CARCINOGENICITY OF SYNCRUDES RELATIVE TO NATURAL PETROLEUM AS ASSESSED BY REPETATIVE MOUSE SKIN APPLICATION

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ABSTRACT

The relative carcinogenicities of coal and shale derived liquid crudes was compared with a composite blend of natural petroleum using discontinuous exposure of mouse skin. All of the syncrudes were carcinogenic while the natural crude composite was negative following three times weekly application of 50% w/v solutions for 22 weeks followed by a 22 week observation period. In addition to eliciting progressive squamous carcinomas the syncrudes were also capable of inducing persistent ulcerative dermatitis. This inflammatory or necrotizing potential appeared to be inversely proportional to the carcinogenicity of the material. A measure of the relative solubility of the materials in mouse skin was obtained by quantitation of native fluorescence in frozen sections of skin. There appeared to be a general, although non-quantitative association between fluorescence intensity in sebaceous glands and carcinogenicity in epidermal cells, however it will be necessary to examine a greater number of samples to establish such a correlation.

The feasibility of using athymic mice to compare the relative in vivo susceptibility of intact human and mouse skin to carcinogenic hydrocarbons has been evaluated. While the approach is technically feasible, the small proportion of grafts which survive indefinitely are evidence that many technical improvements will be necessary before this approach can be exploited.

INTRODUCTION

The production of liquid hydrocarbons from coal or oil shale is inevitable. The only questions are when and by what methodology. Historical evidence suggests that crude shale oil (1) and coal derived liquids (2) are more potent skin irritants and carcinogens than natural petroleum. To determine whether this is equally true for similar materials, produced by contemporary technologies, it will be necessary to quantitatively compare their relative carcinogenicities using experimental animals. In order to apply the animal bioassay data to the question of human occupational exposure it will be necessary to establish the degree to which mouse skin differs from human skin in its responsiveness to topically applied whole crudes. The availability to us of genetically defined athymic nude mice (3) may facilitate this comparison, however many technical problems remain unsolved. The purpose of this presentation is to relate findings of exploratory experiments as well as to describe one approach to the problem of determining the bio-availability of syn-crudes in the intact skin.

MATERIALS AND METHODS

The materials used in these experiments were obtained from various sources. The cooperation of these facilities is gratefully acknowledged. The samples included two coal liquids: Coal liquid A was a centrifuged oil produced by a synthoil process under development and was provided by the Pittsburg Energy Research Center. Coal liquid B was a heavy oil produced by the COED process from Western Kentucky Coal and was provided by the FMC Corporation. In addition to the two prototype coal liquids a shale oil crude produced in the 150 ton simulated in situ retort run was provided by the Laramie Energy Research Center. To place the data in perspective a composite natural petroleum was included that consisted, by volume, of the following natural crudes: 20% Wilmington, California; 20% South Swan Hills, Alberta, Canada; 20% Prudhoe Bay, Alaska; 20% Gach Sach, Iran; 10% Louisiana-Mississippi Sweet and 10% Arabian light. The composite natural crude was provided by the Laramie Energy Research Center.

Animal Exposure

A mixed solvent consisting, by volume, of 70% Acetone 30% cyclohexane (AC) was found that would satisfactorily suspend (with sonication) or dissolve all of the materials. For the skin fluorescence studies solutions of each of the materials were placed in preweighed glass vials and the AC solvent evaporated to constant weight with a gentle stream of air under ambient conditions. The weight of "non-volatile" solids was

determined and an appropriate volume of solvent added to yield solutions containing 20 mg/ml, total solids. Skin carcinogenesis studies were done with the native materials (without adjusting for total solids) by the addition of the AC solvent to a final solute concentration of 50% (w/v). The resulting dilute crudes were sonicated briefly to achieve dispersion. Fresh solutions were prepared weekly.

For the fluorescence studies 10-12 week-old male C3Hf/Bd mice were shaved and those determined not to be in active hair growth were selected. Fifty ul of the 20 mg/ml solution was applied to the dorsal skin. In separate experiments this amount of AC solvent covered an area of approximately 5 cm^2. At 1, 4, 24 hours and 8 and 14 days after exposure three mice treated with each material were killed by CO_2 inhalation. Exposed skin was excised, frozen in liquid nitrogen and sectioned by cryostat. Sections were mounted in non-fluorescing medium and fluorescence intensity was determined using a procedure described elsewhere in greater detail (4).

Dermatologic effects were evaluated by Monday, Wednesday, Friday application of 50 ul of the 50% solutions to the shaved dorsal skin of 30 C3Hf/Bd mice. Separate groups were treated with each material commencing at 10-12 weeks of age. Exposure continued for 22 weeks after which time the mice were held for an additional 22 weeks without additional manipulation other than periodic shaving. During both the exposure period and the follow-up, the time of first skin tumor observation and

character of non-neoplastic skin changes was recorded. Progression of individual lesions was monitored and the location, relative size and character of individual lesions recorded on scale drawings. Mice dying throughout the study or killed at its termination were necropsied. Tissues were subjected to histologic examination to establish the following: (1) Identity and specificity of neoplasms arising in the treated skin; (2) The proportion of mice with tumor, metastatic either to regional lymphnodes or distant sites; (3) The detection of systemic injury arising as a consequence of percutaneous absorption of the applied materials.

RESULTS

Equivalent amounts of the materials applied at the same dose rate resulted in widely differing skin responses. The data summarizing the clinical and anatomic responses are given in Table 1. Of interest is T-1 the lethality of the squamous epidermal tumors induced. Only a portion of this lethality could be attributed to systemic metastasis. Of 30 mice exposed to shale oil, 14 developed progressive epidermal neoplasms and these 10 died before the end of the study, four with histologically confirmed metastasis. From first observation of tumor, average time to death was similar for all materials.

In addition to inducing autonomous and progressive epidermal neoplasms the materials were also capable of inducing dermatitis with

varying degrees of efficiency. In its mildest form skin irritation was expressed by a transient epiliation and well demarkated depigmentation of the exposed skin. This was immediately followed by stimulation of the hair follicle and a subsequent persistent hypertrichosis out of phase with the systemic hair cycle. These changes were manifest in mice exposed to coal liquid A and the composite natural crude. More severe irritation occurred with shale oil and coal liquid B. With these materials hair growth stimulation progressed into frank epidermal ulceration, permanent epiliation and cicatrization. Table 1 also indicates the number of mice, out of the starting group of 30, that developed ulcerative dermatitis following exposure to each material. The incidence is given at the end of exposure (22 weeks) and again at the end of the clinical follow-up (44 weeks). Histologic studies of the ulcerative lesion revealed no evidence of occult malignancy. The central, denuded area consisted of a granulation tissue covered by a thin, insipissated, crust. Adenexal structures were absent from the dermis. Based on the absence of hair follicles, we propose that failure of the lesions to heal was the result of loss of epithelial stem cells which normally would participate in reepitheliazation. The denuded dermis has responded by fibrosis. Healing, when and if it eventually occurs is dependent upon contraction of connective tissue which brings the wound margins into opposition.

It is likely that composition of these materials would influence their solubility in skin and consequently their biological activity. To obtain a measure of the skin penetrability, distribution and persistence of these materials we have used their natural fluorescence to monitor their tissue distribution. Our working hypothesis is that biological activity should be positively correlated with the magnitude and persistence of fluorescence and possibly also localization.

The results of these measurements are given in Table 2. Several differences are readily apparent. Coal liquid A had greater initial fluorescence than any of the other materials and was also observed to persist for at least 14 days following a single application of approximately 200 ug/cm^2 total solids. At the same concentration clearance of both shale oil and the composite crude appeared to be more rapid than either coal liquid. While there appears to be a positive association between total fluorescence, persistence of fluorescence and carcinogenicity, a quantitative correlation cannot be inferred on the basis of these limited data. For example, shale oil and natural petroleum exhibited similar fluorescence intensity as well as skin clearance but shale oil was much more carcinogenic in mouse skin. The most probable explanation for these discrepancies is that fluorescence is not totally correlated with carcinogenicity. Obviously, if fluorescence is to be used as a method for monitoring the work place (5) it will be necessary to determine the degree to which it is correlated and thus predictive of carcinogenicity for each specific composite exposure.

T-2

In order to compare human and mouse dose response relationships for known carcinogens it has been necessary to compare experimental data with epidemiologic data, a comparison that is never easy and usually retrospective. To avoid these limitations we propose determining the species response variable directly. Our approach will be to graft normal human skin onto athymic nude mice. After healing is complete the grafted human skin will be challenged with pure carcinogenic hydrocarbons as well as syncrudes in order to compare the relative sensitivity of human skin to that of similarly grafted mouse skin *in vivo*.

Two exploratory experiments have been done to determine the feasibility of this approach. Groups of specific pathogen free athymic mice were grafted with approximately 2 cm elliptical pieces of adult human skin. In one experiment full thickness skin was used. The only preparation was removal of the subcutaneous fat. In the second experiment skin thickness was reduced using an electro-keratome to approximately 1 mm. The mice were anesthetized with 0.1% pentobarbital, graft beds prepared and human skin implanted under asceptic conditions. Bandages were removed at 11 days and the status of the grafts monitored periodically. After an initial slough of the original donor tissue re-epithelization was observed. Mouse and human skin could be easily distinguished in that the mouse skin was albino and the human skin was pigmented. Over the succeeding weeks the majority of human skin grafts

underwent progressive contracture and eventually were lost. Disappearance of the grafts was not accompanied by clinical evidence of an active or inflammatory rejection process. Our current hypothesis to account for loss of the grafts is that connective tissue, induced during the initial healing process, contracts and decreases the blood supply to the graft which, deprived of nutrition, slowly recedes. An assessment of the persistence and integrity of the grafts from each of the initial studies is given in Table 3. It should be noted that in spite of many known and unknown technical problems, a small percentage of the grafts in each of the experiments have persisted indefinitely. T-3

DISCUSSION

The present paper demonstrates that syncrudes differ greatly from one another as well as from natural petroleums, both as skin irritants and carcinogens, in vivo. The index of relative carcinogenicity (6), in order of decreasing potency was 42, 30, 19 and 0 for coal liquid A, shale oil crude, coal liquid B and natural petroleums, respectively. These are considered to be minimum estimates since they are based upon a single, high dose. It is possible that at the 50% concentration used to induce tumors, expression of neoplasms could have been prevented or delayed in one or more of the test groups by toxic constituents of the mixtures.

The observed lethality of the tumors was unexpected. In previous experiments in which various hydrocarbon and non-hydrocarbon carcinogens have been applied to the same mice under identical conditions, the epidermal tumors induced, while locally malignant, seldom contributed to death (7). An important difference between this and previous experiments was that in the present experiment exposure to the skin carcinogen was discontinuous. While there are a number of possible explanations for the observed lethality the hypothesis that we favor is that discontinuous or short term exposure, coupled with an extended clinical follow up, encourages more rapid clinical progression of the incipient neoplasms. This could be because toxic or inhibitory constituents (which may or may not include the active carcinogens) of the mixtures may kill or inhibit the growth of neoplastic epidermal cells. If future experiments verify this observation then discontinuous exposure may be the preferred means to assess the carcinogenicity of complex mixtures.

Fluorescence of the materials in skin has proven to be a convenient and relatively precise method to detect the presence as well as demonstrate the distribution and persistence of the various petroleum crudes in mouse skin. Many refinements of the approach used are possible and are being actively pursued. We are interested in calibrating the method using known reference carcinogens such as benzo[a]pyrene. It may also be possible to use selective, narrow band pass filters, to achieve more specificity.

It would appear to be feasible to use athymic nude mice grafts with human skin to address the question of species dependent differences in target tissue susceptibility. However, based upon somewhat disappointing long term graft retentions we anticipate that many technical issues remain to be resolved before meaningful data can be obtained.

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Table 1. In Vivo Effects of Continuous Topical Application of Various Crude Petroleums

Compound	Skin Tumor Incidence* (%)	Tumor Latency (days ± S.E.)	Deaths	% Survival		Average Time to Death with Tumor (days ± S.E.)	Number of Animals with Ulcerative Dermatitis	
				Tumor Bearer	Non Tumor Bearer		At 22 weeks	At 44 weeks
Shale oil	47	154 ± 9	11	28	94	117 ± 10	9	2
Coal liquid A	63	149 ± 8	6	68	100	103 ± 11	0	0
Coal liquid B	37	191 ± 14	1	91	100	90	20	5
Composite crude	0	0	0	—	100	—	0	0

*Squamous epidermal tumors present either at death or the end of the experiments.

Table 2. The Intensity of Fluorescence in Sebaceous Glands at Various Times after Topical Application of Petroleum Liquids[1]

Time after application	Average Fluorescence Intensity[2] (±95% confidence limit)			
	Coal liquid A	Coal liquid B	Shale oil	Natural crude
1 hr	540(123)	172(49)	70(21)	137(44)
4 hrs	422(101)	185(61)	55(12)	140(88)
24 hrs	454(114)	190(24)	109(21)	135(40)
8 days	112(21)	43(6)	29(1)	27(1)
14 days	42(11)	23(1)	25(1)	25(1)

[1] $\sim$200 ug/cm^2

[2] 1/seconds X 1000

Table 3. Survival of Full Thickness Human Skin Grafts on Specific Pathogen Free Athymic Nude Mice

Experiment	Time of observation (days)	Effective no. of mice	No. of Animals Whose Grafts Were: Retained	Questionable	Rejected
1	11	44	44	—	—
	22	44	—	43	1
	49	44	12	26	6
	131	44	8	—	36
	225	16	7(2)[a]	—	9
2	11	50	50	—	—
	94	50	20(4)[a]	—	30

[a]The numbers in parentheses refer to the subset of animals who retained human epidermis, as well as dermis.