lowed by an indirect assay based on the ability of diol epoxide to nick supercoiled Col E1 DNA. The half-life reported here is substantially longer than values obtained by Wood *et al.* who used mutagenesis and cytotoxicity assays [A. W. Wood, P. G. Wislocki, R. L. Chang, W. Levin, A. Y. H. Lu, H. Yagi, O. Hernandez, D. M. Jerina, A. H. Conney, *Cancer Res.* **36**, 3358 (1976)].
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20. Col E1 DNA was obtained from Professor J. Hearst of the University of California at Berkeley. ^{14}C-Labeled *anti*-diol epoxide (53.7 mc/mmole) was obtained from Midwest Research Institute. We thank Dr. T. Meehan for critical reading of the manuscript. Supported by National Cancer Institute contract YOI-CP50203 and the Division of Biomedical and Environmental Research of the U.S. Energy Research and Development Administration.

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Interferon: An Inducer of Macrophage Activation by Polyanions

Abstract. *Purified mouse fibroblast interferon (IF) directly rendered resting macrophages tumoricidal. The physicochemical properties and species specificity of the stimulatory agent fall within the present definition of IF. Since a number of polyanions induce macrophage IF, the antitumor and antimicrobial activities may result from the ability of newly released IF to modify macrophage activity.*

Macrophages appear to have an extremely important if not crucial role in maintaining the purity of the internal environment, in surveillance against spontaneously arising malignant cells (*1*), and in controlling tumor cell growth and dissemination [for review, see (*2*)]. A number of agents allow the macrophage to reach an "activated" state whereby the cell exhibits increased phagocytosis (*3*), elevated degradative enzyme activities (*4*), and enhanced cytostatic and cytocidal effects on transformed cells (*5–7*). We have previously shown that nonspecific macrophage activation correlates well with the capacity to enhance host resistance against neoplasia (*6*). Macrophages recovered from these treated animals were selectively cytotoxic for tumor cells by a nonphagocytic contact mechanism which is poorly understood.

In view of these findings on macrophage activity following drug administration in vivo, it seemed appropriate to study further whether these agents might directly activate macrophages in vitro. This information would greatly aid in understanding the mechanism of macrophage activation and may allow for the identification of pharmacologic agents which possess the ability to specifically modify the functional activity of the reticuloendothelial system.

We used a modification of the method of Alexander and Evans (*8*) to measure the ability of drugs to directly activate macrophages in vitro (*9*). Male CD_2F1 mice were obtained from the Mammalian Genetics and Animal Production Section of the National Institutes of Health, Bethesda, Maryland. Noninduced peritoneal exudates were harvested with heparinized medium, and macrophages were purified by adherence. Approximately 1×10^6 macrophages were seeded in 3.5-cm Falcon plastic dishes in 1 ml of standard growth medium consisting of RPMI-1640 supplemented with 20 percent fetal calf serum. An established line of MBL-2 leukemia cells was adjusted to 5×10^4 cells per milliliter of growth medium, and 2-ml portions were immediately admixed with the macrophage cultures. Drugs were made up in tenfold concentrations, and 0.3 ml was added to the cell mixtures. All cultures were incubated at 37°C in an atmosphere of 5 percent CO_2 in air, and viable leukemia cells were counted daily with a hemacytometer. The ratio of macrophages to target cells was 10:1 at the beginning of each experiment. The percentage of growth inhibition of MBL-2 cells due to macrophage-drug interaction was calculated by comparison to MBL-2 cells grown in the presence of normal macrophages alone.

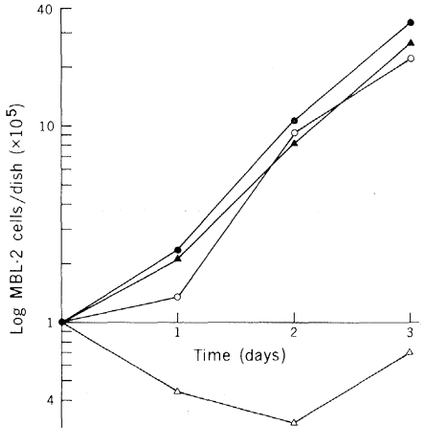

Fig. 1. Effect of peritoneal macrophages and purified mouse interferon on the growth of MBL-2 leukemia cells in vitro. MBL-2 cells (10^5) were grown in 3 ml of RPMI-1640 growth medium. (•) Leukemia cells alone; (○) leukemia cells in the presence of 10^6 normal macrophages; (▲) leukemia cells in the presence of IF (1000 units per milliliter of growth medium); (△) leukemia cells plus 10^6 macrophages plus 1000 units of IF per milliliter of growth medium.

Using the system described above, we showed that pyran copolymer, poly(I) · poly(C) (polyinosinylate · polycytidylate) and dextran sulfate rendered macrophages cytotoxic for leukemia cells in vitro (*9*). The effect was dose-dependent and required greater than 24 hours after exposure to drug. Similarly, the administration in vivo of these agents produces a highly dose-dependent macrophage activation (*10*). The growth inhibition of tumor cells appeared to result from a modification of the macrophages themselves, since neither macrophages nor drug alone interfered with MBL-2 proliferation. The primary mechanism of cytotoxicity did not involve toxic drug metabolites or cytotoxins liberated by the macrophage cultures (*9*). Cell-to-cell contact appeared to be involved in cytostasis since tumor cells were observed to aggregate around and adhere firmly to drug-activated macrophages.

The mechanism of direct activation of macrophages by these nonspecific agents appeared to be related to the common polyanionic character of these molecules. We therefore examined a variety of polyanions for the ability to render macrophages cytotoxic (Table 1). Whereas heparin and poly(A) · poly(U) (polyadenylate · polyuridylate) had a similar stimulatory effect ($P < .001$) on macrophage function, single-stranded RNA was not effective. Control agents consisting of DEAE-dextran, levamisole, and bovine serum albumin were without significant effect.

In view of these observations and the fact that macrophages elaborate and secrete interferon (IF) after polyanion treatment (*11*), we examined the question of whether IF was involved in macrophage activation. Recent observations that crude IF preparations can enhance macrophage phagocytosis in vivo (*12*) and in vitro (*13*) and that the lymphokines (migration inhibition factor and type II IF) cannot be physically separated (*14*) support the concept that IF may regulate macrophage activity. Of particular interest are the findings of Gresser *et al.* that (i) the antitumor effects of IF are host-mediated and not due to direct inhibitory effects on tumor cell growth (*15*) and (ii) that phagocytosis of tumor cells by macrophages was ob-

served from smears obtained from the peritoneal cavities of tumor-bearing mice treated with mouse brain IF, but not from untreated mice or mice treated with normal brain extract (*16*).

Purified mouse fibroblast IF (specific activity, 2×10^7 units per milligram of protein) and human leukocyte IF were provided by Dr. K. Paucker (Medical College of Pennsylvania, Philadelphia). Interferons were purified by affinity chromatography on antibody globulin to IF supported on Sepharose (*17*). Purified human fibroblast IF (specific activity, 5×10^7 units per milligram of protein) was a gift from Dr. J. S. Horoszewicz (Roswell Park Memorial Institute). Crude fibroblast IF (mouse) was purchased from Litton Bionetics.

The presence of purified mouse IF at 1000 unit/ml in the culture medium rendered peritoneal macrophages cytotoxic for MBL-2 cells (Fig. 1). The IF preparations alone did not inhibit MBL-2 cell growth. In contrast to that of polyanions, IF's effect on macrophages was observed as early as 24 hours after incubation began. Similar results were obtained with crude IF prepared from a heterologous mouse fibroblast line. Macrophages treated with IF showed accelerated spreading on plastic and had prominent granulation of the cytoplasm, as compared to control cultures (Fig. 2, a and b). Phagocytosis was not primarily involved in the tumoricidal process. To test whether the inhibition of MBL-2 proliferation resulted from the release from macrophages of a soluble factor into the culture medium, cultures of macrophages were incubated first with IF (1000 unit/ml). The medium was decanted after 48 hours and used as a culture medium for fresh leukemia cells. These conditioned media were without effect on MBL-2 cell growth, an indication that toxic drug metabolites or cytotoxins were not elaborated into the culture medium by macrophages exposed to IF.

To test the dose-dependency of macrophage activation, we added mouse fibroblast IF to macrophage cultures at doses from 10^4 to 10^1 unit/ml, and the macrophages were immediately challenged with leukemia cells. Growth inhibition was assessed 48 hours later. Inhibition was greatest at 10^4 and 10^3 unit/ml (89 and 93 percent, respectively), but was still present at 100 unit/ml (29 percent). A significant effect was not evident at 10 unit/ml.

Finally, to ascertain whether the physicochemical characteristics of our macrophage stimulating factor in the IF preparation were consistent with the antiviral properties of IF, we conducted temperature-sensitivity studies with the purified mouse IF for the purpose of assessing whether heat-inactivation of the antiviral properties of IF correlated with the ability to render macrophages cytotoxic. Preparations of IF were exposed to either 60°, 80°, or 100°C for 30 minutes. Both activities showed similar kinetics with >60°C required for inactivation. Similarly, activity was retained at *p*H 2.0, but was abolished by trypsin treatment (100 μg/ml for 30 minutes at 37°C). Moreover, there was apparently a species-specific requirement for the source of IF, since human IF preparations showed a reduction of approximately two logs of activity when tested on mouse macrophages. Significant activation required at least 10^4 units per milliliter of human IF. These observations are consistent with the characteristics of antiviral activity of IF.

Previous studies show that polyanions or IF-containing preparations have direct antimitotic effects on tumor cells (*18*). Primary tumor preparations frequently contain a high percentage of resident macrophages (*19*). Our results suggest that if primary cultures of tumor cells are used, contaminating macrophages could be activated by polyanions or IF-containing preparations in vitro and could account for the reported cytostatic effect.

An alternative mechanism of macrophage activation results after macrophages are "armed" by a soluble product from T (thymus-dependent) lymphocytes. The macrophages require subsequent exposure to sensitizing antigen to exert their tumoricidal effect. Such a pathway is required for macrophage activation by bacillus Calmette-Guerin (BCG) (*20*). It was shown that macrophages harvested from mice that had been inoculated with BCG 10 days previously were not cytotoxic, but could be

Table 1. Ability of various agents to activate murine peritoneal macrophages in vitro.

Drug	Source	Growth inhibition* at 48 hours of			
		100 μg	10 μg	1 μg	100 ng
Pyran copolymer	Hercules Research Center†	+	++	−	−
Heparin	Sigma‡	−	++	++	−
Poly(A)·Poly(U)	P-L Biochemicals§	−	+	−	−
Dextran sulfate	Pharmacia‖	−	+	−	−
Poly(I)·Poly(C)	Miles Laboratories¶	−	−	++	−
Poly(I)	Calbiochem#	−	−	−	−
Poly(C)	Calbiochem#	−	−	−	−
DEAE-dextran	Pharmacia‖	Toxic	−	−	−
Levamisole	Janssen Pharmaceutica**	−	−	−	−
Bovine serum albumin	Calbiochem#	−	−	−	−

*The minus sign (−) indicates MBL-2 growth inhibition at <25 percent; +, between 25 and 50 percent; ++, >50 percent of control value. The data summarized results from at least two individual experiments involving triplicate determinations each. †Wilmington, Del. ‡St. Louis, Mo. §Milwaukee, Wis. ‖Uppsala, Sweden. ¶Elkhart, Ind. #Los Angeles, Calif. **Beerse, Belgium.

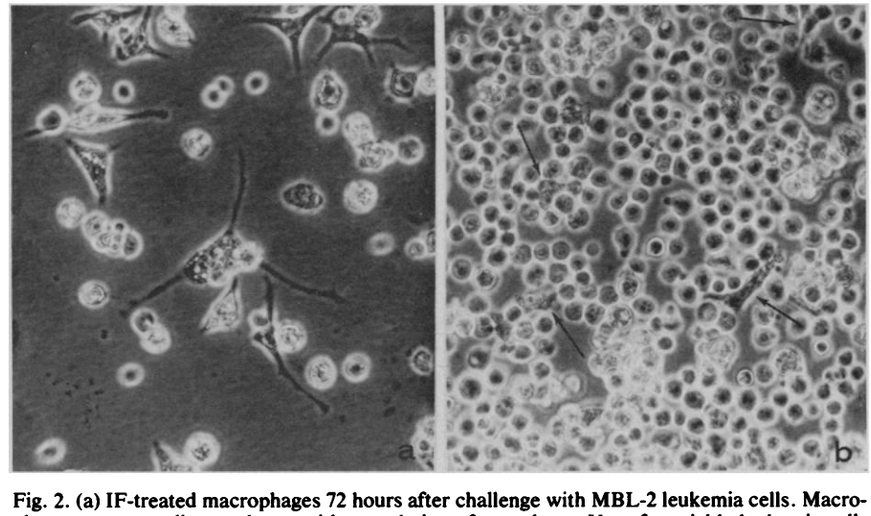

Fig. 2. (a) IF-treated macrophages 72 hours after challenge with MBL-2 leukemia cells. Macrophages appear well spread out, with granulation of cytoplasm. Very few viable leukemia cells were present (phase contrast, ×250). (b) Normal control macrophages 72 hours after challenge with MBL-2 leukemia cells. Granulation was less marked in macrophages (arrows) with no evidence of dead cells or phagocytized debris. Leukemia (rounded) cells grow unhindered (phase contrast, ×250).

rendered cytotoxic by exposure in vitro to either BCG or PPD (purified protein derivative). It is interesting that these challenged cultures give rise to an immune IF (*21*). A simple explanation for the immunologically specific induction of nonspecifically cytotoxic macrophages could be that this IF modifies macrophage activity.

The abilities of IF and IF-inducers to enhance resistance to nonviral intracellular pathogens (*22*) and to cancer (*7, 10, 23*) go well beyond IF's previously known role in the inhibition of virus replication at the molecular level. The evidence presented here broadens the scope of IF as an agent with discrete pharmacologic activity on macrophage function.

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Polymorphism and Geographic Variation in the Feeding Behavior of the Garter Snake *Thamnophis elegans*

Abstract. *Geographic variation in behaviors that influence resource utilization is a key component in current ecological theory, but the phenomenon has been poorly documented. Propensity to attack slugs varies geographically in a western garter snake,* Thamnophis elegans. *Many naive, newborn snakes from inland localities in California refuse to eat slugs. In contrast, virtually all naive young from coastal localities eat slugs. There is, however, no demonstrable polymorphism or geographic variation for propensity to eat anurans. Both coastal and inland snakes consistently eat anurans.*

We know relatively little about the extent of genetic variability for feeding behavior within and between animal populations. Consequently we can only guess at the behavioral alternatives that are vulnerable to natural selection and speculate about the response of populations to selection for resource utilization. Those rare demonstrations of polymorphism and geographic variation in behavior usually deal with behaviors that are unlikely to be modified by experience, for example, social signals (*1*). Because of inherent difficulties in controlling for effects of experience, geographic variation in behaviors that influence resource utilization is seldom studied. One promising approach to the problem is to assay behaviors in inexperienced animals. Studies of newborn snakes indicate that feeding behavior may show genotypic differences within and between populations (*2*).

I now present evidence for a bimodal distribution of behavioral phenotypes in a snake population. The bimodality appears in tests of naive, newborn snakes and cannot easily be attributed to differences in experience. In addition, distributions of behavioral phenotypes vary geographically.

The garter snake *Thamnophis elegans* has a wide range in western North America (*3, 4*). Within California, this species shows geographic variation in its natural diet. Coastal populations feed predominantly on slugs, mice, plethodontid salamanders, and anurans. Inland populations, inhabiting the Sierra Nevada and southern Cascade mountain ranges, prey mainly on fish, anurans and their larvae, and mice (*3, 5–7*). Differences in foraging behavior and morphology coincide with this dietary variation. Inland snakes are more aquatic than coastal snakes and differ in scalation, body proportions, and coloration (*3*). The functional significance of this morphological differentiation is not known.

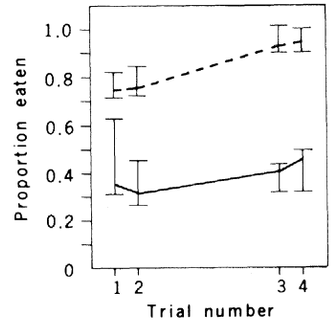

Fig. 1. The feeding responses of newborn coastal (dotted line) and inland (solid line) *T. elegans* to the slug *A. californicus*. Vertical bars indicate the ranges for three inland and two coastal localities (*10*). Sample sizes at the four trials were: coastal snakes, $N = 293$, 292, 173, 169; inland snakes, $N = 101$, 101, 92, 91.

The response of newborn snakes to slugs was studied in three laboratory experiments. The native slug *Ariolimax californicus* was used as a test prey. Slugs of the genus *Ariolimax* are commonly eaten by coastal populations of *T. elegans* in nature (*3, 8*). In California, *Ariolimax* is restricted to the coast and to the western foothills of the southern Cascade Range (*9*). Most inland populations of *T. elegans* are allopatric with this slug. Other slug genera are uncommon in the inland mountain ranges of California, probably because of dry summers, and are virtually absent from the natural diets of inland *T. elegans* (*5, 7*).

Gravid snakes were captured at three inland localities outside the range of *Ariolimax* and at two coastal localities within the range of this slug (*10*). The subjects of this report were the captive-born progeny of 42 such wild-caught, gravid females (*11*). Relatively large samples were obtained from one inland locality, Eagle Lake (64 progeny from 9 females) and from one coastal locality, Scott Creek (221 progeny from 16 females).

In experiment 1, each newborn snake was offered a small piece of *A. californicus* at each of four trials (*12*). For trial 1, all snakes were 14 days old, and the *Ariolimax* presentation represented their first exposure to prey; for trial 2, all snakes were 20 days old. All snakes were tested on the same date for trial 3 in order to simplify logistics. Due to dif-