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Consequences of the AF-2 Incident in Japan

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The discovery of the potent mutagenicity of AF-2, which was once used in Japan as a food preservative, has exerted a great influence not only on screening procedures for carcinogenic compounds but also on legislative approaches to mutagenic substances. It promoted the synthesis of exceedingly sensitive and reliable tester strains in *Salmonella* and supported the hypothesis of a common mechanism between mutagenicity and carcinogenicity. Thus preliminary screening for carcinogenic substances has become feasible using mutagenicity as an index. It also contributed greatly to the formulation of legislative measures for chemical substances which for the first time gave due attention to mutagenicity. Furthermore, the exposure of a large population to such a potent mutagen raised a question as to what extent the genetic constitution of the Japanese population might have been damaged. This suggested the urgent need for a system to monitor the total genetic damage to a human genome.

AF-2 was used as a food preservative in Japan during the years 1965–1974. It was widely used in foods like soybean curd (*tôfu*), ham, sausage, fish ham, fish sausage, and fish paste (*kamaboko*) to prevent contamination by microorganisms. Its chemical structure is 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, abbreviated furylfuramide.

The discovery of the potent mutagenicity of this compound initially produced extraordinarily heated discussion in Japan. It is not pleasant for me to recollect those days, but the discovery brought about several far-reaching effects not only upon legislative approaches to mutagenic substances but also upon scientific development in relevant fields.

Just before the establishment of the Environmental Mutagen Society of Japan a research group was organized for the promotion of studies on environmental mutagens. Each member, using his most familiar test system, focused on the development of sensitive test systems for weak mutagens and chose a few nitrofuran derivatives, including AF-2, as standard test chemicals for comparing different test systems. AF-2 was included because it was widely used in Japan at that time and because mutagenicity and carcinogenicity had already been demonstrated for several other nitrofuran compounds. However, we did not know that a producer of AF-2 had instituted legal proceedings against an author of a book claiming damages caused by the latter's severe criticism of this compound. This added to the complexity of our dispute when we discovered and reported the potent mutagenicity of AF-2.

In what follows I shall describe some consequences of the discovery of the potent mutagenicity of AF-2.

Scientific Contributions

Promotion of the Improvement of the *Salmonella* Test System

At the first joint meeting of the US-Japan Mutagenesis and Carcinogenesis Panel held in Tokyo in August of 1972, Ames (*1*) talked about the usefulness of the *Salmonella* test strains TA1535, TA1536, TA1537, and TA1538, which were capable of detecting the mutagenicity of a wide range of chemicals. In the meantime our research group discovered the strong mutagenicity of AF-2 with several test systems: colon bacteria (*2*, *3*), yeast (*4*), silkworm (*5*), and human cells in culture (*6*). Soon afterwards Sugimura et al. (*7*) noticed that the available *Salmonella* TA systems were unable to detect the mutagenicity of AF-2 as well as other nitrofuran compounds. This finding was communicated by Sugimura at the second joint meeting of the US-Japan Panel on Mutagenesis and Carcinogenesis held at Charleston in 1973. Since then,

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improvement of the screening capacity of the *Salmonella* system was energetically pushed by Ames' group. At the Honolulu Workshop held in December of 1974, McCann (*8*) reported success in the synthesis of strains TA98 and TA100, which do respond to AF-2, to other nitrofurans, and to other compounds which had previously given a negative response, such as benzyl chloride, benzo(α)pyrene, 7,12-dimethylbenzanthracene, and acetoxysafrol. It may be too much to say that this improvement of the *Salmonella* strains was due to the AF-2 incident alone, but it appears certain that the compound promoted the synthesis of these strains.

Utility of Mutagenicity as an Index for Screening Carcinogenicity

Extensive research was carried out by Miyaji (*9, 10*) at Osaka University to test the toxicity and carcinogenicity of AF-2 on rats; but the results were reported to be negative. On the basis of these and some other results, the Food Sanitary Council permitted the use of AF-2 as a food preservative. However, since strong mutagenicity was revealed with this compound, later careful examinations were conducted by many investigators to test for carcinogenicity. Several positive results were obtained, and nowadays there is no room for doubt that the compound is carcinogenic. The differences in experimental conditions between studies carried out at early and late stages were in dose and use of sensitive conditions.

Dose. In Miyaji's experiments, doses were relatively low, less than half of the later experimenter's, because Miyaji thought highly of doses administered at food-additive concentrations. The higher doses used in later experiments enabled the demonstration of the carcinogenicity of AF-2 (*11–14*).

Highly Sensitive Conditions. In later experiments testing was performed under highly sensitive conditions, i.e., using young animals (*15, 16*), under elevated enzyme activity induced by phenobarbital, PCB (*8, 17, 18*), or transplacental administration of test compounds to fetuses *in utero* (*19*). The results clearly indicated that mutagenicity could be used as an appropriate index for the screening of carcinogenicity. This view was completely supported by testing the mutagenicity of several carcinogens with *Salmonella*.

Using improved *Salmonella* strains, in combination with S9 when necessary, Ames et al. (*20*) tested the mutagenicity of many compounds whose carcinogenicity was already known. The results were positive for the majority of compounds tested. This led Ames to conclude that carcinogens are mutagens. For the purpose of screening for carcinogenicity, experimentation on a large scale is required consuming large amounts of money, manpower and time. The exploitation of the improved *Salmonella* system made it feasible to preselect suspicious substances for carcinogenicity testing by prior mutagenicity testing.

Impact on Legislative Requirements

For safety testing of food additives, the following had already been legislated as essential: acute toxicity, chronic toxicity, teratogenicity, and carcinogenicity. In addition, it has often been pointed out that mutagenicity should also be taken into consideration. No country has as yet designated mutagenicity tests as compulsory. In Japan, however, mutagenicity is now regarded as a quasi-compulsory item for safety evaluation of food additives.

As mentioned before, soon after the start of our research group in 1972, Tonomura and Sasaki (*6*) observed the strong chromosome breaking capacity of AF-2 using a cytogenetic test system with human lymphocytes. Furamizole, Nitrofurylacrylamide (Z-furan), Furypyrinole, and Furylfuramide (AF-2) were active in decreasing order. The most astonishing finding was that AF-2 was included in the active group. Tonomura immediately informed the group members of these findings. Thereafter, the mutagenicity of this compound was demonstrated by other members of the group with various test systems: *E. coli* (*2, 3*), *B. subtilis* (*3*), yeast (*4*), and the silkworm (*5*). Independently of this group, Sugimura and his collaborators (*7*) confirmed the strong mutagenicity of AF-2 in bacteria using both the *Salmonella* repair test and the *E. coli* WP-2 test. Although mutagenic activity had not yet been detected by *in vivo* experiments with mammals, it was conjectured that the compound might also be mutagenic to man since mutagenicity had been observed in such a wide range of test systems.

We therefore warned our government of the possible genetic danger of this compound and requested them to perform careful safety tests on mammals. The authorities, however, did not listen to our caution. Half a year later, we released our results to the press on the occasion of the annual meeting of the Environmental Mutagen Society of Japan held in September of 1973. Thereafter, long and disagreeable disputes arose between government authorities and ourselves (*21, 22*). As a representative of the Society, I was threatened by a company that produces AF-2, saying that they would take legal action for damages. We were even

criticized by some scientists for causing a sensation. *In vivo* mutagenicity data on mammals were keenly sought.

Nakagawa, working under Miyaji, reported negative results from his *in vivo* cytogenetic study on mice following orally administered AF-2 (*23*). Similar tests carried out on mice by Yosida, head of the Cytogenetics Department of our Institute, was also yielding negative results. However, the predicted positive results were obtained by Sugiyama et al. (*15*). By administering AF-2 intraperitoneally and/or orally to young rats of the Long-Evans strain he clearly demonstrated that AF-2 breaks bone marrow chromosomes *in vivo*, and he even suggested that it might well be carcinogenic. Sugiyama's success was completely due to the use of young animal. Their results were soon confirmed by my colleague Yosida (*16*). Thus our belief that AF-2 is harmful to human beings was reinforced.

In the meantime, Japanese governmental authorities became aware of developing attitudes in western countries where genetic safety was taken into consideration in the safety examination of food additives. They reorganized the Food Sanitary Council, an advisory organ to the Minister of Health and Welfare, and set up a committee for the evaluation of genetic safety (July 1974). The committee, after formulating principles and standard test systems for the evaluation of genetic safety, evaluated the mutagenicity of AF-2 with the data thus far obtained.

The compound was negative in the dominant lethal test in mice. These tests were carried out in three laboratories: the National Institute of Hygienic Sciences, Tokyo (*24*); the National Institute of Genetics, Mishima (*25*); and, in the United States, the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina (*26*). Midway data collected at that time from these institutes were all negative. The final NIEHS report is now available. In their experiment five groups were run, each comprising more than 500 fertile matings and collecting data for six consecutive weeks. AF-2 doses were 0, 300, 350, 400, and 450 mg/kg. Again there was no indication of AF-2-induced dominant lethality.

The host-mediated assay, carried out by Tutikawa and Kada (*25*) by injecting *B. subtilis* into the peritoneum of mice, gave clearly positive results, indicating that the genetic toxicity of AF-2 was maintained for at least a few hours after oral administration.

Taking this evidence together with the aforementioned *in vivo* cytogenetic data, the committee concluded that AF-2 was very likely to be mutagenic to man. The committee submitted their report to the Minister, recommending that the use of AF-2 as a food preservative should be prohibited (September, 1974). By this time preliminary data demonstrating the carcinogenicity of AF-2 had been obtained by a research team headed by Ikeda (*11*) at the National Institute of Hygienic Sciences. The Government immediately took action to ban the use of this compound. In the work by Ikeda et al., diets containing AF-2 were given continuously to ddY/SLC mice. Squamous cell carcinoma was induced in the forestomach within a year.

Since then, positive results have been obtained by many workers in both mutagenicity and carcinogenicity tests.

Nomura (1975) (*27*) reported that three subcutaneous injections of AF-2 (50 μg/g body weight) into pregnant mice resulted in lung adenoma in 17.1% of their offspring. This was significantly higher than the 2.7% observed in the control group. Takayama and Kuwabara (*13*) observed the induction of mammary tumors in Wister rats by feeding AF-2. Sano et al. (*12*) reported the production of squamous cell carcinomas in golden hamster cheek pouches by feeding a diet containing 0.25% AF-2. They also reported the induction of similar carcinomas in the forestomachs of mice. Yokoro et al. (*14*) were able to produce squamous cell carcinomas in the forestomachs of mice by feeding. Inui et al. (*19*) reported the neoplastic transformation of embryonic hamster cells after transplacental administration of AF-2 in the pregnant mother. Aoki et al. (*28*) reported hepatic changes in the teleost *Oryzias latipes* when they were raised for 2–3 months in water containing 2–5 μg/ml AF-2.

Regarding the mutagenicity of AF-2, further positive results were obtained by many investigators: in *Neurospora crassa* by Ong et al. (*29*), in *Saccharomyces cerevisiae* by Shahin et al. (*30*), in human diploid cells by Kuroda (*31*), in Chinese hamster cells by Wild (*32*) and by Ban et al. (*33*), in mouse lymphoma cells by Nakamura et al. (*18*), and in the mouse micronucleus test by Goodman et al. (*34*).

These results justified the decision of the Food Sanitary Council. It must be noted, however, that their reason for banning AF-2 was not due to mutagenicity, but to carcinogenicity. On the other hand, if mutagenicity had not been detected, this compound would have continued in use.

It is strongly urged that mutagenicity should be regarded as one of the compulsory items for safety evaluation of chemicals.

Suggestion for Future Research

The discovery of the potent mutagenicity of AF-2 raised the question of the extent to which the genetic makeup of the Japanese population might have

been damaged. For the past 20 years we Japanese have been exposed to such mutagens, namely AF-2 for 9 years (1965-1974) and Z-furan (nitrofurylacrylamide), a more potent mutagen than the former, for 11 years (1954–1965). Although daily intakes of these mutagens were very small, the accumulated dose was thought to reach appreciable levels.

The magnitude of this exposure can be roughly estimated as follows in terms of the radiation equivalent chemical. The annual production of AF-2, although not made public, is guessed to be about 3.5 tons in 1973. The biological activity of AF-2 is known to decline in foodstuffs while they are on the shelf in the market. The average decay factor is assumed to 0.95 per total foodstuffs from production to ingestion (*2*). The substantially active amount is, therefore, calculated to be 3.5 ton × (1 − 0.95) = 175 kg/year. The mutagenic activity of AF-2 in reproductive cells of mammals has not been determined yet. Hence, data obtained in the silkworm will be used instead (*35*). In this insect the mutation rate was obtained by oocytes by injecting the chemical into the body cavities of female pupa. RECs obtained in the silkworm are 20 to 70 rem for an administered dose 1 μg per insect, the average body weight of which is 1 g. Hence 40 rem will be taken as a representative value.

A dose of 1μg/g for the silkworm is assumed to correspond to 50 mg/50 kg for the average Japanese. Therefore, 1 mg of AF-2 can be equated to 0.8 rem exposure to γ-rays per average person. We also assume that the mutagenic efficiency of AF-2 in man is 100 to 1000 times lower than in the silkworm, taking several factors into consideration: injection vs. ingestion, difference in time during which the chemical remains active in the body, differential sensitivity of germ cells, etc. In the silkworm AF-2 was injected into the body cavity and it, if not decomposed, remained in the pupal body until emergence of the moth. The mutation assay system used for the silkworm experiment was a specific loci method in which wild type oocytes were treated. The system is very sensitive and gives mutation frequencies at least 10 times higher than the spermatogenic cell system in the same insect.

With these assumptions, the average exposure equivalent per Japanese is calculated as follows: 0.8 rem × 175 kg/mg × $(10^{-2}$–$10^{-3})/10^{8}$ = 14.0–1.4 mrem/year. This is not large, and the genetic effects of such a small dose of exposure to AF-2 may not be detectable in practice. One may notice, however, the vagueness in assuming a conversion factor from the silkworm to man. Based on their data on the mouse sarcoma assay system, Nakamura et al. (*18*) calculated RECs of AF-2 to be 40 mrem per person per year, taking into due consideration the proportion of ingested AF-2 incorporated into gonads. Still, several ambiguous factors were involved. Since an AF-2-induced mutation rate is not available even in the mouse, there is an urgent need for work to estimate the genetic risk to man. In this connection, de Serres (*36*) stressed the urgency of exploiting a system to monitor the total genetic damage inflicted upon the human genome.

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