the Spanish government in June 1981. Many oils were returned on the basis of fear and were not related to the occurrence of cases of TOS. Thus, the *oleoteca* (oil repository) is a systematically compiled and organized collection of oils relevant or possibly relevant to further scientific investigation (*24*).

Additional evidence indicated that one particular set of oils was the point source of the epidemic (*24*). It had been produced at the Industria Trianera de Hidrogenación (ITH) refinery in Seville and distributed by a firm in Madrid whose acronym was RAELCA.

During the 1990s, stored case-related oils and other suspected oils have been analyzed with new, more sophisticated methods, particularly with liquid chromatography combined with atmospheric pressure ionization tandem mass spectrometry (*25*). A new group of chemicals associated with the risk of disease were so identified (*26*): the fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol (PAP)—its 1-oleyl-ester (O PAP) and 1,2-di-oleyl ester (OO PAP) (Figure 1). Indeed, their presence in case-related oils was reported as early as 1984 (*27*). In reanalyses of the oils used in the two toxi-epi case–control studies, OO PAP turned out to be a more specific marker of case relatedness than did OA. In both toxi-epi studies, among oils returned from nonaffected families, the proportion of those that were devoid of OO PAP was higher than the proportion of those that were devoid of OA: respectively, 59/64 and 41/64 in the first study and 70/70 and 54/70 in the second study. Therefore, in statistical terms, the association was stronger for OO PAP than for OA (*28*). OO PAP was also found in a sample of oil retrieved from the ITH refinery, but not in an oil derived from aniline-denatured rapeseed oil illegally produced at other refineries nor in unrefined aniline-denatured samples of rapeseed oil sent to ITH (*26*). Something unique must have occurred to a subset of aniline-denatured oils at ITH that both rendered them toxic and led to the appearance of OO PAP; these two consequences are not necessarily interrelated.

Finding Out the Dynamics of the "Accident"

During refining, the degumming and neutralization steps remove phospholipids and free fatty acids, bleaching takes off the color, and odoriferous compounds are eliminated through distillation at high temperature under vacuum, using steam as stripping gas (*27*). Reconstructing what went wrong in one or more of these phases of the process that eventually led to the production of a highly toxic oil in 1981 would be of much benefit for both the identification of the causal agent of TOS and the prevention of other similar "accidents."

In fact, not until 1993 was it possible to trace (with difficulty) and interview workers in ITH and other refineries (Danesa-Bau) known to have been involved in the circuits of illegal oils in Spain in the early 1980s. Oils from the latter contained no PAP esters and less OA than did case-related oils (*26*). This finally led to firmer hypotheses on how the refining stages could have been implemented when the toxic oil was produced.

Such hypotheses were then tested through the experimental processing of ad hoc denatured rapeseed oils, and a number of possibly toxic oils have been produced in the laboratory. These oils have been assayed for toxicity in laboratory animals, with essentially negative results (see below). They have also been chemically analyzed in the laboratory, with more interesting results. It is reasonable to believe that the processes more likely to reproduce what happened at the time of the accident are those leading to the production of oils containing aniline derivatives at concentrations as high or higher than those estimated in the sample of oil originating from ITH oil (OA ≥ 1,900 ppm; OO PAP ≥ 150 ppm).

In 1995, PAP esters were found in two samples of rapeseed oil after, but not before, bringing them to a temperature of 300°C for 4 hr (*26*). Subsequent experiments at the Instituto de la Grasa in Seville (*29,30*) expanded these findings: Heating at 300°C but not at 250°C led to the formation of PAP esters. However, PAP esters formed at 300°C were lost during processing at that temperature. The level of PAP esters maintained during the operation time at 300°C was higher in denatured samples stored for 3

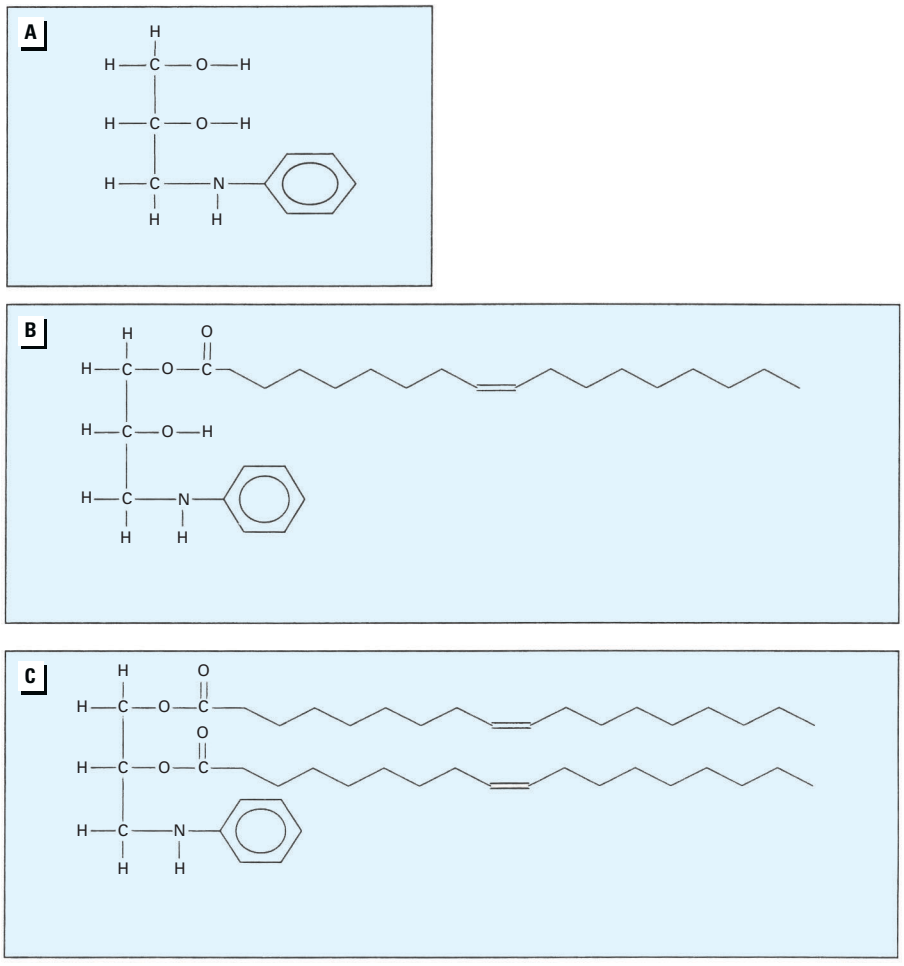

Figure 1. Structural formulas of derivatives of (*A*) PAP and its esters (*B*) O PAP and (*C*) OO PAP.

weeks before refining than in denatured samples stored for only 1 week. Over the years, it had become obvious that anilides form spontaneously from the aniline originally added to denature the oil, when in contact with oil constituent fatty acids, with no role of any step of the refining process; and that these preformed anilides decrease very little with distillation time upon refining (*29*). Thus, in addition to the identification of new, more specific markers of toxicity (PAP esters), new perspectives were opened by experiments using different combinations of temperature, speed of attaining a high temperature, and pressure in the deodorization step (*30*). Transferring findings from lab-scale experiments to the industrial setting leaves a narrow margin for these variables to generate PAP esters. Therefore, the chemical events leading to the formation of toxic compounds must have occurred only under exceptional and accidental circumstances. This may explain why toxicity seems to be linked only to one batch of oil produced in one refinery.

The Pathogenesis of TOS

A basis for the disease. During the 1980s, the vascular and histologic changes typical of TOS clearly showed an inflammatory reaction within areas of fibrosis. Because of the chemical structure of the fatty acid anilides, this could be mediated by an early peroxidative free-radical mechanism involving these compounds (*2*). Although at this stage this hypothesis cannot be discarded, more recent studies have focused on the immunopathologic mechanism of the disease.

In 1990, a comparison was carried out (*31*) on sera collected from TOS patients in the early 1980s and sera retrieved a few years later from healthy blood bank donors in the United States. Levels of eosinophil granule major basic protein (MBP), which is one of the mediators of eosinophil toxicity, were more than two standard deviations above those of the controls in 45%, 32%, and 23% of sera collected from patients in, respectively the acute (n = 159), subacute (n = 100), and chronic phases (n = 64) of TOS. In TOS patients dying in the acute phase, MBP was identified in 6 of 27 lung specimens, which suggests a degranulation of eosinophils in the target organs with subsequent damage (*31*).

Closely related to this finding was the demonstration of interleukin-5 (IL-5) mRNA in paraffin-embedded lung tissue. This interleukin is directly related with eosinophil increase (*32*). An investigation on cytokines (*33*) analyzed 26 lungs from TOS patients who died during 1981–1982 with signs typical of TOS, which were compared with 15 patients with no immunologic diseases who died in 1992–1993, with a similar age and sex distribution. Lung specimens could be traced for specific mRNA for IL-4 and IL-5 from the T_H2 subset of T lymphocytes and for IL-2 and γ-interferon (γ-IFN) from the T_H1 subset. The study also analyzed mRNA for IL-1α, CD25, CD23, and granulocyte macrophage colony–stimulating factor. The study found T_H2 cytokines (IL-4 and IL-5) in a significantly higher proportion of cases than controls (18/26 vs. 3/15, p = 0.003), and also T_H1 cytokines (IL-2 and γ-IFN) in a higher proportion of cases than controls (8/26 vs. 0/15, p = 0.006). The increment of T_H2 versus T_H1 in TOS patients was statistically significant at p = 0.03 (*33*).

Parallel observations, not reaching statistical significance, were the increases in the mean immunoglobulin E (IgE) levels (277 vs. 113 UI/mL) and in the mean values of sCD23 (soluble low-affinity receptor for IgE; 157.1 vs. 50.1 UI/mL) (*34*). The increased expression of IL-5 and IL-4 could explain the early eosinophilia and the elevated IgE levels, respectively. These findings point to a T-cell activation, and this is reinforced by the demonstration of high levels of soluble interleukin-2 receptor (sIL-2R) in the sera of 98 TOS patients in the acute phase compared with 28 controls attending the same hospitals for other conditions (1,810 vs. 892 U/mL; $p < 0.0001$) (*34*).

It has been demonstrated experimentally that one of the accepted markers of oil toxicity, lynolenylanilide, induced apoptosis in human lymphocytes in a concentration- and time-dependent manner. In parallel experiments, neither aniline nor some PAP derivatives produced the same effect (*35*).

In conclusion, the fact that sIL-2R correlates with blood eosinophilia (*36*) strengthens the hypothesis of an immunopathologic mechanism of the disease. The possible cascade of events in TOS patients is as follows: T-cell activation, IL-4 and IL-5 release, eosinophilia, deposition of MBP in tissues, and organ impairment.

The role of genetic susceptibility factors. Previous studies have investigated the role of major histocompatibility complex (MHC). MHC antigens such as DR3, DR4, and DQ8 were more frequent among TOS patients than in a number of control samples of Spanish people (*37*). Nevertheless, the representativeness of the latter is debatable.

Recently, the role of human leukocyte antigen (HLA) class II antigens was investigated in well-designed case–control studies (*38*). One study (*38*) included 117 chronic TOS patients, 71 nonaffected family members, and 77 unrelated controls. In addition, the study compared HLA class II antigens between 34 deceased TOS patients and 13 controls (TOS patients dying from causes unrelated to TOS). The study found no statistically significant association between the disease and any HLA antigen, including those described above: DR3, DR4, and DQ8. However, an increase in the phenotypic frequency of DR2 antigen was found in 73.5% patients dying from TOS. This was significantly different from the proportion of 38.4% among people dying from causes other than TOS. The corresponding proportion was even lower (25–30%) in three groups of people who were not known to be dying from any condition: patients with TOS, nonaffected family members, and unrelated controls ($p < 0.001$ for the three comparisons) (*38*). Arnaiz-Villena et al. (*39*) reported a major DR2 increase in patients who first presented with atypical pneumonia (only in half of the patients with chronic disease). This suggested that DR2 could be a possible *protective* antigen for the evolution to the chronic stage (*39*). The studies of Cárdaba et al. (*38*) did not support the later hypothesis but rather point to the fact that DR2 is an aggravating factor associated with the severity of the disease. Underrepresentation in chronic patients may simply reflect the fact that DR2 patients were prone to die from the disease.

Similar to other conditions caused by toxic chemicals, a role played by the individual's ability to biotransform and eliminate the toxic agent(s) is plausible, regarding susceptibility both to the onset of the disease and to its evolution. A recent study (*40*) found the NAT2 mutation in both alleles (corresponding to the phenotype "slow-acetylators") in 66 of 70 cases versus 59 of 70 unrelated controls living in the same area [crude odds ratio (OR), 3.3; 95% confidence interval (CI), 0.9–12.1; OR estimated under conditional logistic regression, adjusted for a number of potential confounders, 7.0; 95% CI, 1.4–33.2]. Other investigated metabolic genes were not associated to any risk (*40*).

TOS and eosinophilia–myalgia syndrome (EMS). The investigation of TOS was a key factor in the early recognition and study of eosinophilia-myalgia syndrome (EMS) in the United States in 1989. Another new illness affecting some 1,500 patients, EMS was eventually traced to ingestion of L-tryptophan produced by the Showa Denko Company of Japan (*41,42*). The clinical features of EMS (*43,44*) have much in common with TOS, although the clinical onset of EMS was usually less dramatic, and fewer EMS patients exhibited serious pulmonary findings. Like TOS, EMS was more frequent among women, but it was not limited to a single geographic area in the United States. Chemical investigations have postulated similarities between

chemicals ingested by EMS and TOS patients (*45,46*).

Testing Oils in Laboratory Animals

Since the early 1990s, the WHO/CISAT scientific committee implemented the rule that—in addition to suspected chemicals—only oils of proven case relatedness and oils refined in attempts to reproduce the "accident" (heretofore indicated as refined or reconstituted oils) were to be tested for toxicity in projects supported by the committee. Moreover, testing was done in laboratories that were blinded with respect to the origin of the oil.

As yet, six case-related oils, 18 refined oils, and nine defined chemicals (PAP esters and fatty acid anilides) have been tested in short-term experiments, mainly by the oral route, in more than 3,000 laboratory animals (Tables 1 and 2) in 15 different laboratories. The human and financial resources implied in this undertaking can be inferred from these tables. For the most part, these were short-term experiments, carried out either in the acute mode (one single maximum tolerated dose, observation period of 5 days), intermediate mode (7 days at variable doses, observation period of additional 7 days), or subacute mode (longer treatment with up to 14 days of observation). In constructing Tables 1 and 2, we defined an experiment as the simultaneous treatment of a number of animals of the same species and strain, by the same route, with the same material, at one or more dose levels and/or with the same or different schedules.

In principle, in interpreting these data, which essentially result from a screening process, progressive categories of biologic significance of the results of each experiment could be as follows: *a*) no difference from control animals, *b*) some effect, *c*) internal consistency within the experiment (i.e., dose–response); and *d*) consistency with results of other experiments. A detailed presentation of the results is not the purpose of the present review. Nevertheless, despite the dimensions of the screening process, results so far have been meager. Two promising findings, in two separate subacute experiments, are the induction of eosinophilia in Balb/c mice and the induction of an increased IgE in DBA/2 mice with a refined oil containing 1,366 ppm OA and 1,065 ppm OO PAP (*47*). Although these experiments were not designed to detect dose–response effects, if any, this oil induced more eosinophilia and higher IgE levels than did an oil containing only 412 ppm. Similar findings have been reported in both Balb/c and DBA/s strains using the refined oil containing 5,681 ppm OA and 412 ppm OO PAP (*47*). Interestingly, in these investigations, eosinophilia was measured through flow cytometry, whereas manual (less sensitive and less reliable) methods were used in other experiments (*47*).

By the oral route, neither PAP nor its monoester was toxic to Lewis and LAC rats or to MF1 mice. Nevertheless, 14 intraperitoneal daily doses of 150–350 mg PAP per kilogram of body weight induced mesenteric and pulmonary thromboembolism in rats. Intravenous administration showed that the compound was not directly vasotoxic (*48*). None of the other experiments with individual compounds showed any effect.

Metabolic Studies

In general, studies along these lines are difficult to interpret. For the most part they represent a collection of interesting but loosely knit observations, in need of a unifying theory to bring them all together into the explanation of the etiopathogenesis of TOS.

Animal metabolism. Most of the work carried out in this area dates from 1983–1985. A few reports exist in the literature after this period, but these are scarce and do not present clear-cut conclusions. Of the half dozen publications that appeared after the WHO publication in 1992 (*2*), the following observations should be considered.

Studies on rat liver metabolism (*49,50*) show that *N*-phenyllinoleamide produces free aniline and linoleic acid, suggesting a hepatic amidase activity. Lack of this enzymatic activity in humans could be related to their susceptibility to TOS. Other work on toxicity responses in male and female rats examined the effect of anilides and their heat-generated compounds on various physiologic parameters (*51,52*). Several enzyme activities have been found to be decreased at various time points (*53*), but other parameters varied widely, which is difficult to rationalize into a common toxic mechanism. The problem with some of these studies is that metabolic effects have been also observed for non-case-related oils (*54*).

We know little about PAP ester absorption, distribution, and metabolism. In mice administered PAP intraperitoneally, oxidized chemical species derived from aldehyde

Table 1. Summary of experiments carried out in animals treated with oils.

Animal, strain	Oil type[a]	Total no. of experiments[b]	Total no. of animals	Median no. of days	Anilides[c]	OO PAP[d]
Mice						
A/J	Case oils	4	19	5	11.8	662.5
	Reconstituted oils	55	266	5	21.9	42.5
Balb/c	Reconstituted oils	49	257	5	34.2	12.5
C57BL/6	Reconstituted oils	44	217	5	26.8	4.6
CBA	Case oils	1	10	42	323.4	1302.0
DBA/2	Case oils	4	19	5	11.8	662.5
	Reconstituted oils	57	296	5	22.8	42.5
MRL/lpr	Case oils	12	338	7	17.4	346.5
	Reconstituted oils	24	676	7	161.8	1044.0
Subtotal		246	1,802			
Rats						
Brown Norway	Case oils	1	5	90	277.2	1116.0
Lewis	Case oils	3	18	14	13.5	142.3
Sprague-Dawley	Case oils	4	78	7	6.8	134.8
	Reconstituted oils	8	156	7	61.0	1033.5
Subtotal		16	257			
Guinea Pigs						
GOHI	Case oils	4	78	7	6.8	134.8
	Reconstituted oils	8	156	7	66.9	1034.0
NZ	Case oils	2	16	28	32.4	341.6
Not specified	Case oils	1	3	21	168.0	5250.0
Subtotal		15	253			
Chicks						
Not specified	Case oils	1	5	21	22.4	700.0
Dogs						
Beagles	Case oils	5	11	5	13.5	525.0
Minipigs						
Not specified	Reconstituted oils	2	4	7	181.7	7213.5
Monkeys						
Maccaca fascicularis	Case oils	3	18	21	21.0	221.0
Subtotal		11	38			
Total		288	2,350			

[a]Case oils are oils returned from families where some members have been affected by TOS. Reconstituted oils are oils synthesized in the laboratory. [b]An experiment corresponds to a study carried out with a specific sample of oil using a given strain and following a specific schedule. [c]Median of concentration (mg/kg body weight) of oleylanilides ingested by gavage among all experiments with this type of oils and this strain. [d]Median of concentration (µg/kg body weight) of the 1,2-di-oleyl-ester of 3-(*N*-phenylamino)-1,2-propanediol ingested by gavage among all experiments with this type of oils and this strain.

intermediates or from hydroxylation at the aromatic ring have been identified; the detection of some of these species suggests the *in vivo* formation of quinoneimine PAP derivatives (*55*). Closa et al. (*56*) have reported on the capacity of PAP fatty acid esters to be absorbed by the gastrointestinal tract of experimental rats and metabolized in a similar manner as phospholipids. The study showed that the fatty acids present in the PAP molecule can be hydrolyzed and that different fatty acids can then be incorporated by reesterification during the process of gastrointestinal absorption. After absorption from the gastrointestinal tract, the various PAP species are distributed throughout rat tissues by binding to different organs and can further be modified by changes in the fatty acids present in the molecule. Finally, the fraction of these products not taken up by various organs and tissues is catabolized and excreted in urine. Biotransformation in the gastrointestinal tract involves resynthesis of new PAPs with different patterns of constituent fatty acids depending on the vehicle of administration. These are then distributed and stored in different organs, particularly in the liver and in brown adipose tissue. A similar effect was also reported on guinea pig liver microsomes, where changes could be attributable to the free fatty acid contents of ingested oil (*57*). Intestinal hydrolysis of ingested PAP diesters to monoesters and free PAP has also been reported in Wistar rats (*58*). The experiments of Closa et al. (*56*) also showed that some PAP esters inhibit the synthesis of an endogenous systemic mediator, such as platelet-activating factor. This could have a bearing into the widespread systemic effects of TOS.

In vitro *studies.* Several groups have worked in the past on the study of the effects elicited on various types of cell preparations (mouse peritoneal macrophages, human tissue polyps, human polymorphonuclear leukocytes, and human endothelial cells), mostly by fatty acid anilides and later by PAP esters.

Work with *N*-phenyllinoleamide has shown that this anilide potentiates the release of endogenous arachidonic acid from cell membrane phospholipids and enhances the metabolic pathways of the latter, increasing the biosyntheses of its vasoactive endogenous metabolites (*59*). However, Pich et al. (*60*), using fatty acid anilides, described a significantly reduced release of arachidonic acid from cell membranes and impairment of stimulated prostacyclin synthesis accompanied by an antifibrinolytic activity. This apparently contrasting observation was explained by a dual stimulatory and inhibitory effect on the conversion of exogenous arachidonic acid as a function of preincubation time with *N*-phenyllinoleamide alone, because the oleic acid anilide did not show any inhibitory effect with incubation time.

Table 2. Summary of experiments carried out in animals using pure compounds contained in case oils.

Animal, strain	Compound[a]	Total no. of experiments[b]	Total no. of animals	Median no. of days	Anilides[c]	OO PAP[c]
Mice						
A/J	LA	5	50	6	936.0	—
	LL PAP	5	50	6	—	936.0
	OA	5	50	6	936.0	—
	OO PAP	5	50	6	—	936.0
B10.S	LA	1	10	6	1875.0	—
	LL PAP	1	10	14	—	2100.0
	OA	2	22	24	13537.0	—
	OO PAP	1	10	14	—	2100.0
MF1	O PAP	3	18	14	—	5250.0
	PAP	3	18	14	—	3500.0
Swiss CFLP	LA	6	157	5	2500.0	—
Subtotal		37	445			
Rats						
Lewis	O PAP	3	18	14	—	5250.0
	PAP	3	18	14	—	3500.0
Sprague-Dawley	HLLA	1	45	7	1750.0	—
	LLA	1	45	7	1750.0	—
LAC:P	O PAP	3	18	14	—	5250.0
	PAP	8	32	12	—	2800.0
Guinea pigs (Hartley)						
	LA	3	18	30	1500.0	—
	OA	3	18	30	1500.0	—
	OANT	3	18	30	1500.0	—
Subtotal		28	230			
Total		65	675			

[a]Compounds: LA, linoleyl anilide; LLA, linolenyl anilide; LL PAP, diester (dilinoleyl) of phenyl amine propanediol; HLLA, heated linolenyl anilide; OANT, oleyl anilide synthetized by other groups (Purity not checked by the committee). [b]Experiment = study carried out with a specific sample of oil using a strain following a specific schedule. [c]Median of dose (μg/kg body weight) intraperitoneal or intravenous, among all experiments carried out with this material in this strain.

N-phenyllinoleamide also undergoes lipid oxidative metabolism, producing hydroxylated and epoxylated compounds structurally related to the metabolites arising from linoleic acid but bearing the amide moiety. This proves that *N*-phenyllinoleamide can be metabolized via the same hydroperoxidative processes acting upon linoleic acid (*61*). These observations would be in line with the peroxidative hypotheses mediated by fatty acid anilides in TOS. Work on polymorphonuclear leukocytes showed that, as in rat liver, *N*-phenyllinoleamide is metabolized to free aniline and oxidative metabolites of linoleic acid (*62*).

Heiskanen et al. (*63*) have shown that the anilides from palmitic, oleic, and linoleic acids enhance reactive oxygen metabolites in human polymorph nucleated leukocytes, but this effect was also observed for the free oleic and linoleic acids. The expression on leukocyte adhesion molecules by linoleic acid, its anilide, and arachidonic acid was not affected in a whole blood assay (*64*). In addition, these authors also worked with mono- and dioleyl esters of PAP showing that, whereas dioleyl esters of PAP slightly increased the production of reactive oxygen metabolites in polymorph nucleated leukocytes, PAP and its oleyl mono ester had no effect (*65*).

Although extensive in their scope, most of these studies do not provide a clear picture of the mechanism of TOS pathogenesis, other than supporting in general the lipid peroxidative hypothesis initially put forward by various authors.

Discussion and Strategies for Future Research

During the 1990s, substantial progress has been made regarding both the identification of the causal agent and the interpretation of the biologic mechanisms of TOS. Admittedly, 20 years after the outbreak, neither of these goals has been achieved, but in the history of studies on the mechanisms of environmentally induced disease, a delay of decades is not surprising (a century and a half elapsed between Percival Pott's description of scrotal cancer in chimney sweepers and Kennaway's identification of carcinogenic polycyclic hydrocarbons in soot).

- We have yet to determine the long-term consequences of the TOS. It is reassuring that no late excess of mortality in the cohort has been detected. Up to the early 1990s (but not later), TOS victims died as a consequence of the progression of the disease, through a clinical continuum starting in 1981.
- Fatty acid esters of PAP have been found to a higher extent in oils consumed in 1981 by families in which TOS cases had been recorded than in control oils. They

have also been detected in oils synthesized through industrial processes intended to experimentally reproduce those implemented at the time of the "accident."

- PAP esters are an important group of chemicals to which humans may be exposed. Their toxicity has been investigated to a limited extent, and current efforts address their metabolism in laboratory animals and *in vitro*. Their postulated ability to interfere with immunologic changes is of interest.
- No etiologic theory alternative to the adulterated oil (such as an infection or a role for organophosphorus compounds) is based on scientific findings.
- Substantial evidence has been added to the suggestion that TOS is the expression of an immune disorder, most likely an autoimmune condition. The autoimmune hypothesis is strengthened by a number of observations: a postulated cascade of events in TOS patients could be T-cell activation, eosinophilia, release of major basic proteins in tissues, and organ impairment.
- Understanding the biologic mechanisms of TOS has been hindered by the unavailability of an animal model—that is, the "natural" occurrence of a similar condition in animals. However, other conditions (such as allergy) also lack a proper animal model. Research on TOS has also been hindered by the failure of all attempts to reproduce TOS in experimental animals either with oils believed to be case related or with oils synthesized in the laboratory and containing high concentrations of aniline derivatives. A possibility currently being pursued is the use of SCID mice to which human peripheral blood mononuclear cells have been administered.
- Knowledge of the metabolism of chemicals implicated in TOS is diverse, and its significance is difficult to evaluate.

Despite progress, many points still need to be clarified whose relevance surpasses that of TOS itself. The implication of an unknown toxic agent and the similarities of the disease with other autoimmune pathologies make TOS research an outstanding scientific challenge. Priority areas for investigation include immunologic and metabolic factors and the study of the distribution and toxicokinetics of candidate toxicants in animal models. At the same time, developing an animal model should be one of the most important efforts, considering the difficulty in reproducing an autoimmune disease in animals. Finally, another basic objective in the clinical research on TOS is the mortality follow-up and the appearance of new outcomes in those who remain alive.

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