fied by a simple alternative pathway using methylamine-treated plasma.

The homogeneous form of the isolated preparation of pig C3 is documented in figure 3. In SDS-PAGE in 8.5% the pig C3 has a molecular weight of 185,000 ± 18,000 under non-reductive conditions; under reductive conditions, the C3 consists of 2 non-identical polypeptide chains with M_r 112,000 ± 10,000 and 74,000 ± 7,000. Using analytical ultracentrifugation, we have obtained the molecular weight of native pig C3 as 191,000 ± 6,000. The average yield from this method was about 12–17 mg/100 ml of fresh serum. The isolation and characterization of C3 followed the preparation of monospecific rabbit IgG. The antibody obtained did not cross-react with the human, guinea pig, mouse or rat C3 and vice versa. Using this antibody, we tried to detect the C3b receptor on the surface of the macrophages. We found that the C3b receptor is present on the plasma membrane of tested macrophages (fig. 4). The percentage of C3b receptor-bearing macrophages in the peritoneal cavity of stimulated mice is 84.2%. The C3b receptors on the macrophage plasma membrane are spread in the form of clusters, which is documented by figure 4 and also by our electronmicroscopical observation[20]. The distribution of the C3b receptor seems to be independent of temperature, because it has not changed during a 90 min incubation of cell suspension at 37 °C.

Discussion. The pig C3 was isolated in an immunochemically pure, homogeneous and native form and was characterized. Our data agree with the findings of other authors[11,7] and show a high level of homogeneity of the third component of the complement during its evolution. The described method of isolation provided a high yield of pure C3 from the available porcine serum. In spite of the similarity of the C3 structure from various sources, a monospecific IgG was prepared. An interesting finding was that the murine macrophages are able to bind pig C3b, probably due to the high degree of relationship among the C3 components from various species. C3b fragment, previously prepared by limited trypsin cleavage, as described by Tack et al.[14], was also used for studies of C3b receptor. In both cases virtually identical counts of C3b receptor-positive cells were noted and therefore the C3 component only was used in further experiments. The process of the cleavage of C3 to C3b by endogenous macrophage proteases and subsequent binding of C3b on their receptors was blocked by the protease inhibitors PMSF and ε-amino caproic acid. As expected, the native C3 is incapable of binding to the C3b receptors, as has been recently proved by other authors in lymphocytes too[21]. The temperature-independent low lateral redistribution of these receptors seems very likely to have been caused by low mobility of the membrane components of the stimulated macrophages[22]. However, the low rate of C3b receptor redistribution is in contrast to the results of Michl et al.[23] and Griffin and Mullinax[24], who found a normal lateral mobility in stimulated macrophages.

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Immunogenicity of fatty acid anilides in rabbits and the pathogenesis of the Spanish toxic oil syndrome[1]

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Summary. Fatty acid anilides, the major xenobiotic found in the cooking oils responsible for the Spanish toxic oil syndrome, are immunogenic for rabbits as ascertained by a skin test reaction, the characterization of specific antibodies against anilides and the immunofluorescent detection of 'anilide dependent antigens' in tissue slices from treated animals.
Key words. Spanish toxic oil syndrome; oleylanilide; linoleylanilide; hypersensitivity; immunofluorescence; immunopathogenesis.

The Spanish toxic oil syndrome (TOS) resulted from the large scale manufacture and distribution of cooking oil mixtures containing the refining product of imported rape-seed oil denaturated with 2% aniline[5]. Although the identity of the toxic or toxic components in these oil mixtures is yet to be definitely established, fatty acid anilides are likely candidates in view of their high concentration (up to 0.2%) in most samples of suspicious oil[6]. A study of the potential immunogenic properties of fatty acid anilides was undertaken after the consideration of the early clinical features of TOS[7], such as fever, eosinophilia, high IgE titres, skin rash, myalgia, arthralgia, dyspnoea and enlargement of lymph nodes, which were reminiscent of known allergic drug reactions[8]. The rationale for this study was also based on the structural relationships between fatty acid ani-

lides and pentadecyl catechol, the agent of the delayed hypersensitivity to poison ivy[9]. Later features of TOS, such as a disabling neuromyopathy and scleroderma-like lesions have been related to known autoimmune processes[10] that can be induced by drugs[8]. In this communication we show the immunogenicity of fatty acid anilides in rabbits, as ascertained by a skin test reaction, the characterization of specific antibodies against anilides by immunoadsorption and solid phase radioimmunoassay of sera from rabbits treated with anilides, and the immunofluorescent detection of 'anilide specific antigens' in tissue slices from these animals. These findings, together with the neural and muscle lesions induced by the anilide treatment in rabbits[11], led us to suggest the possible implication of immunopathomechanisms related to fatty acid anilides in TOS.

Materials and methods. Oleic and linoleic acid anilides were provided by E. Fernández and M. Studt, from the Centro Nacional de Química Orgánica, C.S.I.C. (Madrid), and their purity ascertained by IR and NMR spectra[12] and liquid chromatography[13]. Radiolabeled anilides (24 mCi/mmol) were prepared with ^{3}H aniline from Amersham. Fatty acid anilides were incorporated into phosphatidylcholine liposomes (0.027 mg of anilides, 14 mg of phosphatidylcholine in 1 ml of 30 mM KCl). Chloroform evaporation and sonication of the phospholipid/anilide suspension were carried out under nitrogen in order to avoid oxidations. A homogeneous preparation of liposomes was obtained by exclusion chromatography on Sepharose 4B previously equilibrated with phosphatidylcholine.

21 New Zealand rabbits, weighing 3–5 kg, were treated with liposomes containing anilides (oleyl, linoleyl or their 80:20 mixture) either i.p. (1 ml of the liposome suspension weekly for 2 weeks, which represents a total dose of 0.055 mg of fatty acid anilides) or by the oral route (1 ml daily, 5 days a week for 2 weeks, amounting to a total dose of 270 µg of anilides). The immunized animals were challenged intradermically with liposomes containing anilides (20 µg) and with samples (20 µl) of suspicious oils thought to contain anilides. 7 animals used as controls received the same treatment but the liposomes did not contain anilides. Blood was collected from the ear vein in order to prepare the sera for antibody characterization.

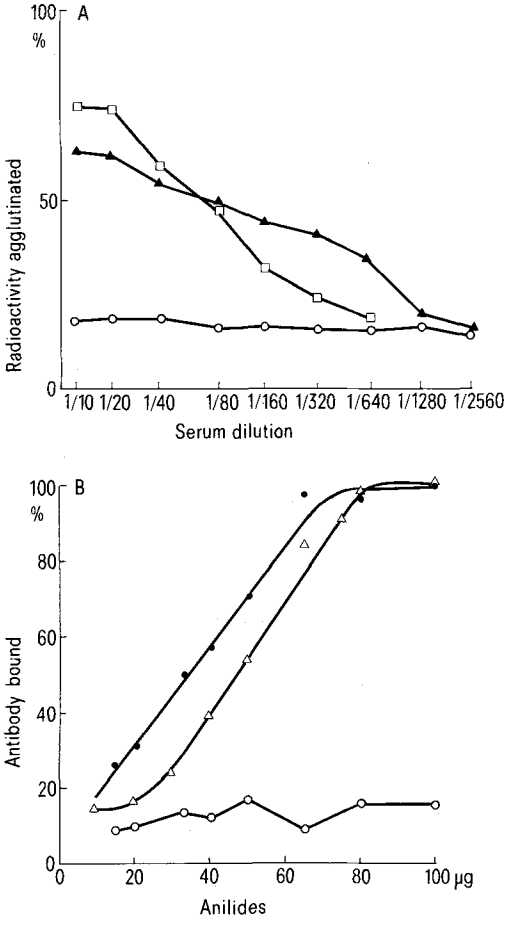

Figure 1. Anilide-specific antibodies in rabbit sera. *A* Agglutination of (^{3}H-anilino) radiolabeled anilides in liposomes by sera from rabbits treated with fatty acid anilides (aleyl:linoleyl, 80:20) either p.o. (▲) or i.p. (□) as described in 'Methods', and by serum from a control animal (○). *B* Solid phase radioimmunoassay of sera from anilide-treated rabbits assayed with increasing concentrations of oleyl (●) or 80:20 oleyl:linoleyl (△) anilides as described in 'Methods' and of a control serum assayed with oleyl anilide coated wells (○).

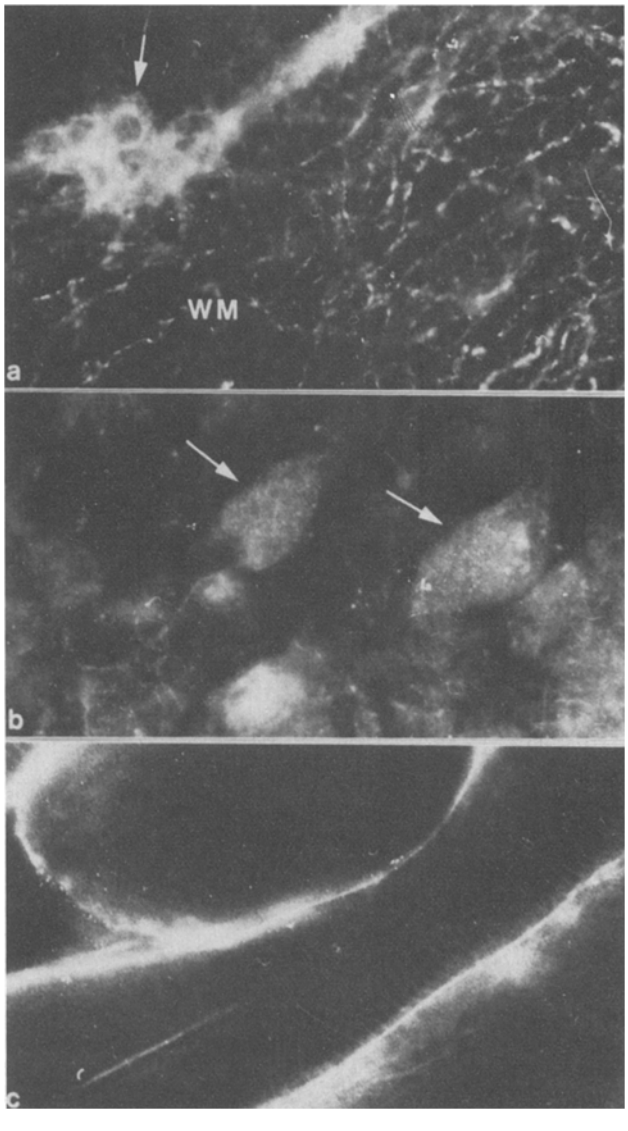

Figure 2. Immunofluorescent detection of anilide-dependent antigens in rabbit tissues. The preparation of tissue slices and incubations with immune serum and fluorescein conjugated antiserum are described in 'Methods'. *a* Cerebellar white matter. The arrow indicate a fluorescent round cell infiltrate. *b* Fluorescent Purkinje cells are indicated by the arrows. *c* Striated muscle sections showing fluorescent deposits in the perimissium. Microphotographs were taken with a Zeiss epifluorescence miscroscope using Tri-X panfilm (Kodak).

For the agglutination reaction, the standard reaction mixture contained 0.07 ml of Tris HCl pH 7.5, 0.03 ml of serum and 0.1 ml of the liposomes containing (^{3}H-anilino)labeled anilides (25,000 cpm). The mixture was incubated for 4 h at room temperature with occasional shaking followed by 18 h incubation at 4 °C. The precipitates were recovered by centrifugation at 100 × g for 1 min and the radioactivity was measured in the supernatant.

Anilide-specific antibodies were purified by a modification of the agglutination reaction. In short, liposomes containing anilides were incubated with immune sera for 4 h at room temperature and subsequently overnight at 4 °C. The liposomes with the adsorbed antibodies were sedimented by centrifugation as indicated above, washed and resuspended in phosphate-saline buffer. The liposomes were dissolved with n-butanol and the antibodies were recovered in the aqueous phase (C. Gitler, personal communication).

Solid phase radioimmunoassay was performed in polyvinyl chloride microtiter plates coated with increasing amounts of fatty acid anilides following the technique of Smolarsky[14]. A constant amount of the serum was added to the wells and the incubation was for 4 h at room temperature. After exhaustive washing with saline-phosphate, ^{125}I labeled goat anti rabbit serum (GAR/Ig) was added to each well (200,000 cpm per well) and the incubation of the mixture was continued overnight. The supernatant was removed and the wells were washed 5 times with saline-phosphate buffer and the plate was dried under vacuum. The wells were cut and mounted in a Beckman 5500 gamma counter. Controls were carried out with the same procedure but without anilides, as well as with preimmune sera in anilide-coated wells. All the assays were performed in duplicate.

For the immunofluorescent studies, tissues from treated and control animals, killed by exsanguination under anesthesia, were immediately frozen in liquid nitrogen and sliced with a cryostat in 4–5-µm-thick slices. Samples were briefly fixed in absolute ethanol and were subsequently washed twice with goat control serum in order to reduce unspecific antibody binding. A 2nd incubation with serum from control or anilide-treated rabbits was followed by incubation with FITC goat antirabbit serum (1/50 dilution). All the incubations were for 1 h at room temperature and were followed by 3 washes with saline-phosphate buffer. Microphotographs were taken with a Zeiss epifluorescence microscope using Tri-X panfilm from Kodak.

Sensitizing effect of fatty acid anilides in rabbits

Treatment[a]	Challenge[b]	Skin reaction[c]
Anilide liposomes i.p. (3)	Anilide liposomes	+++
Anilide liposomes i.p. (3)	Liposomes alone	–
Anilide liposomes p.o. (4)	Anilide liposomes	+++
Anilide liposomes p.o. (4)	Liposomes alone	–
Liposomes alone i.p. (1) or p.o. (1)	Anilide liposomes	–
Anilide liposomes p.o. (2)	Anilide containing oils (6)	++
Anilide liposomes p.o. (2)	Oils without anilides (4)	±

[a] Rabbits were immunized with anilide-containing liposomes or liposomes lacking anilides as described in 'Materials and methods'. Figures in brackets represent the number of animals for each set of experiments.
[b] Intradermal challenge after 2 weeks of treatment. Each animal received in parallel a dose of liposomes containing anilide and a control shot with liposomes lacking anilides. Suspicious oils containing anilides (6 different batches) and control oils or suspicious oils lacking anilides (4 samples) were administered in a dose of 20 µl.
[c] Skin reactions were as follows: +++ represent lesions at least 2 cm in diameter, which evolved in 24 h to papula and necrosis. ++ represents those lesions which do not go beyond the papula stage. +, small erythema, less than 0.5 cm in diameter.

Results. The putative sensitizing effect of anilides was examined by intradermal challenging with 0.8 ml of anilide-containing liposomes. As shown in the table, those animals treated with anilides developed an Arthus-like reaction, with strong hyperemia starting 5–10 h after the injection, followed by a papula and with the histological appearance of an eosinophilic infiltrate. No differences were observed between rabbits treated p.o. or i.p. Negative results were obtained in control animals challenged with anilide-containing liposomes as well as in anilide-treated animals when challenged with liposomes lacking anilides. It is worth noting that the skin tests with suspicious oil samples only gave a positive reaction with those samples containing fatty acid anilides. This was an indication that anilides in oils consumed by humans behave like the synthetic ones, incorporated into liposomes.

The results of the skin test strongly suggest that anilides behave as lipid haptens able to elicit an immunological response. In order to ascertain the presence of specific antibodies, we first examined the ability of rabbit sera to agglutinate liposomes containing radiolabeled (^{3}H-anilino) anilides. In this approach we observed that the sera from anilide-treated animals were able to precipitate 60–80% of the liposomes, with titres ranging between 1/80 and 1/320 (see fig. 1A). Similar assays carried out with sera from control animals failed to agglutinate any liposomes above a 20% background of unspecific precipitation. Anilide-specific antibodies were also detected by solid phase radioimmunoassay (fig. 1B) in which variable amounts of fatty acid anilides were assayed with a fixed dose of sera from control an anilide-treated animals. Maximum antibody binding was usually obtained with 50–80 µg of anilides for 1/10 serum dilutions from treated animals. 100% binding in figure 1B corresponds to 10–12.000 cpm, compared with 1500–2000 cpm with control era. This represents about 10% of the total immunoglobulin content in the specific sera. The results obtained with sera fom control animals were always within the background levels.

In the course of these immunological studies we observed that the animals treated with fatty acid anilides developed some signs of neuromuscular effects; a variable degree of stiffnes (present in 100% of the treated animals), ataxia and hind leg paralysis (in 50% of the treated animals). No other signs of involvement of neurological functions were observed, although an abolition of the pupillomotor reflex was observed in several cases. These signs started to develop with a latency of 3–6 weeks after the beginning of anilide treatment. Although the animals ate and drunk normally, some of them lost weight and 4 of the most affected died. In one case only, some regression was observed. Light and electron microscopic studies[11] of tissues from these animals revealed histological and ultrastructural abnormalities in the CNS (shrinkage, pyknocytosis and eventual cytolisis of Purkinje neurons together with vacuolar degeneration of the white matter and disruption of the myelin sheaths) and striated muscle (disorganization of the myofibrils and mitochondrial swelling). These findings, together with pharmacokinetic studies carried out in mouse[15], showing that the (^{3}H) anilino group of fatty acid anilides accumulated in brain and muscle (with half lives of 55 h and maximal steady concentrations of 150–170 nmol/g) led us to a search for anilide-dependent antigenic determinants in tissues from rabbits by indirect immunofluorescence.

In figure 2 we show that specific antigenic determinants can be detected in Purkinje cells, myelinated fibers in the cerebellar white matter and in striated muscle fibers of anilide-treated animals when incubated with immune serum followed by a 2nd incubation with the FITC anti rabbit serum. Similar results (not shown) were obtained with the specific antibodies purified by the immunoadsorption technique described in the methods section. Negative results were always obtained in tissue slices from control challenged with control serum or with immune serum after the immunoadsorption with liposomes containing

anilides (data not shown). We have also observed foci of intense direct immunofluorescence in some vascular and perivascular round cell infiltrates in the central nervous system and striated muscle from anilide-treated animals when challenged directly with the FITC goat anti rabbit serum. This unpublished observation suggests the occurrence of immune complexes in certain tissue structures; this feature was never seen in slices from control animals.

Discussion. The specific response to anilides in skin tests and the characterization of anilide-specific antibodies in the sera from anilide-treated rabbits by immunoadsorption and solid phase radioimmunoassay strongly support the immunogenic character of fatty acid anilides when administered to rabbits either p.o. or i.p. Furthermore, the identification of 'anilide-dependent antigens' in different tissue structures, as shown by the immunofluorescence studies, introduces the possibility of several immunopathogenic mechanisms of cell injury, which may be relevant for the pathogenesis of the toxic oil syndrome. For instance, the activation of the complement pathway by immunocomplexes, could have caused acute respiratory distress[16] in toxic oil consumers when challenged with volatile aniline derivatives in the kitchen. The neurological disorders in rabbits[11], which bear a close resemblance to the experimental neurotoxicity of eosinophilic extracts[17], might represent an antibody-directed eosinophilic neurotoxicity, which could have been operative in TOS patients. In addition, the production of antibodies able to recognize components of otherwise normal structures could have been involved in the induction of scleroderma-like autoimmune processes[18] which are characteristic features of the chronic stage in TOS[10].

It remains however, a matter of debate to establish how direct the relationship may be between the experimental pathology and immunogenicity of anilides in rabbits and the clinical manifestations in TOS patients. Although there is no evidence that anilides act as liposomes in the clinical situation, there is epidemiological data showing a high comsumption of emulsified oil (mayonnaise) among affected people. In a preliminary study it was found that 70% of the sera from acute phase TOS patients had specific IgE antibodies for the same synthetic anilides used in our experimental work[19]. Moreover, all these acute phase sera had IgG antibodies specific for the fatty acid anilides (C. Lahoz, personal communication). It is, however, unfortunate that these preliminary findings were not followed by a more comprehensive study of the acute phase sera that might still be available. The only other report related to this issue described the absence of specific IgE antibodies for aniline derivatives in subacute and chronic phase sera[20]. Thus, in the absence of additional confirmatory data from human studies, no clear cut conclusion can be drawn as to the relevance in humans of our experimental findings in rabbits.

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Influence of sulfhydryl reagents on the structural and molecular organization of crayfish photoreceptor microvilli

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Summary. SDS-polyacrylamide gel electrophoresis of isolated photoreceptor membrane from crayfish compound eyes in the absence and presence of the sulfhydryl-reducing agent 2-mercaptoethanol revealed major qualitative and quantitative differences in electrophoretic behaviour of polypeptides. Two peptides of 37 and 69 KD show abnormal migration patterns under the experimental conditions, indicating a possible significance of disulfide bridges for the structural integrity of invertebrate photoreceptor membrane.

Key words. Sulfhydryl; photoreceptor; microvilli; compound eye; crayfish.

The photoreceptors of the compound eyes of arthropods exhibit a highly organized system of microvilli containing the light-harvesting photopigment rhodopsin. These microvilli are supported by a single axial filament crosslinked to the photoreceptor membrane via numerous 3 nm bridges[1,2]. Photoreceptor membrane also undergoes a circadian turnover, most dramatic among crustaceans, possibly involving the breakdown and reassembly of the membrane cytoskeleton[2,3].