7. Living larvae of *Watersipora cucullata* were transferred to seawater containing DAPI (4′,6-diamidino-2-phenylindole) (2 to 5 μg/ml; Sigma) for periods of 30 seconds to 5 minutes. After being rinsed in a large volume of filtered seawater, larvae were prepared as wet mounts and examined by epifluorescence (Olympus Vanolux research microscope with filter) by the method of A. W. Coleman [*Limnol. Oceanogr.* **25**, 948 (1980)]. Photomicrographs (Ektachrome 400) were made of larvae killed with dilute Formalin.
8. R. L. Zimmer and R. M. Woollacott, in *Biology of Bryozoans*, R. M. Woollacott and R. L. Zimmer, Eds. (Academic Press, New York, 1977).
9. We thank M. S. Brancato and C. G. Reed for assistance with several aspects of this project, R. Mitchell for permitting us to use his fluorescence microscope, and P. Boyle, D. Branton, R. Mitchell, R. Cole, and R. Whitcomb for criticisms and suggestions. Supported by ONR contract N00014-78-C-0064 with Harvard University.

12 October 1982

Regulation of the Macrophage Content of Neoplasms by Chemoattractants

Abstract. *Factor chemotactic for mononuclear phagocytes was found in supernatant fluids of cultured human and mouse tumor cells. In 11 mouse tumors there was a correlation observed between chemotactic activity and macrophage content of neoplastic tissues. Tumor-derived chemoattractants appear to participate in the regulation of tumor-associated macrophages.*

Macrophages are a major component of the lymphoreticular infiltrate of tumors in humans and mice, but their significance in vivo is largely a matter of speculation (*1*). How the macrophage content of neoplasms is regulated is not known. A relation between macrophage content of neoplastic tissues and tumor immunogenicity has been reported (*2, 3*), indicating that tumor-directed immune responses would be the major determinant of entry of blood monocytes into neoplastic tissue. However, some observations are not consistent with this view (*4*). Evans (*4*) chemically induced sarcomas in mice and found no relation between immunogenicity and macrophage concentration at the tumor site. Moreover, transplantation into nude or thymus-deprived mice (*4*) did not decrease tumor-associated macrophages (TAM's). Although in some systems specific immunity appears to play a role (*2, 3*), it is not the sole or most important determinant controlling TAM's. The possibility that macrophages accumulate in tumors attracted by neoplastic cells has been the subject of conflicting reports (*5, 6*). We found that neoplastic cells of mouse or human origin produced chemoattractants for macrophages and that there is a relation between production of chemotactic factors by tumor cells and the amount of macrophage infiltration in vivo.

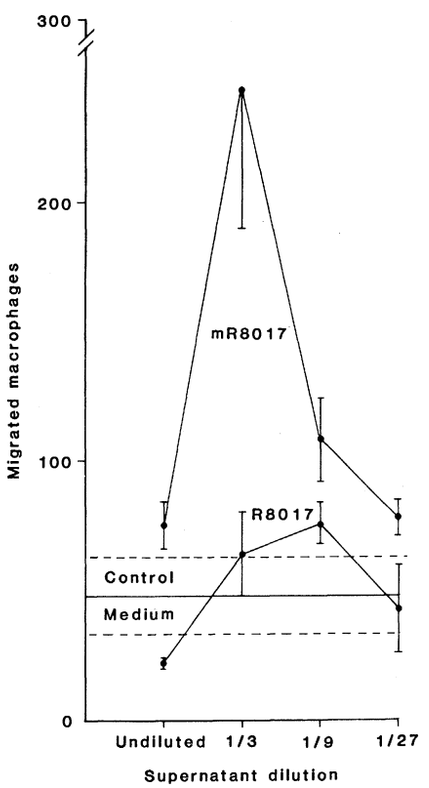

Fig. 1. Correlation between chemotactic activity and tumor-associated macrophages (TAM's) in mice. This is a typical dose-response experiment with supernatants from the low macrophage content (7 percent TAM's) sarcoma R8017 and its subline with a high macrophage content (20 percent TAM's) mR8017. Supernatants were harvested from subconfluent cultures (4.3×10^6 cells per 25-cm^2 flask for R8017 and 4.8×10^6 cells for mR8017).

Chemotaxis of human monocytes or mouse (male C57B1/6) macrophages was measured (*7*) in chemotactic chambers with 5-μm polycarbonate filters (3400253, Neuroprobe). Blood samples taken were from healthy human volunteers and mononuclear cells were separated by sedimentation at 400*g* for 20 minutes on Ficoll-Hypaque. Counts were made on cytocentrifuge smears, and cells were resuspended (1.5×10^6 monocytes per milliliter) in RPMI-1640 or minimum essential medium (MEM) with 10 percent fetal bovine serum (FBS). A mild inflammation was induced in mice by interperitoneal injection of 1 ml of phosphate-buffered saline, and after 24 hours peritoneal exudates were collected. Mouse peritoneal macrophages were resuspended (2×10^6 macrophages per milliliter). Chemotaxis was assessed after 1.5 and 4 hours of incubation at 37°C for human monocytes and mouse macrophages, respectively (Table 1). Cells were washed and seeded in RPM I-1640 or MEM with 10 percent FBS (1×10^5 cells per milliliter) in 25 cm^2 tissue culture flasks (5 ml). Supernatants, collected when cultures were subconfluents, were centrifuged at 600*g* for 15 minutes and tested immediately or stored at −20°C.

A summary of the chemotactic activity of supernatant fluids from various mouse and human tumor cells is presented in Table 1 and the results of typical individual experiments in Fig. 1. Supernatants of mouse carcinomas and sarcomas had appreciable chemotactic activity for mononuclear phagocytes. Production of chemoattractants was observed with long transplanted tumors (Lewis lung carcinoma; 1023, 3T3-B77, mFS6, M4, and M9 sarcomas), early passage chemically induced sarcomas (MN/MCA1, second and fourth transplant generation; mR8017, second and fourth transplant generation), and tumors obtained from autochthonous hosts (two spontaneous mammary carcinomas, R8018, R8016, and R8039). Chemotactic activity was observed with both in vitro established cell lines and with primary cultures (data not shown). The R8001 and R8017 sarcomas and the TLX9 and YAC-1 lymphomas showed no appreciable chemotactic

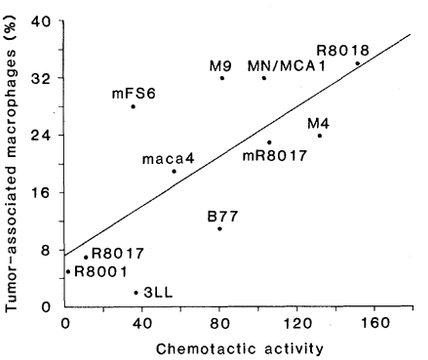

Fig. 2. Correlation between chemotactic activity in supernatants in vitro and tumor-associated macrophages in vivo. Chemotactic activity was calculated as the area under the dose-response curve after subtraction of migration with control medium (see Fig. 1) ($r = 0.71$, $t = 3.083$ (*10*), $P = .013$). We excluded the R8039 and R8016 sarcomas from the analysis because the percentage of TAM's in these tumors varied between experiments (Table 1); mammary carcinoma 1 was excluded as well because viability did not exceed 60 percent, making the TAM evaluation questionable. However, when these three tumors were included (taking the mean percentage of TAM's for the two sarcomas), a statistically significant correlation was still found ($r = 0.58$, $t = 2.49$ (*13*), $P = .028$).

activity. Production of a chemotactic factor or factors was not a specific property of tumor cells because supernatants of mouse embryo fibroblasts showed substantial activity.

Chemotactic activity was also measured in supernatant fluids of human cells. Supernatant of a fibrosarcoma (8387), a melanoma (10080/80), and a mammary carcinoma (SKRB-3) were strongly chemotactic for human monocytes, whereas a breast carcinoma cell line (MCF-7) had little activity. Little or no activity was found in supernatants of K562 leukemia, Raji lymphoma, and CEM leukemia. Human lung embryo fibroblasts (He and MRC5) yielded supernatants with chemotactic activity.

To assess whether induction of migration of mononuclear phagocytes was related to chemotactic factors rather than to chemokinesis, we conducted checkerboard experiments with varying dilutions of supernatants in the upper and lower compartments of the chemotaxis chamber (*7, 8*). These data (not shown) indicate that macrophages migrated only in the presence of a positive stimulatory gradient. Additional factors about tumor-derived chemoattractants are that (i) they are produced in serum-free conditions, (ii) their production is inhibited in vitro by protein synthesis inhibitors such as cycloheximide but not by mitomycin C, (iii) they are present in mouse tumor homogenates (M4 and MN/MCA1), (iv) tumor supernatants have little or no chemotactic activity for polymorphonuclear leukocytes, and (v) chemotactic activity is inactivated by proteolytic enzymes (Pronase and trypsyn). Biochemical characterization of tumor-derived chemoattractants is not complete but fractionation on Sephadex G100 columns suggests a molecular weight of 11,000.

To assess the possible significance in vivo of the tumor-derived chemoattractants, we looked for a relation between macrophage content of mouse tumors and activity in supernatants. When the percentage of TAM was plotted as a function of the area under the titration curve of the chemotactic activity, a statistically significant correlation ($r = .71$, $P = .013$) was found (Fig. 2). In contrast, the correlation with the peak number of migrated macrophages or with the highest active dilution was not significant. This may be related to the fact that we found supernatants with relatively high peak activity (for example, 3T3-B77) rapidly disappearing with dilution and some with low peak activity maintained with dilution (for example, mFS6) (Table 1).

In relation to the possible correlation between chemotactic activity and TAM's, the data obtained on the related sarcomas R8017 and mR8017 are of interest. The mR8017 subline was derived from a spontaneous lung metastasis of the R8017 sarcoma found in the autochthonous host and showed consistently a higher percentage of TAM's (20 percent) than the parent tumor (7 percent). The mR8017 also consistently produced

Table 1. Chemotactic activity of supernatant fluids cultured from mouse and human tumor cells. Mouse tumors included long passaged lines [Lewis lung carcinoma; TLX9 and YAC-1 lymphomas; mFS6 sarcoma and its sublines M4 and M9 (*14*); Rous sarcoma virus–transformed 3T3-B77 (*15*); and the 1023 sarcoma, (*5*)], early passage methylcholanthrene-induced tumors [MN/MCA1, second through sixth transplant generations (*10*) and mR8017, second through fourth transplant generations], and tumors obtained from autochthonous hosts (two spontaneous mammary carcinomas from C3H/HeN mice and the methylcholanthrene-induced R8001, R8016, R8017, R8018, and R8039 sarcomas). All tumors are of C57B1/6 origin except the 1023 sarcoma (C3H/HeNicr), the mammary carcinomas (C3H/HeN), the 3T3-B77 (BALB/c), and the YAC-1 lymphoma (A/Sn). Autochthonous tumors were tested immediately when obtained from the primary host and in the first three trials in syngeneic mice. Results were consistent for repeated tests except for the percentage of tumor-associated macrophages (TAM's) for R8016 and R8039, which showed considerable variation; selected determinations are presented for these tumors. In chemotaxis experiments three filters per experimental group were used and 10 (human) and 20 (mouse) oil immersion fields were counted in each filter. Samples were coded before counting. Results represent mean (± standard deviation) number of migrated cells in 10 human monocytes or 20 oil fields (mouse macrophages). Mouse tumor supernatants were routinely tested for chemotactic activity after one and more than five in vitro transfers without appreciable alteration of the pattern of activity. The Lewis lung and mammary carcinomas were tested only as primary cultures. In experiments with mouse macrophages a positive control of endotoxin-activated mouse serum (1/100) was included, and the average number of migrated macrophages was 792. With human monocytes, lymphocyte-derived chemotactic factor (*9*) was used as a positive control, with an average of 623 migrated monocytes. Tumors were disaggregated by exposure to collagenase (*12*) and the percentage of TAM's was assessed by counting adherent cells (*12*) and, in parallel, the number of cells forming rosettes with antibody-coated sheep erythrocytes (*4, 12*). The results were superimposable, and data presented refer to rosette-forming macrophages. The viability of disaggregated tumor cell suspensions exceeded 80 percent except for mammary carcinoma 1, which had 40 percent dead cells, indicating that results for this tumor should be regarded with caution. Abbreviations: N.D., not diluted; N.T., not tested.

Cell	Macrophages with control medium	Macrophages with supernatant (optimal dilution)	Highest dilution with significant activity	TAM (%)
		Mouse		
Sarcoma				
1023	11 ± 5	180 ± 15 (1/3)*	1/9	N.T.†
R8018	15 ± 5	361 ± 10 (N.D.)*	1/9	34
MN/MCA1	55 ±	183 ± 37.9 (1/3)*	1/27	32
M9	47 ± 15	159 ± 22 (N.D.)*	1/9	32
mFS6	2 ± 1	52 ± 2 (1/9)*	1/27	28
M4	2 ± 1	72 ± 17 (N.D.)*	1/9	24
R8039	31 ± 7	101 ± 7 (1/3)*	1/27	31, 18, 11
mR8017	48 ± 15	243 ± 54 (1/3)*	1/27	20
R8016	31 ± 7	165 ± 7 (N.D.)*	1/27	8, 14, 23
3T3-B77	81 ± 45	167 ± 20 (N.D.)*	N.D.	11
R8017	48 ± 15	76 ± 9 (1/3)	Inactive	7
R8001	22 ± 12	26 ± 22 (1/3)	Inactive	5
Carcinoma				
Mammary 1	21 ± 3	167 ± 17.8 (N.D.)*	1/9	25
Mammary 4	31 ± 7	103 ± 13 (1/3)*	1/27	19
Lewis lung	55 ± 2	98 ± 11 (N.D.)*	1/3	2
Fibroblasts				
3049 embryo (C3H/HeN)	41 ± 3.1	121 ± 18 (N.D.)*	N.D.	
181/1 embryo (C57B1/6)	55 ± 23	229 ± 6 (1/3)*	1/9	
Lymphoma				
TLX9	7 ± 2	21 ± 0.6 (1/3)	Inactive	
YAC-1	31 ± 8	45 ± 4 (1/3)	Inactive	
		Human		
Sarcoma 8387	55 ± 3	724 ± 7 (N.D.)*	1/27	
Melanoma 10080/80	60 ± 10	488 ± 17 (1/3)*	1/81	
Breast carcinoma SKBR-3	71 ± 11	221 ± 17 (N.D.)*	1/27	
Breast carcinoma MCF-7	56 ± 7	94 ± 10 (1/3)*	1/3	
Fibroblasts				
He lung embryo	95 ± 11	405 ± 35 (N.D.)*	1/9	
MRC5 embryo	26 ± 4	304 ± 18 (N.D.)*	1/27	
K562 leukemia	46 ± 3	118 ± 9 (N.D.)	N.D.	
Raji lymphoma	34 ± 1	93 ± 14 (1/3)	1/9	
CEM leukemia	151 ± 2	153 ± 5 (1/3)	Inactive	

*Significantly above number of macrophages migrated with control medium, $P < .05$. †Not tested.

much higher levels of chemotactic activity in the supernatants (Table 1 and Fig. 1).

Our investigation may provide an explanation for some negative findings (*6*). Chemotactic activity varied widely among cell lines, with negative activity observed in leukemias or lymphomas and some solid tumors (Table 1). Moreover, a prozone phenomenon was frequently encountered (Table 1 and Fig. 1). Defective in vitro chemotaxis and in vivo inflammatory responses, possibly related to soluble inhibitors, have been reported in tumor-bearing animals and humans (*9, 10*). Our results are not necessarily in conflict with these findings. For instance in mice with advanced M4 and M9 sarcomas the accumulation of macrophages in the peritoneal cavity was reduced after intraperitoneal inoculation of phytohemagglutinin. Systemic defective chemotaxis does not mean that, at the tumor site, neoplastic cells could not be attracting mononuclear phagocytes into the tissue to stimulate directly or indirectly tumor growth (*1, 11*).

Macrophage infiltration in tumors is likely to be a complex process and depend on more than one factor. Local proliferation could contribute to TAM's, and host cells (for example, fibroblasts and macrophages) could also be a source of chemotactic materials. Tumor-derived chemotactic factors may play a role in determining the entry of blood monocytes into neoplastic tissues, a suggestion supported by the statistically significant, although not strict, correlation found between chemotactic activity and TAM content of mouse tumors.

Macrophages from two sarcomas (mFS6 and MN/MCA1) enhanced tumor cell proliferation in vitro, at least at low macrophage to target cell ratios (*11, 12*). The observation of chemoattractants from these tumors supports the proposal that TAM's may stimulate tumor growth in vivo, at least at the primary tumor site (*1, 11–13*).

BARBARA BOTTAZZI
NADIA POLENTARUTTI
RAFFAELLA ACERO
Istituto di Ricerche Farmacologiche "Mario Negri," Via Eritrea 62, 20157 Milan, Italy
ANDREA BALSARI
Istituto de Microbiologia Medica, University of Milan, Milan, Italy
DIANA BORASCHI
PIETRO GHEZZI
MARIO SALMONA
ALBERTO MANTOVANI
Istituto di Ricerche Farmacologiche "Maria Negri"

References and Notes

1. R. Evans, *J. Reticuloendothel. Soc.* **26**, 427 (1979).
2. S. A. Eccles and P. Alexander, *Nature (London)* **250**, 667 (1974).
3. K. Moore and M. Moore, *Int. J. Cancer* **19**, 803 (1977).
4. R. Evans and D. M. Eidlen, *J. Reticuloendothel. Soc.* **30**, 425 (1981); R. Evans and E. M. Lawler, *Int. J. Cancer* **26**, 831 (1980); S. Szymaniec and K. James, *Br. J. Cancer* **33**, 36 (1976); J. E. Talmadge, M. Key, I. J. Fidler, *J. Immunol.* **126**, 2245 (1981).
5. M. S. Meltzer, M. M. Stevenson, E. J. Leonard, *Cancer Res.* **37**, 721 (1977).
6. S. J. Norman and E. Sorkin, *ibid.*, p. 705.
7. M. K. Lohr and R. Snyderman, in *Manual of Macrophage Methodology*, H. B. Herscowitz et al., Eds. (Dekker, New York, 1981), p. 303.
8. S. H. Zigmond and J. G. Hirsch, *J. Exp. Med.* **137**, 387 (1973).
9. R. Snyderman, M. C. Pike, G. J. Cianciolo, in *Mononuclear Phagocytes: Functional Aspects*, R. van Furth, Ed. (Nijhoff, The Hague, 1980), p. 569.
10. R. Snyderman and M. C. Pike, *Science* **192**, 370 (1976).
11. S. E. Salmon and A. W. Hamburger, *Lancet* **1978-I**, 1289 (1978).
12. A. Mantovani, *Int. J. Cancer* **27**, 221 (1981).
13. _____, R. Giavazzi, N. Polentarutti, F. Spreafico, S. Garattini, *ibid.* **25**, 617 (1980); A. Mantovani, *ibid.* **22**, 741 (1978).
14. A. Mantovani, R. Giavazzi, G. Alessandri, F. Spreafico, S. Garattini, *Eur. J. Cancer* **17**, 71 (1981).
15. M. Prat, G. Tarone, P. M. Comoglio, *Int. J. Cancer* **27**, 797 (1981).
16. Supported by grant R01 CA 12764 from the U.S. National Cancer Institute, and by Finalized Project "Control of neoplastic growth" (81.01403.96) from the National Research Council of Italy.

11 May 1982; 8 September 1982

Taste-Aversion Conditioning of Crows to Control Predation on Eggs

Abstract. *Free-ranging crows (*Corvus brachyrhynchos*) that ate chicken eggs that were painted green and contained a nonlethal toxin subsequently avoided green eggs at various locations, whether or not they contained toxin. The crows also continued to eat unpainted and nontoxic chicken eggs. Illness-induced aversions among predators in nature may be a powerful determiner of the evolution of Batesian mimicry and, in human hands, serve as a practical tool for wildlife ecologists.*

Some predators avoid prey that are harmless to them but resemble noxious prey. Such mimics of the noxious models represent a phenomenon known as Batesian mimicry (*1*). Some mimics appear to be avoided even when the predators have not been exposed to the noxious model (*2*). The majority of mimics, however, are apparently avoided only when predators have been exposed to the models, often becoming ill after consuming one or more model prey (*3*).

Many animal species develop an aversion to the flavor of a food if it makes them sick (*4*); this process is known as taste-aversion conditioning. In agriculture, it has been used to control predation by olfactory predators when they associate the taste and smell of live prey with those of toxic meat baits that they have eaten. The success of the process depends on hiding the toxin in the bait so that it is the taste of the prey that is avoided rather than that of the toxin (*5*). As long as taste is paired with illness, nongustatory senses may also play a role in the avoidance behavior. Thus, olfactory predators avoid by smell (*6*), raptors and pigeons by vision (*7*), and bats by hearing (*8*).

Taste-aversion conditioning has not been generally included in evolutionary models of Batesian mimicry because the research has focused on nonillness-producing noxious events and visual mimics. Research on taste-aversion conditioning has focused on learning theory and, until recently, on olfactory predators. We report two experiments designed to (i) evaluate the usefulness of the taste-aversion agent UC 27867 (*9*) in producing visual avoidance of colored eggs by free-ranging crows (*Corvus brachyrhynchos*) and (ii) measure the degree to which crows would generalize their conditioning from the toxic baits.

In the first experiment (23 June to 13 July 1981) we established five treatment and five control sites near Fargo, North Dakota (*10*), each with a line of 20 straw nests at 15-m intervals on the ground. All nests were visible from the air. Half the nests at each site contained one chicken egg painted green (*11*); the others had one white, unpainted chicken egg. The color sequence was randomized and eggs were rearranged every 3 days. Only green eggs in treatment sites were injected with UC 27867 (30 mg) (*12*). We replaced eggs every morning, noting the location and activities of crows as well as evidence of predation. Each site appeared to have a separate group of crows (*13*).

For three-way analysis of variance (*14*), averages per site were used because measurements at the different sites were considered to be independent. Only days that followed rearrangement of the color sequence were used in this analysis ($N = 7$ and $N = 8$ days for the two experiments, respectively). The statistical significance level for all comparisons was set at $P < .05$.

A total of 400 eggs were available at each treatment and control site during