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Inhibitory Effect of Propolis and Bee Venom on the Mutagenicity of Some Direct- and Indirect-Acting Mutagens

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The antimutagenic effect of ethanolic extract of propolis (EEP) and honeybee (*Apis mellifera*) venom, both collected in the State of São Paulo, Brazil, was assessed by the Salmonella/microsome assay upon direct- and indirect-acting mutagens. EEP had inhibitory effect (in an ascending order) on the mutagenicity power of daunomycin (TA102), benzo(a)pyrene (TA100), and aflatoxin B_1(TA98) and the venom acted against the mutagenicity of 4-nitro-o-phenylenediamine (TA98) and daunomycin (TA102). *Teratogenesis Carcinog. Mutagen. 19:403–413, 1999.* © 1999 Wiley-Liss, Inc.

Key words: propolis; bee venom; antimutagenicity; direct and indirect mutagens

INTRODUCTION

Harmful health effects caused by mutation events involve somatic and germinal tissues and can cause somatic diseases, teratogenic effects, and inherited disorders. Direct evidence of the mutational origin of somatic diseases in man is limited, but may be of great importance in arteriosclerosis, neurological disorders, and aging. Analysis of oncogene activation has indicated that specific alteration in the DNA and chromosomes is intimately involved with the carcinogenic process. The extent of the influence of environmental factors at the mutation level is still unknown because tumor formation is a complex process involving a series of steps, some of which are probably epigenetic. Moreover, experimental data on chemical mutagenic and carcinogenic action show that mutagenic factors are extremely important in car-

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cinogenic action. These data suggest that certain types of cancer in humans may be prevented by identifying the mutagenic agents in the environment and by preventing exposure of individuals to such agents [1].

According to Ferguson [2], the use of antimutagenic and anticarcinogenic agents in daily life would be the most effective procedure for prevention of cancer and genetic diseases in humans. In fact, protection against mutations could be beneficial at least in the initial stages of tumor development. Some natural compounds have recently been described as inhibitors of the mutagenic effect in bacteria [3–8].

Propolis is a natural composite balsam, produced by honeybees from the gum of various plants. Bees collect vegetal exudates and form pellets with their mandibles, mixing the exudates with wax and products of their salivary glands. The resulting material is used to wax and strengthen the nest, to provide protection from microorganisms, and as an embalming substance to cover the carcass of a hive invader [9]. In addition, this material is widely used in popular medicine for a range of treatments. Ethanolic extract of propolis (EEP) is a complex mixture which has been used for medical purposes since antiquity, mainly as an anti-inflammatory and scar healer [10]. But recently there has been considerable interest in the therapeutic properties of propolis, such as its immune-modulator and anti-infectious action [11–14]. The venom, extracted from the same bee species, is known for its anti-inflammatory [15] and radioprotective [16–18] properties. *Apis mellifera* venom has been frequently used to treat rheumatoid arthritis [15,19].

The present study was carried out in order to assess the influence of EEP and honeybee venom on the effects of direct and indirect standard mutagens in assays with *Salmonella typhimurium* (Ames test).

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO-CAS #67-68-5), nicotinamide adenine dinucleotide phosphate sodium salt (NADP-CAS #11-84-16-3), D-glucose-6-phosphate disodium salt (CAS #3671-99-6), L-histidine monohydrate (CAS #7048-02-4), D-biotin (CAS #58-85-5) were purchased from Sigma Chemical Co. (St. Louis, MO).

Standard mutagens: benzo(a)pyrene (B(a)P-CAS #50-32-8), daunomycin (DAU-CAS #23541-50-6), sodium azide (AZS, NaN_3-CAS #26628-22-8), aflatoxin B_1 (AFB_1-CAS #1162-65-8), 2-aminofluorene (AAF-CAS #153-78-6), 2-anthramine (2-AA-CAS #613-13-8), 4 nitro-o-phenylenediamine (NPD-CAS #99-56-9) were also obtained from Sigma. In the preparation of bacterial growth media, Oxoid Nutrient Broth No. 2 (Oxoid, UK), Difco Bacto Agar (Difco, Detroit, MI) were used. All other reagents used for buffers and preparation of media were from Merck (Whitehouse Station, NJ) and Sigma. The S9 fraction from Aroclor 1254 treated rats was obtained from Molecular Toxicology, Inc. (Annapolis, MD).

Tester Strains

The strains of *Salmonella typhimurium* TA98, TA100, and TA102 were kindly provided by Dr. Bruce Ames, University of California at Berkeley. *Salmonella* strain TA98 detect mutagens that cause a frameshift in a G-C basepair region. The *his*D3052 mutation in TA98 is in the hisD gene coding for histidinol dehydrogenase. Strain TA100 detects mutagens that cause basepair substitution at G-C pairs. The *his*G46

mutation in TA100 is in the *his*G gene coding for the first enzyme of histidine biosynthesis. The TA102 strain contains the *ochre*-TAA mutation in the *his*G gene and efficiently detects a variety of mutagens, mainly oxidant compounds [20].

Preparations of EEP and Honeybee Venom

Propolis was collected in beehives in the Araraquara region of São Paulo State, Brazil, where the predominant crops are sugar cane, oranges, and eucalyptus. A solution was prepared by agitating 110 g of propolis in 300 ml of 70% alcohol and left to rest for 7 days. The solution was filtered at the end of this period and 25 ml of sterile distilled water was added to each 0.5 ml of the filtrate. The hydro-ethanolic solution obtained was used in the assays. The venom was extracted from honeybees collected and frozen to remove the stings with the venom sacs. The venom was squeezed out from its sac through the sting spears using light finger pressure and collected in graded capillary tubes. The venom was then diluted in sterile distilled water (1 μl venom/ml water) and used in the assays.

Mutagenicity Testing

Following the methodology of direct incorporation in plates developed by Maron and Ames [20], different concentrations of EEP (0.25, 0.5, 1, 2, and 4 μl/plate) or of *Apis* venom (0.01, 0.02, 0.04, and 0.05 μl/plate) were mixed with 0.1 ml of overnight cultures of *Salmonella typhimurium* tester strains, 2 ml of top agar containing histidine and biotin, and 0.5 ml of the S9 mixture in the metabolism assays. The mixtures were poured onto minimal glucose agar plates. The number of revertants were counted after incubation for 2 days at 37°C. To ensure that *Salmonella* colonies growing on the incubated Ames plates are true (his+) revertants and did not arise due to the presence of excess histidine in the EEP or bee venom samples, 10 colonies from one duplicate assay plate were streaked over histidine-free Ames plates with a sterile inoculating loop and then incubated for 48 h at 37°C. Only his+ organisms grow on the plates.

Antimutagenicity and Toxicity Assays

The procedures for the antimutagenicity assays were similar to those described for the mutagenicity assays. However, the recommended mutagenic agent for the bacterial strain in question was also added to the tube of top agar containing the bacteria, EEP, or bee venom, along with 0.5 ml of S9 mix for the indirect mutagens: TA100, in experiments with AZS, 2-AA, B(a)P; TA98, with AFB_1, 2-AA, B(a)P, NPD; TA102 with AAF, B(a)P, DAU.

For the determination of surviving cells, 0.1 ml of each tested mixture diluted to 10^6 was poured onto nutrient agar plates. The number of viable cells was counted after incubation for 2 days at 37°C. According to Vargas et al. [21], the samples were considered cytotoxic when less than 60% survival was obtained.

Among the tested mutagens, AZS and DAU were dissolved with water, whereas AFB_1, 2-AA, B(a)P, NPD, and AAF were dissolved with DMSO. Each sample was assayed using triplicate plates and each experiment was assayed twice. The calculation of the percentage of mutagenic inhibition was carried out according to Tachino et al. [22] where:

$$\text{inhibition (\%)} = 1 - \frac{\text{induced revertant/plate (with inhibitor)}}{\text{induced revertant/plate (without inhibitor)}} \times 100$$

RESULTS

Some assays were first carried out to assess the mutagenic activity of EEP and bee venom. Table I shows the results obtained for both agents. No mutagenic activity was found in all tests made, since the per-plate his+ revertant frequencies obtained in the presence of different EEP and bee venom concentrations did not differ significantly from the negative controls in the three *S. typhimurium* strains.

Studies on the antimutagenic activity of EEP and bee venom for different standard mutagenic agents were then carried out in assays with *S. typhimurium* (Tables II–VII). Some of the mutagenic agents used have direct action, such as AZS, NPD, and DAU, and others are indirect mutagenic agents and were therefore used with the S9 mixture: AFB_1, 2-AA, B(a)P, and AAF.

Table II shows the results with TA100 strain. No antimutagenic effect was observed with EEP in the dose range used between 0.25 and 4 μl/plate the in presence of AZS. An increase in mutagenic activity was observed in a dose-related fashion in the dose range between 0.25 and 1 μl/plate of EEP, which was followed by an apparent decrease in mutagenic activity between 2 and 4 μl/plate of EEP in presence of 2-AA. A decrease in mutagenic activity was observed in a somewhat dose-related fashion between 0.5 and 4 μl/plate EEP in the presence of B(a)P.

When TA98 was used (Table III), no antimutagenic effect was observed with EEP in presence of NPD. A decrease in mutagenic activity was observed in a dose-related fashion of EEP in the presence of AFB_1. The mutagenic inhibition rate varied from 52% to 90%, according to the increase in EEP concentration. An increase in mutagenic activity was observed in the dose range between 0.25 and 2 μl/plate of EEP, with no decrease in mutagenic activity at the 4 μl/plate of EEP in the presence of 2-AA. Probably in this concentration the antimutagenic effect observed is due to

TABLE I. Mutagenic Effect of Bee Venom and Propolis Ethanolic Extract (EEP) in *Salmonella typhimurium* TA100, TA98, and TA102

		Revertants his^+/plate (±SD)					
	Dose	TA100		TA98		TA102	
Compound	μl/plate	+S9	–S9	–S9	+S9	–S9	+S9
Bee venom	0	153 ± 28	133 ± 21	34 ± 15	31 ± 5	262 ± 62	209 ± 21
	0.01	149 ± 25	120 ± 18	35 ± 10	32 ± 3	266 ± 86	201 ± 2
	0.02	136 ± 25	120 ± 18	32 ± 11	32 ± 10	290 ± 68	203 ± 2
	0.04	96 ± 40	105 ± 20	22 ± 8	27 ± 2	274 ± 24	208 ± 1
	0.05	82 ± 44	79 ± 7	24 ± 5	32 ± 6	272 ± 51	220 ± 26
EEP	0	184 ± 23	140 ± 19	36 ± 6	36 ± 1	294 ± 21	221 ± 53
	0.25	180 ± 38	150 ± 21	36 ± 0	40 ± 7	320 ± 67	339 ± 106
	0.5	168 ± 2	138 ± 24	34 ± 4	45 ± 1	304 ± 58	317 ± 22
	1.0	182 ± 8	138 ± 17	38 ± 5	42 ± 12	323 ± 58	326 ± 15
	2.0	173 ± 13	153 ± 13	36 ± 4	47 ± 2	276 ± 44	353 ± 15
	4.0	196 ± 7	157 ± 30	30 ± 10	43 ± 9	298 ± 24	326 ± 31

The positive controls: TA100 → sodium azide (1.25 μg/plate) → 1,252 ± 179, 2-anthramine (20 μg/plate) → >1500 TA98 → 4-nitro-o-phenylenediamine (5 μg/plate) → 1,034 ± 60 2-anthramine → (20 μg/plate) >1500 TA102 → Daunomycin (1.5 μg/plate) → 1,024 ± 63 2-aminofluorene (10 μg/plate) → 860 ± 10.

The negative controls: 0 → 0.1 ml of distilled water for the venom and 0.1 ml water + 4 μl ethanol for EEP.

TABLE II. Inhibition of Sodium Azide (AZS), 2-Anthramine (2-AA) and Benzo(a)pyrene (B(a)P) Induced Mutagenesis in *Salmonella typhimurium* TA100, by EEP

Compound	Dose (μg/plate)	EEP (μg/plate)	Rev/plate*	I (%)
AZS	1.25	0	1,172 ± 291	—
		0.25	1,264 ± 345	0
		0.5	1,276 ± 383	0
		1.0	1,397 ± 340	0
		2.0	1,210 ± 322	0
		4.0	1,171 ± 267	0
2-AA	0.125	0	687 ± 270	—
		0.25	1,376 ± 229	0
		0.5	1,280 ± 136	0
		1.0	1,084 ± 362	0
		2.0	398 ± 35	42
		4.0	272 ± 22	64
B(a) P	1.0	0	1,150 ± 57	—
		0.25	1,012 ± 58	12
		0.5	857 ± 351	25
		1.0	473 ± 93	59
		2.0	662 ± 155	43
		4.0	328 ± 49	71

*Each value represents the mean ± SD of six replicate plates from two separate experiments (three plates/experiment).
I = inhibitory effect.

toxicity. Antimutagenic effect was observed in a somewhat dose-related fashion between 0.25 and 4 μl/plate EEP in the presence of B(a)P.

When the TA102 strain was used (Table IV) the antimutagenic effect of EEP was observed against the mutations induced by DAU. This effect did not seem to depend on the EEP concentration and the inhibition rate was about 40%. There was a very weak mutagenic inhibition from AAF for the same strain, and if EEP is associated with B(a)P, an apparent increase in the mutagenic activity was observed.

The action of the bee venom as an antimutagen was not observed in association with AZS, 2-AA, for TA100 (Table V). Table VI shows an decrease in mutagenic activity between 0.04 and 0.05 μl/plate of bee venom in the presence of NPD, for TA98, but in the presence of 2-AA this effect was not observed. The bee venom had an inhibition rate for mutagenic activity induced by DAU for the TA102 strain (Table VII) which varied from 25–80%, according to the increase in the venom doses used but in the presence of AAF this effect was not observed.

Toxicity assays were carried out parallel to the antimutagenic activity assays to find if the decrease in the number of revertants was due to the toxic effect of the drugs used. The greatest bee venom concentrations (0.05 μl/plate) when associated with AZS or 2-AA, and 4 μl of EEP/plate associated with DAU, had a weak cytotoxic effect.

DISCUSSION

The mutagenic activity assays permitted us to show that neither propolis nor bee venom induce an increase in the normal frequency of mutations in the three *S.*

TABLE III. Inhibition of Aflatoxin B_1 (AFB_1), 2-Anthramine (2-AA), Benzo(a)pyrene (B(a)P), and 4-Nitro-o-phenylenediamine (NPD) Induced Mutagenesis in *Salmonella typhimurium* TA98, by EEP

Compound	Dose (μg/plate)	EEP (μg/plate)	Rev/plate*	I (%)
AFB_1	0.5	0	1,178 ± 251	—
		0.25	885 ± 404	52
		0.5	465 ± 46	74
		1.0	DNU	DNU
		2.0	418 ± 35	76
		4.0	174 ± 35	90
2-AA	0.125	0	240 ± 53	—
		0.25	469 ± 19	0
		0.5	623 ± 16	0
		1.0	381 ± 128	0
		2.0	395 ± 32	0
		4.0	44 ± 4	82
B (a) P	1.0	0	310 ± 85	—
		0.25	165 ± 25	47
		0.5	144 ± 9	53
		1.0	118 ± 8	62
		2.0	133 ± 6	57
		4.0	174 ± 10	44
NPD	5.0	0	1,273 ± 288	0
		0.25	957 ± 212	0
		0.5	1,088 ± 133	0
		1.0	1,282 ± 253	0
		2.0	1,113 ± 334	0
		4.0	1,077 ± 150	0

*Each value represents the mean ± SD of six replicate plates from two separate experiments (three plates/experiment).
I = inhibitory effect; DNU = data not usable.

typhimurium strains (TA100, TA98, and TA102) with or without metabolic activation. The quantity of histidine supposed to be present in the EEP samples did not influence the results.

These results are relevant if the great numbers of people who use EEP daily are considered and also because this work intended to assess the antimutagenic effect of these two natural products.

Rao et al. [23] showed that a component of propolis identified as caffeic acid phenethyl ester (CAPE) inhibited the mutagenic activity of 3,2-dimethyl-4-aminobiphenyl in the TA100 and TA98 *S. typhimurium* strains. Propolis induced a dose-related reduction of approximately 40% and the bee venom 25–80% on the number of reverse mutations induced by DAU in the TA102 strain.

Among the mutagens that TA102 detects are X-rays, hydrogen peroxide, phenyl-hydrazine, and DAU that generate oxygen radicals which are proven mutagens and/or carcinogens [24].

Mutations in the TA102 strain in our work were induced by DAU, which probably created reactive forms of oxygen. However, the number of these mutations was significantly reduced in the presence of EEP and even more significantly in the presence of bee venom. It may be inferred that EEP and bee venom interfered in some way with the action of these radicals, perhaps acting as free radical scavengers.

TABLE IV. Inhibition of 2-Aminofluorene (AAF), Benzo(a)pyrene (B(a)P), and Daunomycin (DAU) Induced Mutagenesis in *Salmonella typhimurium* TA102, by EEP

Compound	Dose (μg/plate)	EEP (μg/plate)	Rev/plate*	I (%)
AAF	10	0	783 ± 40	—
		0.25	748 ± 85	4
		0.5	614 ± 152	22
		1.0	659 ± 54	16
		2.0	634 ± 63	19
		4.0	484 ± 23	38
B(a)P	1.0	0	597 ± 80	—
		0.25	853 ± 35	0
		0.5	498 ± 21	17
		1.0	636 ± 83	0
		2.0	550 ± 151	8
		4.0	645 ± 41	0
DAU	1.5	0	907 ± 172	0
		0.25	540 ± 54	40
		0.5	487 ± 97	46
		1.0	514 ± 40	43
		2.0	486 ± 88	46
		4.0	465 ± 49	49

*Each value represents the mean ± SD of six replicate plates from two separate experiments (three plates/experiment).
I = inhibitory effect.

Scheller et al. [25] showed that propolis has a radioprotective activity in mice exposed to 6 Gy of cobalt-60 gamma radiation and suggest that an antioxidant component present in EEP is responsible for the radioprotective effect. The antioxidant activity of EEP has been confirmed in other studies [26–28] which also attribute the ability of scavenging free radicals to EEP. Krol et al. [29] suggested that the antioxidative activity of propolis is due to its high flavonoid content, which makes up approximately 25–30% of its dry weight. The antioxidative effect of flavonoids has been attributed to their scavenging ability on peroxide ions, hydrogen peroxide, oxygen active forms, and lipid peroxide radicals.

TABLE V. Inhibitory Effect (I) of Bee Venom on the Mutagenicity of Sodium Azide (AZS) and 2-Anthramine (2-AA) in *Salmonella typhimurium* TA100

Compound	Dose (μg/plate)	Bee Venom (μl/plate)	Rev/plate*	I (%)
AZS	2.5	0	1,339 ± 349	—
		0.01	1,098 ± 179	18
		0.02	1,287 ± 282	4
		0.04	999 ± 135	25
		0.05	1,048 ± 205	22
2-AA	0.625	0	3,880 ± 86	—
		0.01	3,194 ± 246	18
		0.02	3,037 ± 52	22
		0.04	3,184 ± 102	18
		0.05	3,250 ± 65	16

*Each value represents the mean of six replicate plates from two separate experiments (three plates/experiment).

TABLE VI. Inhibititory Effect (I) of Bee Venom on the Mutagenicity of 4-Nitro-o-phenylenediamine (NPD) and 2-Anthramine (2-AA) in *Salmonella typhimurium* TA98

Compound	Dose (μg/plate)	Bee Venom (μl/plate)	Rev/plate*	I (%)
NPD	2.5	0	1,546 ± 382	—
		0.01	1,658 ± 267	0
		0.02	1,600 ± 281	0
		0.04	903 ± 279	42
		0.05	733 ± 291	53
2-AA	0.625	0	2,810 ± 407	—
		0.01	2,596 ± 251	7.6
		0.02	2,506 ± 56	11
		0.04	3,124 ± 124	0
		0.05	2,746 ± 186	2

*Each value represents the mean of six replicate plates from two separate experiments (three plates/experiment).

The activity of EEP as an antioxidant has also been confirmed, but other possible mechanisms are probably involved in its antimutagenic activity. Flavonoids have been show to inhibit the biotransformation of the procarcinogen B(a)P as measured by a decrease in aryl hydrocarbon hydroxylase activity [30,31]. In this study, the best results in terms of antimutagenic activity on indirect-acting mutagens were obtained by the use of EEP associated with B(a)P (TA100) or AFB_1 (TA98). Probably both chemical inactivation and inhibition of the S9 enzymatic activation of the mutagens could be involved. Therefore, based on our present knowledge it is only possible to speculate on the mechanisms of the antimutagenic activity of EEP.

Caffeic and chlorogenic acids are polyphenols which inhibit the mutagenic activity in AFB_1 and B(a)P by different mechanisms. In the case of B(a)P, the inhibitory effect is assumed to be due to the links in the acids with the active metabolite of B(a)P [32], while the phenol compounds do not covalently react with AFB_1 or its metabolite, but probably inhibit inactivating enzymes [33].

TABLE VII. Inhibitory Effect (I) of Bee Venom on the Mutagenicity of Daunomycin (DAU) and 2-Aminofluorene (AFF) in *Salmonella typhimurium* TA102

Compound	Dose (μg/plate)	Bee Venom (μl/plate)	Rev/plate*	I (%)
DAU	1.5	0	779 ± 294	—
		0.01	589 ± 228	25
		0.02	290 ± 111	63
		0.03	222 ± 27	71
		0.04	165 ± 35	79
		0.05	144 ± 45	82
AAF	10	0	426 ± 209	—
		0.01	490 ± 169	0
		0.02	649 ± 123	0
		0.04	540 ± 68	0
		0.05	578 ± 89	0

*Each value represents the mean of six replicate plates from two separate experiments (three plates/experiment).

Another provocative study on flavonoids has demonstrated that they are excellent and rapid substrates for catechol 0-methyltransferase. Quercetin is a common dietary flavonoid that is present in EEP. It is often mutagenic in various in vitro assays but is apparently not in chronically quercetin-fed mice, as it is rapidly detoxified by methylation. It is a better substrate than catecholamines and catecholestrogens [34]. Kaempferol, a related flavonoid which is not a catechol, does not cause mutations in Ames assays unless metabolically activated by S9, perhaps by hydroxylation to a catechol. These findings can certainly help rationalize the very tangled literature on quercetin and other flavonoids as mutagenic/antimutagenic agents [35].

In this study, besides the antimutagenic effect we observed that in some experiments EEP increased mutagenic activity when associated with mutagenic agents.

The chemical composition of propolis is very complex, with more than 160 components, mainly phenolics compounds. The phenolics compounds belong to three main groups: flavonoids aglicones, phenolics acids and their esters, and their relative concentrations depend on the origin of the samples [36,37].

Park et al. [38] studied the composition of propolis collected in Brazil, specifically in São Paulo State, in a region relatively close to where the propolis used in the present study was collected. A high flavonoid total was detected (40.8 mg/g propolis) and some were identified, such as galangin (9%), chrysin (3.8%), and quercetin (2%). Moreira [39] analyzed vitamin and amino acid concentrations in propolis samples commercialized in São Paulo. Histidine is among several amino acids detected and vitamins B1 and B2 but not vitamin C were also detected.

The chemical composition of *A. mellifera* venom has been investigated by several authors [40,41] but little is known about the mechanism responsible for its radioprotective activity. It inhibited DAU and NPD mutagenic activity.

Assays using AFB_1 and B(a)P were only carried out with EEP and not with bee venom, as propolis is a more widely used product.

The best mutagenic inhibition rates were obtained with EEP and AFB_1 (TA98), bee venom and DAU (TA102), and EEP and B(a)P (TA100), respectively, 90%, 80%, and 70%. Gasiorowski et al. [42], using todralazine, obtained a reduction in the mutagenic activity of some standard mutagens with direct and indirect action in the Ames test. Reduction exceeded 80% in some cases.

Ruan et al. [43] found that several natural foods caused different antimutagenic activity in TA100 and TA98 *S. typhimurium* strains exposed to AFB_1 and other mycotoxins. The inhibition rate varied from 37–87%.

Mejía et al. [44] studied the antimutagenic activity of lutein, a pigment for poultry use (oleoresin) and a pigment for human use (xantrophyll plus) against the mutagenic action induced by 1-nitropyrene, and obtained an inhibition percentage of 72%, 92%, and 66.2%, respectively.

Therefore, the mutagenic inhibition rates obtained in the present study are significant when compared with the values shown by other antimutagenic agents. The results obtained with the use of EEP and bee venom encourage investigation to clarify the action mechanisms.

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