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Sensitivity of Mice to Benzo(a)pyrene Skin Cancer following Acclimation to Cool and Warm Temperature[1] (41136)

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Abstract. Skin tumorigenesis induced by benzo(a)pyrene (BaP) in male C3H/HeJ mice was accelerated in a cool (T_a = 16°) and inhibited in a warm (T_a = 32°) environment, compared to the normal T_a = 23°. For example, repetitive, topical application of 0.2 mg BaP/week in 0.08 toluene resulted in (i) the mean tumor appearance time occurring 4–5 weeks earlier in cool and 2–3 weeks later in warm compared with the 21–22 weeks average in normal T_a; (ii) the average number of tumors per mouse increasing twice as fast in cool as in warm T_a; and (iii) shortened survival in cool, lengthened in warm. There were few metastases to internal organs at any temperature. Once initiated, tumors progressed similarly in all T_a's to squamous cell carcinomas. Cool and warm T_a per se had little effect on spontaneous tumors, survival, body weight, rectal temperature, or hair growth. However, skin temperature was 1–2° lower in cool and 1–2° higher in warm T_a than the normal 32–33°. Physiological adaptation was particularly evident in the 66% average higher food intake in cool and 63% lower in warm T_a. These results may help in defining environmental risks in cancer, in increasing the efficiency of cancer screening trials, and in exploring physiological modifiers of carcinogenesis.

The overall relationship of temperature to cancer is unclear. Manipulations which lead to hyperthermia appear to be useful in the treatment of neoplasms (e.g. (1, 2)). However, the picture is contradictory with regard to prolonged but less severe environmental temperature (T_a) changes to which some degree of physiological adaptation may occur. For example, sustained high T_a is reported variously to have no effect (3, 4) or to promote (5, 6) cancer in mice, to promote cancer in man (7), eels (8), and reptiles (9), but to be inhibitory in fish (10, 11); and sustained low T_a is reported to have no effect on mice (3, 4), to both promote (12, 13) and inhibit (14, 15) cancer in rats, to promote cancer in fish (10, 11), but to be inhibitory in eels (8). No two of these studies have utilized precisely the same tumor, T_a, or species. It would appear to be of value to attempt to clarify the effects of high and low T_a on cancer since the environmental conditions reported encompass situations likely to be encountered by man with changes in geographic location, season, or work place. Clarification of the interaction between T_a and neoplasia may also provide useful insight into the mechanisms of carcinogenesis.

Materials and Methods. *Carcinogen.* Benzo(a)pyrene (BaP) powder was obtained from Aldrich Chemical Company and its purity confirmed by spectrophotometry. Solutions of BaP were made up in toluene in concentrations permitting the weekly dose to vary from 2.0 to 0.1 mg. Stock solutions were kept in the dark. Application of the BaP solution was by automatic micropipet to the skin of the clipped area of the back under incandescent light. A day's application consisted of two drops of 0.02 ml each, separated by a few seconds, for a total of 0.04 ml. Two applications were made each week, separated by at least 1 day, for a total volume per week of 0.08 ml. Two groups of control mice were used: one

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received no treatment other than clipping of fur, the other received toluene only in the same amount and schedule as for BaP.

Acclimation. The ambient temperatures (T_a) chosen after some preliminary trials were 16 ± 1° for the cool room (C), and 32 ± 1° for the warm room (W), with the normal room (N) at 23 ± 2°. These T_a's induced adaptative responses without being unduly stressful, as indicated by general maintenance of body weight and colonic temperature (T_c) in the face of altered skin temperature (T_s) and food usage.

Animals. C3H/HeJ male mice were obtained from Jackson Laboratories at 5 to 7 weeks of age. They were housed 5 to a standard, plastic, shoe box cage on ground corncob litter (Anderson's bed-o'-cobs). Ears were punched for identification and hair was trimmed from the back with a fine-tooth electric clipper. Feed (Purina Rodent Laboratory Chow, 5001) and water were *ad lib* at all times. Lighting was clock controlled, 14 hr on−10 hr off in all rooms. After 5−7 days adjustment to normal vivarium conditions (23°), part of the mice population was moved directly to C (preset at 16°) and part to W (preset at 32°). Ten to fourteen days later, treatment with BaP began and continued until the mice died or were sacrificed. Primary treatment groups varied in size from 50 to 150 mice. Observations and measurements were recorded once a week for all mice. Hair was clipped at these times if required by regrowth. A record was kept of the frequency of clipping. Gross examination for metastases was performed at necropsy at which time selected tissues were preserved for histology. Histopathological evaluations were made by board-certified pathologists (K.M.K. or S.E.W.).

Observations and measurements. Weekly data collected for each mouse included the number of tumors visible at the general site of BaP application, the size of the largest tumor (length × width, each estimated to nearest mm), and whether ulcerated and invasive tumors were present. General appearance and behavior, e.g., keratinization, hyperactivity, etc., were also noted. Every other week, body weight was measured to the nearest ½ g on a spring balance. At 6- to 10-week intervals, T_c, T_s, and feed usage were evaluated. Data handling was aided by marking observations directly on computer cards at the animal location.

T_c was measured with either a Yellow Springs Instrument Company No. 402 thermistor probe or a Bailey No. IT-1 thermocouple probe (results were interchangeable). The probes were inserted via the anus approximately 2 cm and readings made within 10 sec. T_s was measured on the back at the site corresponding to where BaP would be applied, using either a YSI No. 409 surface probe or a Bailey No. SST-1 surface probe (results also interchangeable). The surface probes were held against the skin under slight tension with the help of a plastic rod and spring arrangement and readings made within 10 sec. Animals were restrained only by the tail during measurements. Food usage was measured by weighback of the feed container, neglecting wastage. Precautions were taken to ensure that all measurements fell at comparable times of the day for the three T_a's. Controls were used primarily for body wt, T_c, T_s, and food usage comparisons to avoid complications from tumors, but some measurements were made on BaP-treated mice.

Analysis and presentation of data. Visually fitted curves were used to demonstrate T_a effects on incidence of first tumor, number of tumors per mouse, and survival time. Statistically, the visually fitted curves were supplemented by weekly comparisons among T_a's, using χ^2 analysis for incidence and survival and t tests for number of tumors. At the conclusion of a trial, a mean tumor appearance time (MTAT ± SE) and mean survival time (MST ± SE) were calculated from the weekly recordings, and further comparisons made by t test. Linear regression analysis was applied to selected portions of the incidence data and was also used to examine tumor growth.

Results. *Incidence of first tumor.* In Fig. 1 curves have been fitted by eye for the incidence of first tumor in C, N, and W, for repetitive application of BaP at 0.2 mg/week. Tumors first appeared at the 5th week, with no clear difference due to T_a but a separation in incidence was soon evident

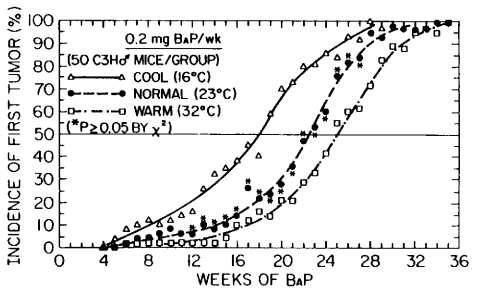

FIG. 1. Visual plot of the effect of cool (16°), normal (23°), and warm (32°) temperatures on the incidence of first skin tumor in C3H male mice, 50/group, treated topically with 0.2 mg BaP in 0.08 ml toluene/week.

with tumorigenesis being accelerated in C and retarded in W. The separation among C, N, and W persisted until close to 100% incidence was reached. From the 12th to the 27th week, 2×3 χ^2 statistics applied to each weeks data indicated that significant differences in incidence existed among the three T_a's. The maximum time separation among T_a's appeared to occur at the 50% incidence level (IT_{50}) which was reached by C about 4½ wks before N and by W 2½ after N (i.e., an estimated IT_{50} of 18 weeks for C, 22½ weeks for N, and 25 weeks for W). Examination of the central portion in the incidence curves of Fig. 1, between 15 and 85% incidence, suggests, and in fact regression analysis confirms, that the three lines are essentially parallel, indicating that the T_a effect is primarily on latency rather than on rate of appearance of first tumor.

Acceleration of tumorigenesis by C and inhibition by W was also seen with lower and higher doses of BaP (0.1 and 1.0 mg/week). In Table I, all trials involving incidence of first tumor are summarized in terms of a calculated mean tumor appearance time (MTAT). Row 3 [dose 0.2(A)] is the trial shown in Fig. 1. It may be seen that the calculated MTATs are within 1 week of the visual estimates of IT_{50}, and that the MTAT for C and W are significantly different from N. It may also be noted that the T_a effect is highly reproducible, since there is close agreement of trial 0.2(A) with trial 0.2(B), carried out a different time and in a different facility. It appears, though, that in terms of separation of the MTATs, the T_a effect becomes less evident as the BaP dose increases, and may be obscured around 2.0 mg/week. T_a had no measurable effect on spontaneous skin tumors: In fact only two to three tumors were observed in over 200 controls.

Average number tumors per mouse. The acceleration of skin tumorigenesis by C and its inhibition by W can perhaps be seen more clearly with the average number of tumors per mouse. An example is shown by the visually fitted curves in Fig. 2 for the 0.2 mg BaP/week dose. Significant difference between C and N began to be seen around

TABLE I. SUMMATION OF THE EFFECT OF ACCLIMATION TO MODERATE CHANGES IN ENVIRONMENTAL TEMPERATURE ON MEAN TUMOR APPEARANCE TIMES (MTAT) IN MALE C3H MICE TREATED REPETITIVELY WITH VARIOUS DOSES OF BaP

BaP dose (mg/wk)	No. mice (initially) and mean tumor appearance times (weeks ± SE)		
	Cool (16°)	Normal (23°)	Warm (32°)
2.0[a]	—	(100) 8.6 ± 0.29	(150) 8.2 ± 0.29
1.0	(69) 7.0 ± 0.38[b]	(74) 8.8 ± 0.48	(75) 10.0 ± 0.54[b]
0.2 (A)	(50) 17.4 ± 0.86[b]	(50) 21.8 ± 0.82	(50) 24.3 ± 0.83[b]
0.2 (B)[a]	—	(99) 21.4 ± 0.77	(150) 24.7 ± 0.72[b]
0.1	(50) 19.5 ± 1.01[b]	(50) 24.9 ± 0.75	(50) 26.5 ± 1.23

[a] Experiments carried out under conditions of facility and time different from those of other trials.
[b] Significantly different from Normal at $P \leq 0.05$.

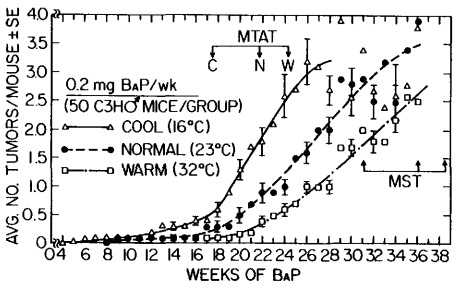

FIG. 2. Visual plot of the effect of cool (16°), normal (23°), and warm (32°) temperatures on the average number of tumors/mouse in C3H male mice, 50/group, treated topically with 0.2 mg BaP in 0.08 ml toluene/week. (MTAT = mean tumor appearance time from Table I and MST = mean survival time from Fig. 3.)

the 13th wk and between W and N around the 17th wk (by t test). In these trials, evaluation of carcinogenesis via average number of tumors per mouse appears useful only until approximately three tumors per mouse was reached; thereafter large week-to-week fluctuations and high variability occur in the count, probably because of concurrent mortality and coalescence of small tumors. It can be estimated from Fig. 2 that W would reach three tumors per mouse about 12 weeks later than C; or that when C reaches three tumors per mouse, at week 26 (actually 3.2 ± 0.40 tumors/mouse), W has less than one-third as many (actually 1.0 ± 0.14 tumors/mouse).

Inspection of Fig. 2 suggests that in addition to simple displacement in time of the curves for average number of tumors per mouse by T_a there might be also a change in slope of the curves. This was confirmed by linear regression analysis, applied to the central portion of each curve starting with the week when average number of tumors per mouse first reached at least one-half and ending with the week near two and one-half to three tumors per mouse when the count became erratic and/or reversed itself. The regression equations are (where Y = no. tumors/mouse and X = weeks): for C, $Y = 0.31X - 5.02$ ($n = 9$, $S_b = 0.012$, and $r = 0.99$); for N, $Y = 0.24X - 4.42$ ($n = 12$, $S_b = 0.017$, and $r = 0.98$); and for W, $Y = 0.16X - 3.30$ ($n = 14$, $S_b = 0.012$, and $r = 0.97$). Thus for this restricted part of the curve, the overall rate of tumor development in C is about twice that in W.

Survival and pathology. Once tumors were initiated, they progressed in all T_a's from the papilloma stage to ulceration and massive, invasive carcinomas, apparently leading finally to death. Survival curves for the 0.2 mg/week dose of BaP are drawn by eye in Fig. 3. They appear to be mirror images of the incidence curves, though displaced some 13–14 weeks in time, with survival prolonged in W and shortened in C. Significant differences are found among the 3 T_a's from about the 27th to the 43rd week. A calculated mean survival time (MST) from a summation of weekly mortality data shows the differences between C and N or W and N to be statistically significant, with values (±SE) of: C, 31.1 ± 0.97; N, 36.1 ± 0.93; and W, 38.5 ± 1.02. The 7.4-week difference between C and W is very close to the 6.9-week W-C Δ for MTAT (Table I). About 90% of the pooled controls are still alive when half the BaP groups are dead, indicating that T_a per se had little effect on survival during the trial interval.

The high mortality with BaP does not appear to be related to metastases to internal organs. Visceral tumors were relatively uncommon in these studies in any T_a. By gross necropsy, tumors were found in liver of six mice, lungs of four, and kidneys of only one out of several hundred examined. Histology by light microscopy placed the majority of skin tumors in the category of squamous cell carcinoma (16).

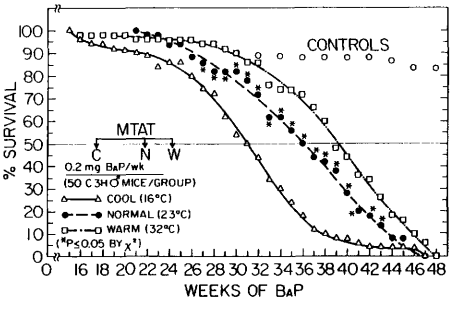

FIG. 3. Visual plot of the effect of cool (16°), normal (23°), and warm (32°) temperatures on percentage survival in C3H male mice, 50/group, treated topically with 0.2 mg BaP in 0.08 ml toluene/week. (Controls were no treatment and toluene only combined, 25 each, MTAT = mean tumor appearance time from Table I.)

Tumor growth. The observation that survival curves for BaP-treated mice in C, N, and W (Fig. 3) bore a parallel relationship to each other similar to the incidence curves (Fig. 1) suggested that once initiated, tumors progress at equivalent rates in the three T_a's. To examine tumor growth quantitatively, a regression analysis was made of tumor area vs time for the largest tumor of the first 10 mice in each T_a to show tumors. The starting point was the week in which a tumor reached an estimated 4 mm^2 and the comparison continued for 14 weeks, when mortality was about 20–30%.

Although the weekly tumor area measurements turned out to be highly variable, correlations between area and weeks were 0.85 or better and the regressions are therefore considered reliable. The respective regression equations are (where Y = tumor area in mm^2 and X = weeks): for C, $Y = 8.1X + 16.5$ ($S_b = 1.02$, $r = 0.92$); for N, $Y = 12.2X - 0.6$ ($S_b = 2.14$, $r = 0.85$); and for W, $Y = 12.0X - 2.5$ ($S_b = 1.32$, $r = 0.93$). Comparison among regression coefficients suggests that contrary to its accelerating effect on induction of tumors, C may actually slow subsequent tumor growth, whereas W has no effect on tumor growth.

Physiological changes. As an indication of the physiological response of the mice to C and W, body weight, food usage, colonic temperature (T_c), and skin temperature (T_s) are presented in Table II for controls. The three periods shown (7–8, 22–24, and 30–34 weeks) bracket roughly the BaP treatment period.

There is little indication from body weight at least (Table IIA) that either C or W adversely affected growth. While there may be a 1- to 2-g gain in W compared to either C or N during the first 2 months, by the 22nd wk, the three groups were less than a gram apart. However, the cost of maintaining normal growth was clearly different, since food usage (Table IIB) was consistently higher in C and lower in W than in N. Overall, C used about 66% more food than N whereas W used about 63% less than N. The average usage of C was about 2.7 times greater than that of W.

T_c (Table IIC) shows a mixed and inconsistent pattern but no real indication of either hyperthermia or hypothermia. At 8 weeks, C and W were identical, but both were a significant 0.9° below N. At 24 weeks, C and W were within 0.3° of N but in opposite directions. At 34 weeks there

TABLE II. SAMPLING OF BODY WEIGHTS, FOOD USAGE, AND BODY TEMPERATURES OF CONTROL C3H MICE (NO TREATMENT AND/OR TOLUENE ONLY) ACCLIMATED FOR VARIOUS PERIODS TO COOL (16°), NORMAL (23°), AND WARM (32°) AMBIENT TEMPERATURES

Weeks	Cool	Normal	Warm
A. Body wt (g)			
7	(25)[a] 28.2 ± 0.34[b]	(25) 28.5 ± 0.31	(25) 29.9 ± 0.39
22	(25) 31.6 ± 0.36	(25) 31.0 ± 0.40	(25) 30.8 ± 0.64
30	(20) 32.6 ± 0.50	(20) 31.9 ± 0.46	(23) 32.1 ± 0.50
B. Food usage (g/mouse/day)			
8	(8) 5.5 ± 0.15	(21) 3.1 ± 0.13	(21) 2.1 ± 0.15
24	(15) 5.9 ± 0.25	(15) 3.8 ± 0.33	(15) 2.1 ± 0.13
34	(18) 6.6 ± 0.27	(24) 4.0 ± 0.30	(18) 2.4 ± 0.12
C. Colonic temperature (°)			
8	(17) 36.4 ± 0.25	(17) 37.3 ± 0.16	(17) 36.4 ± 0.18
24	(20) 37.7 ± 0.24	(20) 37.4 ± 0.20	(20) 37.1 ± 0.18
34	(20) 37.0 ± 0.14	(20) 37.3 ± 0.14	(20) 37.2 ± 0.10
D. Skin (back) temperature (°)			
8	(17) 30.7 ± 0.21	(17) 32.7 ± 0.26	(17) 34.5 ± 0.31
24	(20) 31.4 ± 0.32	(20) 32.0 ± 0.32	(20) 33.8 ± 0.16
34	(20) 30.4 ± 0.12	(20) 32.5 ± 0.23	(20) 33.9 ± 0.15

[a] Numbers in parentheses indicate number of animals.
[b] Data are expressed as the mean ± SE.

was essentially no difference among the three T_a's. Skin temperatures were clearly different, however. T_s (Table IID) was consistently 1–2° lower in C and 1–2° higher in W than in N. Overall, C averaged a highly significant 3° below W.

Body weight, food usage, T_c, and T_s were measured in several BaP-treated groups before tumors had become massive and survival depressed and found to be very similar to control values. As measured by numer of times clipping was performed (roughly once every 4 weeks) hair growth was similar in all T_a's. The only other observation that may be relevant to the T_a effect was that keratinization appeared greater at the site of BaP application in W than in C.

Discussion. These results indicate that BaP-induced skin tumorigenesis in C3H male mice can be accelerated by acclimation to a cool T_a (C, 16°) and inhibited by acclimation to a warm T_a (W, 32°). Mean appearance time of first tumor (i.e., latency) occurred 4–5 weeks earlier in C and 2–3 weeks later in W than in normal T_a (N, 23°). Also after one-half tumor per mouse was reached, average number tumors per mouse (i.e., tumor mass) increased twice as fast in C as in W. Once induced, tumors progressed at approximately equal rates in C, N, and W from papillomas to invasive squamous cell carcinomas. Mortality occurred proportionally sooner in C and later in W, presumably due to the differences in skin neoplasia, since there were few internal metastases in any T_a, and T_a itself had little effect on survival in controls.

As to possible mechanisms for the effect of T_a on tumorigenesis, several factors would seem to be ruled out. One is any connection to the well established inhibitory effects of hyperthermia on tumors (1, 2). The 32° warm T_a did not increase T_c above normal and while T_s was elevated 1–2° over normal, it was still only in the 33–34° range, well below the 40–42° used in the hyperthermic studies. More directly, measurements of tumor size show that once induced, skin tumors in W were not inhibited but rather increased in area at a rate equal to or greater than in N or C. Auerbach *et al.* (3) also noted relatively comparable tumor growth between 15 and 33° T_a for a mammary adenocarcinoma (C755) in mice. Differences in skin blood flow mediated by T_a could possibly affect tumorigenesis, except that Auerbach *et al.* (3) were unable to find confirmatory vascular changes in their acclimated mice.

Since the number of times that fur needed to be clipped was similar in the three T_a's, there is no evidence that altered rate of hair growth influenced the results. It also seems unlikely that differences in persistence of the diluent at the application site could account for the effects in view of the low potential of toluene as a carcinogenic enhancer (17) and the essential absence of tumors in toluene controls at all T_a's. However, some interaction between toluene and T_a on tissue metabolism seems possible (18). Finally, neither C or W appear to have produced any debilitating stress in the mice as suggested by the maintenance of body weight, T_c, and normal survival in controls.

Since BaP must be activated by cellular enzymes before it is effective as a carcinogen, and can also be detoxified by enzymatic activity (19), changes in temperature or metabolism in the mouse may well alter the balance between the activation–detoxification process. Such effects may perhaps be occurring locally in the epidermal cells at the site of application of BaP, in view of the 1–2° lower T_s in C and 1–2° higher T_s in W. Or the activation–detoxification balance may be altered by the acclimation-mediated changes in systemic metabolism, as indicated by the 66% increase in feed usage in C and the 67% decrease in C. Acclimation is known to be associated with a variety of metabolic and enzymatic changes (20).

These results may have some bearing on the assessment of the carcinogenic hazards of man's environment, on the design and efficiency of cancer screening trials, and perhaps in helping delineate endogenous mechanisms which modify carcinogenesis. However, it should be noted that this study was limited to the topical application of BaP to the male C3H/HeJ mouse and therefore broad extrapolations of the effect of T_a on cancer are not warranted. Unfortunately, the literature on the thermal environment and cancer does not help in making generalizations either. Conflicting results

on eels (8), reptiles (9), and fish (10, 11) might be put aside on grounds that T_a is likely to affect carcinogenesis in poikilotherms differently than in mammals. However, results with mammals are equally conflicting. In rats, 0 to 3° increased the incidence of Walker 256 carcinoma (12) and the growth of salivary gland tumors (13), linked in both cases to augmented metabolism. On the opposite side, a 2° environment reduced tumor incidence following injection of methylcoholanthrene (MCA) (14) or irradiation (15), both also linked to increase metabolic activity.

Several reports on mice diverge from our results. Wallace *et al.* (6) found that in comparison to "cold" environment (18°), a tropical environment (33°) aggravated tumorigenesis in male C3H mice. The hot T_a shortened by 2 weeks (statistically significant) the latency of tumors induced by MCA injected sc. Also, an MCA-induced sarcoma, transplanted to C3H mice, grew rapidly in the hot room but slowly or even regressed in 18°. Freeman and Knox (5) found that rate of development of uv-induced squamous cell skin tumors was higher in both male and female albino mice kept at elevated T_a (32°) than at room T_a (24°). The 32° environment per se also markedly increased mortality, including controls. Jochimsen *et al.* (4) reported that percentage takes of Ehrlich ascites tumor cells transplanted sc into Swiss Webster female mice were independent of T_a (2, 21, and 31°). Both the low and high T_a inhibited growth of the mice. In C57Bl/6 mice (3), T_a's of 15 or 33° had little effect on transplanted C755 mammary adenocarcinoma.

Greater incidence of skin cancer is reported for man in warmer (higher) than cooler (lower) latitudes but it is unclear whether this is related to elevated T_a, increased uv exposure, or higher concentration of chemical carcinogenes in the tropics (see comments in (7)). We are unable at this point to reconcile these conflicting results except to note that no two studies have examined precisely the same neoplasm, species, or environmental conditions. It may be that the T_a effect is quite specific with respect to neoplasm and species.

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