in separate experiments, they produced tumors consistent with their original histopathology. Cultures of these transplants by methods identical to those used for the primary human tumor transplants did not result in propagation of the transformed fibroblastoid cells despite several attempts. It thus appears that stromal cell oncogenicity in human tumor transplants is restricted to human tumors that are first established in nude mice. We cannot rule out the possibility that this could result from the interaction between mouse stromal cells and the human stromal cells present in the original transplants, since cultured cancer cells (lacking human stroma) did not give the same results. However, different transplant generations of human tumors devoid of human stroma showed murine stomal cell oncogenicity in nude mice (Tables 1 and 2).

A tumor's stroma has generally been considered to be a supportive structure nourishing the growing neoplasm. Less clear has been whether the tumor has genotypic effects on the stroma, or vice versa, although sarcomatous transformation of the connective tissue stroma of mouse mammary adenocarcinoma has been reported repeatedly since 1905 (*4*). The question of whether malignant cells can transfer malignancy to other cells has been addressed previously (*5*); that such transfer occurs is supported by our observations. Human tumor xenografts in nude mice were found to contain murine host cells which, after short-term culture in vitro, showed evidence of malignant transformation. The transformed cells were then able to produce sarcomas in other nude mice. This has been confirmed by transplanting a human embryonal carcinoma into nude mice; after the human tumor graft was cultured in vitro, the overgrowth of murine cells showed signs of malignancy (*6*). However, these investigations failed to produce tumors in mice with the transformed murine cells. Furthermore, a similar finding was made when a human melanoma was transplanted into nude mice (*6*).

Grafts of human tumor cells to immunodeficient animals can produce tumors of host origin (*7*). We therefore presume that, in our experiments, in vivo oncogenesis of mouse stromal cells in the human tumor grafts occurred but that these cells were unable to grow alongside the human tumor cells as a sarcoma until the malignant stromal cells were propagated in vitro, separated, and inoculated into appropriate recipients. Perhaps oncogenic viruses were activated in this process, since Gautsch *et al.* (*8*) recently found that 1 of 20 human tumors transplanted and passaged in nude mice was associated with the induction of endogenous murine leukemia virus. However, we had one of our murine sarcomas and three human tumor transplants in nude mice tested for virus, and the results were negative (*9*).

The alterations in the murine stromal cells isolated from the human grafts may have occurred in vitro, but this is unlikely because of the very short duration in culture before transformed cells were observed and because such results were not obtained when human tumor grafts made from established human tumor cell lines were cultivated in vitro. Nevertheless, we cannot exclude the possibility that the human tumor cells accelerated the in vitro transformation of murine stromal cells. Another possibility is that the malignant murine stromal cells resulted from in vitro or in vivo hybridization of the human tumor and mouse stromal cells, with subsequent loss of the human genome and retention of the malignant genotype. Indeed, somatic cell fusion in vivo has been reported (*10*), but not involving tumors in nude mice. Although we do not have any evidence to support this explanation, we must conclude that some form of cell-cell interaction results in the alteration and ultimate oncogenesis of normal murine stromal cells contained in human cancer xenografts. Interestingly, this event was only observed with human tumors grafted directly from patient specimens, and not with established human cancer cell lines.

We realize that extrapolation of these findings to human and other primary cancers is limited by our experimental system. Nevertheless, the widespread use of nude mice in cancer research warrants the caution that, under the conditions described here, nude mouse cells can become malignant.

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Eicosapentaenoic and Arachidonic Acids from *Phytophthora infestans* Elicit Fungitoxic Sesquiterpenes in the Potato

Abstract. *Mycelial extracts from* Phytophthora infestans *caused necrosis and elicited the accumulation of antimicrobial stress metabolites in potato tubers. A portion of the material with elicitor activity could be extracted from the mycelium by a mixture of chloroform and methanol. The most active elicitors of stress metabolites in these extracts were eicosapentaenoic and arachidonic acids. These fatty acids were found in either free or esterified form in all active fractions of the mycelial extracts.*

When plants interact with parasites, some resistant cultivars respond hypersensitively to incompatible races of pathogens by accumulating antimicrobial stress metabolites (*1*). These stress metabolites, which may be a defense mechanism against disease, are elicited by phytotoxic compounds that are produced by fungi and bacteria (*2*). Incompatible races of the fungus *Phytophthora infestans* (Mont.) de Bary, which causes late blight disease in the potato, elicit the accumulation in tuber tissue of rishitin and lubimin as well as other fungitoxic

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Table 1. Distribution of elicitor activity in initial fractions of the extraction procedure. All fractions were assayed at 300 μg per tuber slice. Specific activity is measured as micrograms of stress metabolites per microgram of treatment solution. Total activity is measured as micrograms of stress metabolites.

Treatment	Stress metabolites (μg/g fresh weight)	Specific activity	Total activity (μg)	Percentage recovery
Crude sonicate	80 ± 4	0.133	319,000	100
Mycelial residue	42 ± 2	0.070	118,000	37
Chloroform-methanol filtrate	114 ± 3	0.190	136,000	43
Chloroform phase	77 ± 1	0.128	43,600	14
Chloroform phase residue	166 ± 8	0.277	17,600	6
Aqueous phase	29 ± 1	0.048	16,400	5

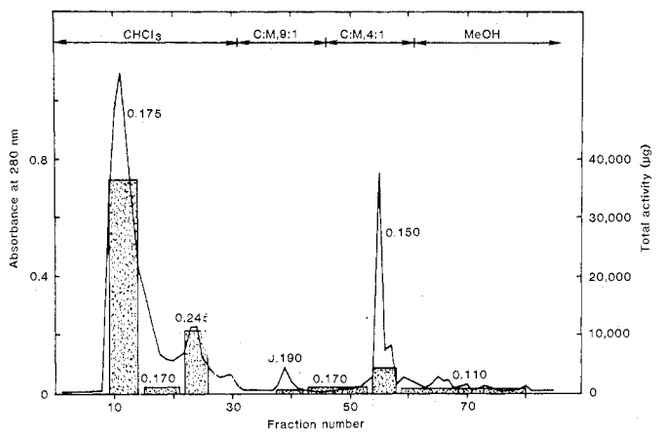

Fig. 1. Chromatography of the chloroform phase (*9*) of the mycelial extract from *P. infestans* on Sephadex LH-20 and resulting distribution of elicitor activity. Total activity (histogram) of each group of fractions is expressed as micrograms of stress metabolites. Specific activity (micrograms of stress metabolites per microgram of applied solutes) is indicated by the number above each bar in the superimposed histogram. All treatment solutions were assayed at 300 μm per tuber slice. *C:M* is ratio of chloroform to methanol.

sesquiterpenes (*3*). Crude suspensions of the mycelium of *P. infestans* can also cause rapid cell death and sesquiterpenoid stress metabolite accumulation in potato tuber tissue.

Our experiments (*4*) and those of others (*5*) have indicated that the active material in mycelial extracts has lipophilic properties. We later found that most of the active material in a crude mycelial suspension could be extracted into a mixture of chloroform and methanol (2 : 1, by volume). By standardizing the bioassay and using increasing specific activity as the criterion for purification, we have now identified the simplest and most active elicitors of stress metabolites in the potato as eicosapentaenoic and arachidonic acids.

Kennebec tubers and mycelium from race 4 of *P. infestans* were used to establish the purification protocol. All organic solvents were removed from the mycelial extracts by a stream of nitrogen, and the material to be assayed was suspended in sterile distilled water by sonication. Kennebec tuber slices were incubated at 20°C in the dark for 96 hours after treatment with mycelial extracts. The sesquiterpenoid stress metabolites were then extracted from the tuber tissue and quantified (*6*). Since rishitin and lubimin are the predominant stress metabolites in the treated Kennebec tuber slices, we have expressed the activity as the sum of rishitin and lubimin accumulations, a value which is consistently correlated with the degree of necrosis in treated tissue slices from stored tubers (*7*).

The chloroform-methanol extracts and the mycelial residues remaining after extraction were assayed at a dilution equivalent to that of a crude cell-free sonicated suspension of mycelium. In seven experiments, the chloroform-methanol extract consistently had higher specific and total activities than the mycelial residue (*8*). After water-soluble contaminants were removed from the total lipid extract (*9*), insoluble material arising after concentration of the chloroform phase was removed by filtration or centrifugation. This residue was insoluble in all solvents tested, including water, and had high specific activity (Table 1). Most of the active material, however, remained soluble in the chloroform-methanol mixture, and this fraction could be subjected to further chromatography (*10*).

Sephadex LH-20 column chromatography was used because of the enriched specific activity and nearly quantitative recovery of active material (Fig. 1). Fractions 22 to 26, corresponding to the second peak had the highest specific activity. These fractions, which were pooled for further study, were subjected to preparative thin-layer chromatography (TLC). All of the activity was associated with a diffuse band centered at a retardation factor (R_f) of 0.46.

Methanolysis of this pooled material and analysis by gas-liquid chromatography (GLC) (*11*) revealed a number of fatty acid methyl esters. No methyl glycosides, amino sugars, or amino acids were detected. Treatment of the pooled material with diazomethane resulted in a chromatogram identical with that obtained after methanolysis, an indication that activity was associated with a free fatty acid fraction. Reverse-phase chromatography on Whatman KC18 thin-layer plates separated the free fatty acid mixture. Most of the activity was associated with a band at R_f = 0.58. Preparative TLC of this material on 5 percent $AgNO_3$ silica gel G yielded a single compound, as judged by GLC (Fig. 2) of the methyl ester (*12*).

The chemical ionization (methane) mass spectrum of the methyl ester (GLC combined with mass spectrometry) revealed a molecular ion at a mass-to-charge ratio (*m/z*) of 316, consistent with that of a 20-carbon fatty acid containing five double bonds. Reduction with hydrogen over platinum oxide yielded methyl arachidate, as shown by a molecular ion with an *m/z* of 326. Since trimethylsilylation (TMS) of the methyl

Table 2. Elictor activity of purified eicosapentaenoic acid from *P. infestans* and authentic fatty acid standards. All were assayed at 100 μg per tuber slice at *p*H 6.6. Specific activity is measured as micrograms of stress metabolites per microgram of fatty acid. The following fatty acids were tested and had no activity: myristic acid, palmitic acid, stearic acid, oleic acid, ricinoleic acid, linoleic acid, linolenic acid, γ-linolenic acid, arachidic acid, eicosaenoic acid, eicosadienoic acid, eicosatrienoic acid (*cis*-8,11,14), eicosatrienoic acid (*cis*-11,14,17), behenic acid, and erucic acid.

Fatty acid	Stress metabolites (μg/g, fresh weight)	Specific activity
Eicosapentaenoic acid from *P. infestans*	136 ± 6	0.680
Eicosapentaenoic acid standard	147 ± 12	0.735
Arachidonic acid	162 ± 8	0.810
Docosahexaenoic acid	47 ± 2	0.235
H_2O control	0	0

ester did not change the retention time or the mass spectrum, there were no other replaceable hydrogens in the molecule. The ethyl ester and TMS derivatives of the free acid gave corresponding increases in the molecular ion (*13*). The TLC behavior, the GLC retention time, and the mass spectrum of the methylated elicitor were identical to those of the methyl ester of *cis*-5,8,11,14,17-eicosapentaenoic acid (*14*). The authentic eicosapentaenoic acid was equally active in the bioassay (Table 2).

To determine whether eicosapentaenoic acid was present in free or esterified form in the other active fractions from Sephadex chromatography (Fig. 1), we treated portions of the active fractions with diazomethane or with 1*N* methanolic HCl for 2 hours at 100°C and analyzed the derivatives by gas chromatography. Fatty acid methyl esters were detected in these other active fractions with methanolic HCl, but not with diazomethane treatment, an indication that the fatty acids were esterified to other compounds. Eicosapentaenoic and arachidonic (*cis*-5,8,11,14-eicosatetraenoic) acids were found in all the active fractions. Arachidonic acid proved to be as active as eicosapentaenoic acid in the bioassay (Table 2). Both eicosapentaenoic and arachidonic acids were active at the lowest concentration tested (5 μg per slice). Subsequent experiments indicated that the methyl esters were also active.

Of 18 commercially available saturated and unsaturated fatty acids that were tested for activity (Table 2), only arachidonic, eicosapentaenoic, and *cis*-4,7,10,13,16,19-docosahexaenoic acids were active in eliciting necrosis and stress metabolite accumulation. Docosahexaenoic acid was not as active as the other two acids, nor have we observed it in mycelial extracts. *cis*-8,11,14-Eicosatrienoic acid was only 10 percent as active as arachidonic acid on new tubers (after 3 months of storage), and was not active on older tubers (after 10 months of storage) (Table 2).

Arachidonic and eicosapentaenoic acids are not present in higher seed plants but are present in lower plants and fungi (*15*). Eicosapentaenoic acid is the predominant fatty acid in *Saprolegnia parasitica*, which, like *P. infestans*, is a member of the Oomycetes; other incompatible Oomycetes are also active in eliciting stress metabolite accumulation in potato tubers (*16*).

Other studies of plant pathogenic fungi have attributed elicitor activity to glucans and glycoproteins associted with the cell wall (*17*). In our studies, arachidonic and eicosapentaenoic acids were present in small amounts in cell wall preparations. Even after extraction in chloroform-methanol, the mycelial residue contained these fatty acids which could be removed by extraction with methanolic KOH.

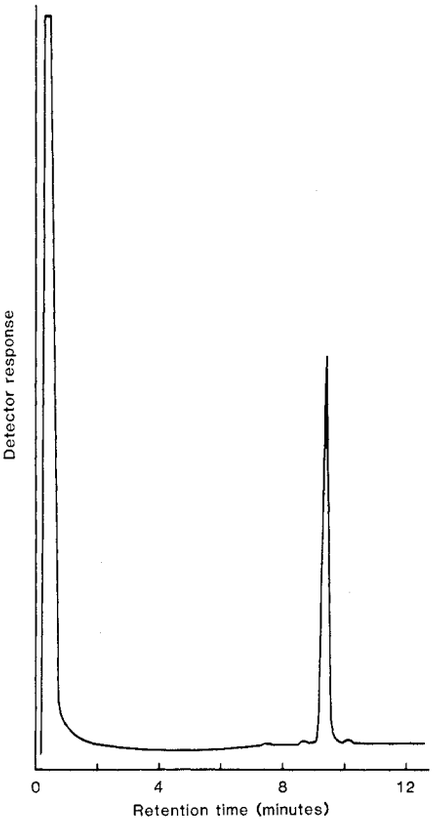

Fig. 2. Gas-liquid chromatogram of purified eicosapentaenoic acid from *P. infestans*. Chromatography was performed as described in (*11*). The oven temperature was increased from 150° to 250°C at 8°C per minute.

Zimmerman and Coudron (*18*) reported that the wound hormone, traumatin, is an oxidation product of linoleic or linolenic acids, and other fatty acids may have growth-regulating properties in higher plants (*19*). To our knowledge, this is the first report of these polyunsaturated fatty acids eliciting responses associated with hypersensitivity in higher plants.

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8. Mycelial mats from 14-day-old cultures of *P. infestans* were stored at −20°C until extraction. The mycelium was thawed and chopped, then homogenized in a mixture of chloroform and methanol (2 : 1, by volume) with a Polytron homogenizer (Brinkmann). A standard lipid extraction method was followed throughout this study (*9*). The concentrations of the test solutions were determined by drying 50- to 100-μl portions on previously weighed aluminum pans at 60°C for 1 hour, and then weighing them on a microbalance (Perkin-Elmer). Specific activities are expressed as micrograms of stress metabolites (rishitin plus lubimin) per microgram of dry weight of the treatment solutes. Total activity is the product of specific activity and total dry weight of a given fraction.
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10. The lipid extract was applied to a Sephadex LH-20 (Pharmacia) column (2.7 by 33 cm) and eluted with chloroform (250 ml), chloroform-methanol 9 : 1 (125 ml), chloroform-methanol 4 : 1 (125 ml), and methanol (200 ml). The column effluent was monitored by absorbance at 280 nm and by TLC on silica gel G Uniplates (Analtech). A mixture of chloroform, methanol, and NH_4OH (65 : 25 : 5) was used as a developing solvent, and spots were visualized by spraying with 50 percent H_2SO_4 and heating. Preparative TLC was performed on 1000-μm-layer silica gel G plates with the same solvent mixture.
11. The partially purified elicitor was treated with 1*N* methanolic HCl at 100°C for 2 hours. The methanolyzed material was partitioned three times against redistilled hexane. Fatty acid methyl esters in the hexane phase were analyzed on a Varian 1400 gas chromatograph equipped with a flame ionization detector and a 1.8 m by 2 mm glass column packed with 10 percent SP-2330 on Chromosorb W AW (Supelco). Trimethylsilylation of the methanolic phase and analysis by gas chromatography on 3 percent OV-1 on Gas-Chrom Q (Applied Science) revealed no TMS-methyl glycosides. To check for amino sugars, aliquots were hydrolyzed in 6*N* HCl at 100°C for 4 hours, trimethylsilylated, and analyzed by gas chromatography.
12. The developing solvent used for reverse phase TLC was a mixture of acetonitrile, tetrahydrofuran, and acetic acid (80 : 15 : 5). The developing solvent used for argentation TLC was a mixture of hexane, diethyl ether, and acetic acid (80 : 40 : 3).
13. Chemical ionization mass spectra were obtained on an automated gas chromatography–mass spectrometer (Finnigan, model 3300). Methane was the ionization gas.
14. *cis*-5,8,11,14,17-Eicosapentaenoic acid was a gift of J. Gellerman, Hormel Institute, University of Minnesota, Austin.
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