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Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
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Macrophage Activation by a Series of Unique Polnanionic Polymers

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To cite this Article Ottenbrite, Raphael M. , Kuus, Kristine and Kaplan, Alan M.(1988) 'Macrophage Activation by a Series of Unique Polnanionic Polymers', Journal of Macromolecular Science, Part A, 25: 5, 873 — 893
To link to this Article: DOI: 10.1080/00222338808053404
URL: http://dx.doi.org/10.1080/00222338808053404

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J. MACROMOL. SCI.—CHEM., A25(5-7), pp. 873-893 (1988)

MACROPHAGE ACTIVATION BY A SERIES OF UNIQUE POLYANIONIC POLYMERS

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ABSTRACT

Polyanionic polymers with differing molecular weight, lipophilicity, and charge density were synthesized and their ability to nonspecifically "activate" macrophages was evaluated. The level of activation of test polymer-elicited macrophages was monitored by their nonspecific tumoricidal capacity and ectoenzyme profiles. In addition, the ability of test polymers to inhibit growth of Lewis lung *in vivo* was evaluated. We have determined that the activation of macrophages by test polymers differs from that of the conventional activating agents pyran and *Clostridium parvum.*

INTRODUCTION

Macrophages may be activated to tumoricidal capacity, *in vivo,* by a variety of agents including pyran, *Clostridium parvum, Micrococcus bovis* (strain BCG), muramyl dipeptide, and polynucleotides. Synthetic polyanions have the potential for size and structural modifications which have been shown to alter

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biologic activity and toxicity selectively [1, 8, 9]. Although the exact mechanism of antitumor activity of the polyanions is unclear, several mechanisms have been actively considered, including: a) macrophage (Mϕ) activation [1, 2, 7, 8, 10, 11], b) stimulation of natural killer cell activity [12, 13], and c) stimulation of specific tumor immunity [14, 15].

Over the last 5 years a great deal of interest has been shown in a particular series of polyanions, MVE-2-MVE-5 (pyran copolymer), due to their ability to activate macrophages to enhanced microbicidal and tumoricidal activity [9] The difference in biologic activity of various molecular weight fractions of pyran, and in compounds structurally related to pyran, suggested that it would be worthwhile to synthesize a series of compounds modeled on maleic anhydride-divinyl ether to analyze structure-function relationships with a goal toward obtaining a compound with higher efficacy.

We report the structures and antitumor activities of murine peritoneal macrophages (PEC) elicited with this series of compounds. In addition, the ectoenzyme profiles and cellular compositions of the polyanion elicited PEC and the cellular composition of the peripheral blood from mice which have received these compounds are described.

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MATERIALS AND METHODS

Animals

Female C57BL/6 mice from various sources were used at 6 and 8 weeks of age. Mice were housed in standard cages and maintained on standard kibbles (Charles River, New York) and tap water *ad libitum.* Mice were free of endo- and ectoparasites, maintained in clean conditions, housed according to vendor, and free of clinical evidence of virologic disease during the course of these experiments.

Media and Target Cell Cultures

All tissue cultures were performed in complete medium, RPMI-1640 (Gibco, Grand Island, New York) containing 100 μg penicillin/mL, 100 μg streptomycin/mL 2 m*M* glutamine, and 10% Hy-Clone fetal calf serum (FCS) (Sterile Systems, Ogden, Utah). Lewis lung (LL) carcinoma was maintained by *in vivo* passage or *in vitro* as a target cell for cytotoxic assays as previously described [1] and was mycoplasma free.

Peritoneal Macrophage Eliciting Agents

The series of polyanionic compounds were synthesized as previously described [16, 17]. Discrete molecular weight fractions were obtained by filtration through appropriate Amicon membranes (Amicon, Lexington, Massachusetts). The purified polymers were evaluated by ^{1}H and ^{13}C NMR spectroscopy and carbon-hydrogen analysis [17]. The test polymers and pyran copolymer (NSC-46015, National Cancer Institute, Bethesda, Maryland) were hydrolyzed in sterile 1 *N* NaOH, neutralized with sterile 1 *N* HCl, and diluted in pH 7.4 sterile phosphate-buffered saline (PBS) to a final concentration of 5 mg/mL. Mice were inoculated i.p. with 0.2 mL of the polymer preparations, corresponding to a dosage of 50 mg/kg, and peritoneal exudate cells (PEC) harvested 7 d later, unless otherwise specified. A 10% solution of Brewer's thioglycollate (Gibco, Grand Island, New York) was prepared by dissolving 10 g of the powder in 100 mL H_2O and autoclaving. Mice were inoculated i.p. with 1 mL of the solution and PEC harvested 3 d later. *C. parvum*, 7 mg/mL (Burroughs-Wellcome Research Laboratories, Research Triangle Park, North Carolina) was diluted 1:3 in PBS (pH 7.4), 0.2 mL (17.25 mg/kg) inoculated i.p., and PEC harvested on Day 7, unless otherwise noted.

Macrophage Cytotoxicity Assays

Macrophages (Mϕ) were obtained by peritoneal lavage with 5 mL cold Hanks balanced salt solution (HBSS, Gibco, Grand Island, New York) containing 100 μg penicillin/mL, 100 μg streptomycin/mL, and 5 μg lipoheparin/mL (Riker Laboratories, Northridge, California). Cytotoxic assays were carried out in RPMI 1640 containing 100 μg penicillin/mL, 100 μg streptomycin, 2 m*M* glutamine, and 10% FCS (Sterile Systems, Ogden, Utah, <1 ng/mL endotoxin). Cell suspensions were collected in 50-mL polystyrene tubes, maintained on ice, pelleted, washed three times in HBSS, and resuspended in cold complete medium. Morphologic cytotoxicity was assayed in 8-chambered Lab Tek tissue-culture chambers (Lab-Tek Products, Naperville, Illinois) at effector (PMO) to target (LL) ratios of 20:1, 10:1, 5:1, and 1:1 and quantified at 60-70 h as previously described [18]. A ^{3}H-thymidine release cytotoxicity assay was performed in 96-well flat-bottom microtiter plates (Falcon, Oxnard, California) as previously described [19]. Effector cells and target cells were incubated for 48 h, after which the plates were centrifuged and the amount of ^{3}H-thymidine released determined.

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Macrophage Ectoenzyme Assays

Mϕ were prepared by peritoneal lavage, as described for cytotoxicity assays, plated at 4-6 X 10^6 cells/2 mL complete medium into 35 mm^2 wells of Costar plates (Coster, Cambridge, Massachusetts), incubated for 2 h at 37°C, washed three times with HBSS to remove nonadherent cells, and cultured for varying time periods in complete medium. At the time of harvest, the wells were washed three times with 4°C PBS and 200 μL of freshly prepared 0.1% Triton-X100 added. The plates were swirled to cover the entire surface with liquid, maintained on ice for 10 min, the wells scraped (Cell Scraper, Costar, Cambridge, Massachusetts), and the lysates transferred into ice-cold 1.5-mL microfuge tubes (Eppendorf, Westbury, New York). Following centrifugation at 10 000 X *g* for 5 min, the supernatant was aliquoted for 5-nucleotidase (5N), leucine amino peptidase (LAP), alkaline phosphodiesterase (APD), and protein assays. Samples were stored in covered vials at 4°C and assayed within 48 h of collection. Protein in each lysate was determined by the Bio-Rad Laboratories, Richmond, California). Alkaline phosphodiesterase was assayed spectrophotometrically at 400 nm with *p*-nitrophenylthymidine-5′-monophosphate (Sigma, St. Louis, Missouri) substrate as previously described [20, 21]. Leucine aminopeptidase was measured spectrophotometrically at 405 nm with leucine *p*-nitroanaline (Sigma, St. Louis, Missouri) substrate as previously described [21, 22]. 5-Nucleotidase was measured by the hydrolysis of adenosine-2-^3H-5 monophosphate (NEN, Westwood, Massachusetts) as previously described [21, 23]. Enzyme activities are expressed as mean ±S.E. of specific activity (*n* moles of product formed per minute at 37°C per mg protein) of triplicate samples in three experiments.

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Cytochemistry

Lysosomal peroxidase activity was determined by counting a minimum of 500 cells stained with Sigma Histozyme Kit #390.A (Sigma, St. Louis, Missouri). Nonspecific esterase activity was determined with Sigma Histozyme Kit #90.al (Sigma, St. Louis, Missouri).

Statistical Analysis

Analysis of the data was performed using Student's two-tailed *t*-distribution.

Anionic Polymer Synthesis

The procedures for the synthesis of the polycarboxylic acid polymers used in this study were as follows: Maleic anhydride 9.8 g (0.1 mol) was dissolved

in 100 mL of freshly distilled acetone containing an equivalent amount of appropriate monomer and 5 wt% AIBN initiator. After bubbling dry N_2 through the solution for 10 min, the polymerization flask was sealed and placed into an oil bath held at 80°C for 24 h. The reaction mixture was then cooled and added dropwise to 400 mL dry ether. The polymer was collected by filtration, redissolved in purified acetone, and reprecipitated in dry ether. The polymer was recovered by filtration, dried, and analyzed by ^{1}H NMR, IR, and elemental analysis.

The molecular weight of polyelectrolyte polymers was controlled by using the conventional polymerization techniques such as: a) altering monomer concentrations, b) variation of initiator concentration, c) changing the temperature of the reaction mixture, d) adding chain transfer agents or inhibitors, and e) choice of solvent.

Hydrolysis was carried out by stirring 10 g polymer in 50 mL of 1 *N* NaOH at 60°C for 3 h. Fractionation of the polymer was carried out by diluting the hydrolized sample to 1 L, adjusting the pH to 7, and passing the solution sequentially through PM 30 (to collect the fraction above 30 000 MW), PM 10 (to collect the fraction between 10 000 and 30 000 MW), and UM-2 (to collect the fraction between 1 000 and 10 000 MW) Amicon Diaflo membrane filters (Amicon Corp., Lexington, Massachusetts).

The polyethacrylic acid was supplied by David Tirrell (University of Massachusetts, Amherst, Massachusetts). Poly(icatonic acid-*co*-styrene) was prepared by the above method by substituting itaconic acid for maleic anhydride.

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RESULTS

Test Polymers

A series of polyanionic polymers which varied in chemical properties was synthesized. The rationale for the syntheses was that pyran, a polyanionic copolymer of maleic anhydride and divinyl ether, had previously been shown to have antitumor activity and activate macrophages to tumoricidal capacity. The test polymers consisted of six copolymers of maleic anhydride; cyclohexyl-1,3-dixoepin/maleic anhydride copolymer (CDA-MA), styrene maleic anhydride copolymer (S-MA), ethylene/maleic anhydride copolymer (EMA), vinylacetate/maleic anhydride copolymer (MAVA), allylurea/maleic anhydride copolymer (AU-MA), 4-methyl-2-penten-4-one-2/maleic anhydride copolymer (MP-MA), ethacrylic acid homopolymer (EA), and itaconic acid/styrene copolymer (IAS) (Fig. 1).

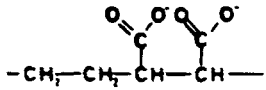
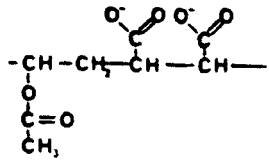
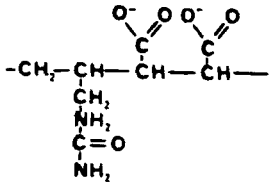
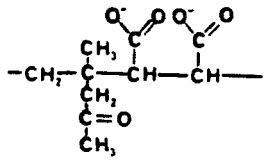
POLYANION	STRUCTURE	CODE
ETHYLENE - MALEIC ANHYDRIDE COPOLYMER		E - MA
VINYL ACETATE - MALEIC ANHYDRIDE COPOLYMER		VA - MA
ALLYL- UREA - MALEIC ANHYDRIDE COPOLYMER		AV - MA
4-METHYL-2-PENTENONYL- MALEIC ANHYDRIDE COPOLYMER		MP - MA

FIG. 1. Structure of test polymers used in this study.

Induction of Cytotoxic Macrophages with Polyanionic Polymers

The test polymers were screened for their ability to elicit tumoricidal macrophages in the peritoneal cavities of C57Bl/6 mice. The polymers were administered intraperitoneally at a dosage of 50 mg/kg body weight. Peritoneal exudate macrophages were routinely harvested 7 d after the administration of the test agent and tested for their cytotoxic capacity against Lewis lung carcinoma in both morphologic and ^{3}H-thymidine release cytotoxicity assays. Pyran (50 mg/kg) and *C. parvum* (17.25 mg/kg) activated macrophages were included in each experiment as positive controls. Negative controls consisted of thioglycol-

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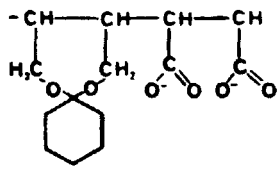
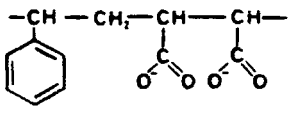
CYCLOHEXYL-1,3-DIOXEPIN - MALEIC ANHYDRIDE COPOLYMER



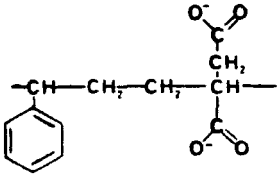
CDA - MA

STYRENE - MALEIC ANHYDRIDE COPOLYMER



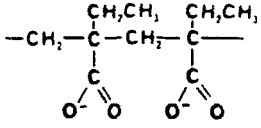
S - MA

ITACONIC ACID - STYRENE COPOLYMER



IAS

α - ETHACRYLIC ACID HOMOPOLYMER



EA

FIG. 1 (continued)

late (1 mL of a 10% solution, 3 d postinjection) and normal (wash-out) or saline-elicited macrophages (24-48 h postinjection). The ability of the test polymers to induce cytotoxic macrophages is shown in Figs. 2 and 3. Figure 2 depicts results obtained with the ^{3}H-thymidine release assay, and Fig. 3 the morphologic cytotoxicity assay. Both figures show cytotoxic data for effector:target cell ratios of 20:1, 10:1, 5:1, and 1:1. Similar trends in the cytotoxicity data are obtained for both assays. The cytotoxicity observed with CDA-MA and MP-MA elicited macrophages in the ^{3}H-thymidine release assay were significantly increased compared to normal macrophages ($p < 0.005$). Cytotoxicity values obtained for pyran and IAS-UM2-elicited

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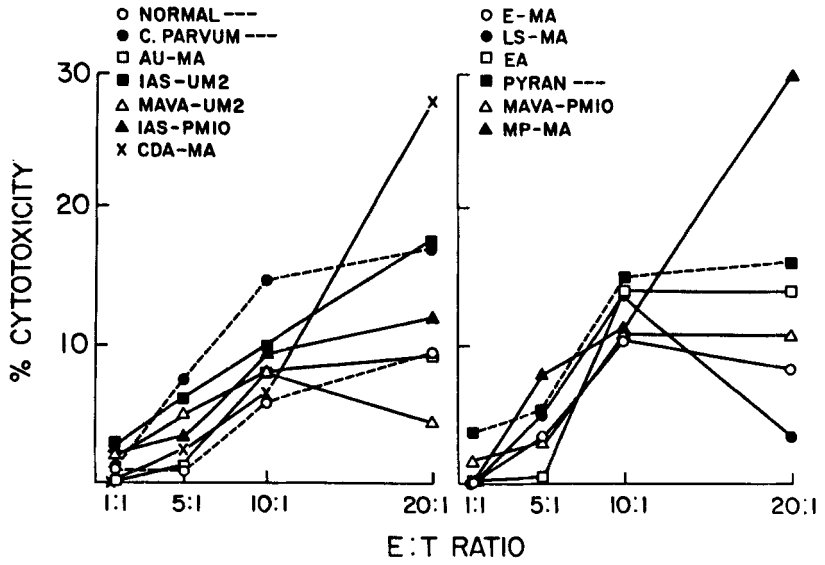

FIG. 2. Cytotoxicity of peritoneal exudate cells elicited by test polymers as measured by ^{3}H-thymidine release from Lewis lung tumor cells. PEC were obtained from mice that received 50 mg/kg of test polymers or pyran, or 17.25 mg/kg of *C. parvum*, 7 d prior to harvest. "Normal" indicates that cytotoxicity of nonelicited resident PEC. Thioglycollate PEC were obtained from mice that received 1 mL of a 10% solution of Brewers thioglycollate 3 d prior to harvest. The data represent the mean ± SEM (standard error of the mean) of triplicate samples in three experiments ($n = 3$) minus the percent specific cytotoxicity obtained with thioglycollate PEC (13 ± 2.6). The data presented represent an effector:target cell ratio of 20:1. Percent specific cytotoxicity was calculated from

$$\% \text{ Cytotoxicity} = \frac{\text{test cpm} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100.$$

macrophages were also increased ($p < .10$ and $p < .02$, respectively) compared to normal macrophages in the ^{3}H-thymidine release assay. In the morphologic cytotoxicity assay, pyran, *C. parvum*, MP-MA, CDA-MA, IAS-UM2, IAS, and MAVA elicited macrophage populations were significantly more cytotoxic than either normal or thioglycollate-elicited macrophages ($p < .001$). In the test

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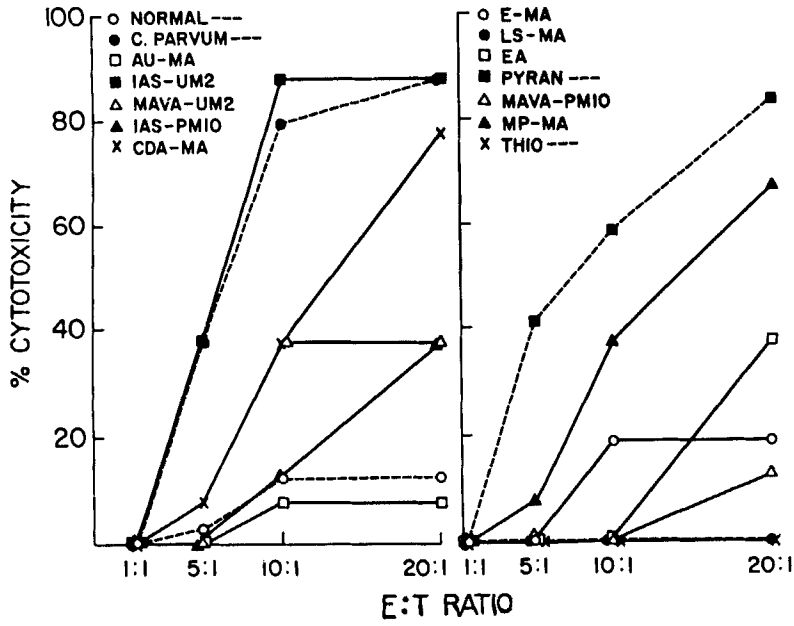

FIG. 3. Cytotoxicity of test polymer-elicited peritoneal exudate cells against Lewis lung tumor cells in a morphologic cytotoxicity assay. PEC were obtained from mice that received 50 mg/kg test polymers or pyran, or 17.25 mg/kg *C. parvum* i.p. 7 d prior to harvest. Thioglycollate PEC were obtained from mice that received 1 mL of a 10% solution of Brewers thioglycollate i.p. 3 d prior to harvest. "Normal" indicates the cytotoxicity observed with unelicited resident PEC. Data represent the mean ± SEM of duplicate samples in three experiments ($n = 3$) at an effector:target cell ratio of 20:1. Cytotoxicity was calculated based on macroscopic observation of cultures of PEC with tumor cells compared to tumor cells alone. The value used for the purposes of calculation was 87.5%, corresponding to 4+ cytotoxicity or a culture chamber essentially devoid of all cells. The value used for 3+ cytotoxicity was 67.5%, the value used for 2+ cytotoxicity was 37.5%, the value used for 1+ cytotoxicity was 12.5%, and 0% for no cytotoxicity, which corresponded to a completely confluent tumor cell monolayer.

polymer groups, IAS-UM2, IAS-PM10, CDA-MA, and MP-MA elicited high cytotoxic activity, MAVA-PM10 and EA elicited moderate cytotoxic activity, while S-MA, AU-MA, and E-MA did not elicit cytotoxic activity in PMϕ harvested 7 d after polymer injection.

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Although the two cytotoxicity assays differ somewhat in their sensitivity, it may be concluded that CDA-MA, MP-MA, and IAS have the greatest potential for inducing cytotoxic macrophages. In addition, test polymer as well as pyran and *C. parvum*-elicited peritoneal macrophages were not cytotoxic to normal cells (data not shown).

Ectoenzyme Profile of Test Polymer-Elicited PM

The enzyme profile described by Morahan and Edelson [21] was used to evaluate the activation/differentiation state of test polymer-elicited macrophages.

The ectoenzyme activities in polymer-elicited macrophage populations, at 48 h in culture, is shown in Fig. 4. The 5′-nucleotidase (5′N) activity was elevated in all test polymer-elicited macrophage populations compared to those elicited with thioglycollate, *C. parvum,* and pyran ($p < .05$ for all groups). Indeed, the levels detected in 48-h cultures of CDA-MA and MP-MA elicited macrophages, which also possessed enhanced tumor cytotoxicity, approached the level of resident populations. Thus, cell populations which were activated to tumoricidal capacity by test polymers did not possess the low 5′N phenotype exhibited by macrophages elicited with the conventional activating agents pyran and *C. parvum.*

Polymer-elicited macrophages cultured for 48 h did not share the elevated alkaline phosphadiesterase (APD) phenotype of inflammatory/thioglycollate macrophage populations. APD levels detected in test polymer-elicited macrophage populations were not significantly different from those obtained with normal and pyran or *C. parvum*-elicited macrophages. The leucine aminopeptidase (LAP) activity was not helpful in discriminating activation states between any of the macrophage populations tested.

Effect of Test Polymers on Peripheral Leukocyte and Peritoneal Exudate Cell Number and Composition

Based on the previous observations it was of interest to further characterize the cell populations involved in the induction of tumoricidal activity. A study of the magnitude and composition of peripheral blood leukocytes (PBL) and peritoneal exudate cells after administration of polymers that induced good tumoricidal activity (CDA, MP, IAS) and one that did not (LS) were performed.

Mice were injected with eliciting agents and killed on Days 3, 5, and 7 after administration. In separate experiments, samples were taken for the

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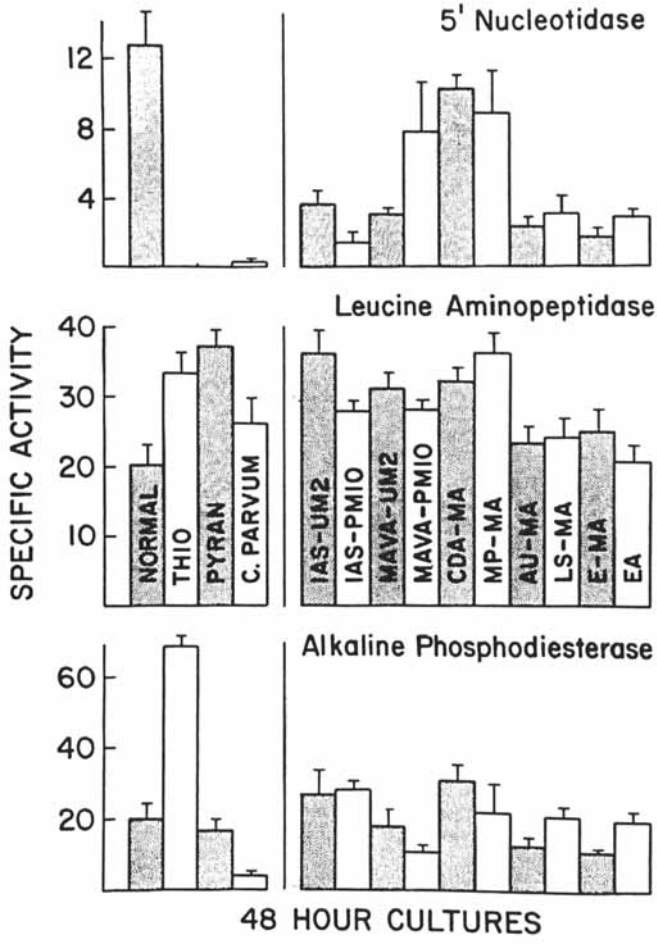

FIG. 4. *In vitro* ectoenzyme activity of polymer-elicited 48-h macrophage cultures. Peritoneal macrophages were obtained from mice that received 50 mg/kg test polymers i.p. 7 d prior to harvest or control eliciting agents. Macrophages were prepared and assayed for ectoenzyme activity as described (see Materials and Methods). Cells were cultured for 48 h prior to assay. Data represent the mean ± SEM of triplicate samples in three experiments ($n = 3$).

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TABLE 1. Effect of Test Polymers on Peripheral Leukocyte Counts[a,b]

Agent	Day 3	Day 5	Day 7
CDA	13.40 ± 2.35	11.31 ± 0.64	8.7 ± 1.3
MP	9.17 ± 2.24	12.93 ± 0.38	11.0 ± 0.8
IAS	16.36 ± 1.71	10.03 ± 0.86	11.7 ± 2.5
LS	21.12 ± 3.48	13.13 ± 1.6	10.4 ± 1.3
Pyran	7.6 ± 0.94	11.40 ± 1.67	12.6 ± 2.2
C. parvum	9.19 ± 1.82	10.83 ± 0.39	13.3 ± 2.6
Thio	10.64 ± 1.41	8.77 ± 0.88	9.0 ± 2.2
None	6.60 ± 1.0	6.60 ± 1.0	6.60 ± 1.0

[a]Mice were injected with test polymers or pyran (50 mg/kg), *C. parvum* (17.25 mg/kg), or 1 mL of a 10% solution of thioglycollate, i.p., 3, 5, and 7 d prior to sampling. "None" refers to mice which received no treatment. Blood samples were obtained by cardiac puncture, using heparin as an anticoagulant. Samples were diluted in isotonic saline for counting. Triplicate counts were performed on each sample using a Coulter, Model B, particle counter.

[b]Data represent the mean ± SEM (standard error of the mean) of triplicate experiments ($n = 3$). Three mice in each group were evaluated in each experiment. Data are expressed as cells/mm^3 × 10^5.

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determination of peripheral leukocyte and peritoneal exudate cell number and cell differentials.

Shown in Table 1 is the effect of agent administration on peripheral blood leukocyte (PBL) number. PBL's were increased in all mice which received eliciting agents when compared to normal uninjected mice. Pyran and *C. parvum* stimulated an elevation in the PBL response, which increased with time after administration. In contrast, the PBL response of mice that received CDA-MA, IAS, and S-MA was elevated on Day 3 and decreased at later time points. The PBL counts in mice that received MP were relatively stable but were increased compared to normal mice.

In Table 2 are shown the peripheral blood differentials of mice that received eliciting agents. The PBL in normal mice consisted of about 80% lymphocytes. Mice treated with reagents MP-MA, IAS, LS-MA, and *C. parvum* demonstrated an initial increase in percent PMN while the PBL response to CDA-MA remained near normal at all time points tested. The PBL differentials in response to

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TABLE 2. Effect of Polymer Administration on the Composition of Peripheral Blood Leukocytes[a,b]

Eliciting agent	Day 3			Day 5			Day 7		
	PMN	Lymph	Mono	PMN	Lymph	Mono	PMN	Lymph	Mono
CDA	20 ± 1.5	78 ± 2.5	3 ± 1.0	12 ± 3.5	84 ± 5.2	4 ± 1.7	12 ± 0.5	85 ± 2.0	4 ± 2.0
MP	29 ± 5.5	66 ± 6.5	4 ± 1.0	30 ± 6.4	59 ± 5.2	11 ± 2.3	15 ± 1.5	81 ± 2.5	5 ± 1.5
IAS	31 ± 8.5	63 ± 10.5	6 ± 3.0	32 ± 6.9	65 ± 7.5	3 ± 0.6	22 ± 3.0	75 ± 4.0	3 ± 1.0
LS	31 ± 3.0	67 ± 3.5	3 ± 1.0	26 ± 2.9	63 ± 7.5	10 ± 1.2	26 ± 2.0	68 ± 3.0	6 ± 3.0
Pyran	20 ± 2.0	62 ± 4.5	18 ± 4.0	33 ± 0.6	47 ± 4.0	19 ± 3.5	32 ± 3.5	48 ± 2.5	21 ± 4.5
C. parvum	27 ± 3.5	68 ± 6.0	11 ± 4.5	30 ± 4.0	57 ± 7.5	12 ± 3.5	28 ± 3.0	54 ± 6.5	19 ± 5.5
Thio	19 ± 0.5	74 ± 1.0	7 ± 0.5	18 ± 1.2	77 ± 2.3	5 ± 1.2	27 ± 5.5	69 ± 5.0	4 ± 0.5
None	18 ± 2.0	80 ± 3.0	2 ± 1.0	18 ± 2.3	80 ± 3.5	2 ± 1.2	18 ± 3.0	80 ± 3.0	2 ± 1.0

[a]Cells were obtained from mice which were treated as previously described, see Table 1. Blood smears were prepared from individual mice and stained with Camco Quick stain for microscopic evaluation.

[b]Data represent the mean ± SEM for 3 experiments on Day 5 (n = 3), and 4 experiments on Days 3 and 7 (n = 4), where 200 cells from each sample were evaluated.

TABLE 3. Effect of Test Polymers on Peritoneal Exudate Cell Number[a,b]

Agent	Day 3	Day 5	Day 7	Day 10
CDA	2.50 ± 0.58	2.47 ± 0.81	3.33 ± 0.55	2.79 ± 0.32
MP	2.40 ± 0.43	2.34 ± 0.11	2.49 ± 0.14	2.77 ± 0.28
IAS	1.73 ± 0.10	1.98 ± 0.16	3.23 ± 0.42	3.04 ± 0.07
LS	2.30 ± 0.17	2.69 ± 0.68	3.06 ± 0.28	2.51 ± 0.17
Pyran	3.27 ± 0.57	3.90 ± 0.31	5.08 ± 0.39	7.17 ± 1.40
C. parvum	2.33 ± 0.15	2.61 ± 0.21	3.28 ± 0.56	5.90 ± 1.16
Thio	21.19 ± 0.31	ND[c]	13.24 ± 2.68	ND[c]
None	1.87 ± 0.31	1.87 ± 0.31	1.87 ± 0.31	1.87 ± 0.31

[a]Mice were injected i.p. with 50 mg/kg of CDA, MP, LS, IAS, or pyran, 17.25 mg/kg of *C. parvum*, 1 mL of a 10% solution of thioglycollate or nothing 3, 5, 7, and 10 d prior to PEC collection. Whole PEC were obtained from individual mice and diluted in isotonic saline for counting. Three counts were performed on each sample, and the mean taken as the value for an individual mouse.

[b]Data represent the mean ± SEM of triplicate experiments of three individual mice/group ($n = 3$). Data are expressed as total PEC/mouse $\times 10^6$.

[c]ND = not done.

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MP-MA, IAS, and S-MA returned to normal by Day 7. In contrast, the PBL response to pyran and *C. parvum* showed an increase in monocytes, which was particularly evident on Day 7 when compared to polymer-treated or normal mice. The PBL in pyran and *C. parvum*-treated mice consisted of about 20% monocytes on Day 7 while PBL differentials in all other groups showed only about 4% monocytes. It is important to note, however, that monocytes in the pyran and *C. parvum* groups appeared atypical and were difficult to read. This reactivity pattern was not observed in test polymer-treated mice.

This effect of agent administration on peritoneal exudate call (PEC) number is shown in Table 3. An increase in total PEC number with time was observed following administration of pyran and *C. parvum*, suggesting that cells from the periphery were migrating to the peritoneal cavity in an inflammatory response. In contrast, the PEC response to test polymers, while slightly elevated compared to resident populations, remained relatively constant over the time course of the experiment.

The composition of PEC after administration is shown in Table 4. A marked

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TABLE 4. Composition of Peritoneal Exudates after Administration of Eliciting Agents[a,b]

	Day 3			Day 5			Day 7		
Agent	PMN	Lymph	Mono	PMN	Lymph	Mono	PMN	Lymph	Mono
CDA	13 ± 2.8	44 ± 2.8	43 ± 7.0	10 ± 3.5	65 ± 8.5	26 ± 4.9	8 ± 3.5	71 ± 4.3	21 ± 3.5
MP	16 ± 4.9	32 ± 4.3	52 ± 8.5	8 ± 4.9	67 ± 2.8	26 ± 2.8	6 ± 1.4	67 ± 7.0	28 ± 6.4
IAS	12 ± 3.5	35 ± 2.1	53 ± 4.9	7 ± 2.8	54 ± 4.3	40 ± 2.3	5 ± 1.4	53 ± 8.5	42 ± 9.2
LS	13 ± 9.2	36 ± 4.3	48 ± 12.8	10 ± 2.3	21 ± 2.8	71 ± 3.5	7 ± 2.8	51 ± 9.9	42 ± 9.9
Pyran	19 ± 3.5	13 ± 4.3	68 ± 7.0	7 ± 2.3	16 ± 3.5	77 ± 4.3	9 ± 4.3	9 ± 3.5	82 ± 2.3
C. parvum	23 ± 1.4	16 ± 1.4	62 ± 0.7	18 ± 4.9	28 ± 8.5	55 ± 7.0	19 ± 4.9	17 ± 2.8	65 ± 4.3
Thio	15 ± 5.6	4 ± 2.1	81 ± 7.0	8 ± 1.4	3 ± 2.8	90 ± 4.3	8 ± 2.3	10 ± 2.3	82 ± 1.4
None	2 ± 2.8	66 ± 7.0	32 ± 4.3	2 ± 2.8	66 ± 7.0	32 ± 4.3	2 ± 2.8	66 ± 7.0	32 ± 4.3

[a]PEC were obtained as previously described. After aliquots of PEC were taken for counting, the samples were centrifuged, and smears for differentials were prepared from the cell pellet. Smears were stained with Camco Quick stain for microscopic evaluation.

[b]Data represent the mean ± SEM of two experiments in which 200 cells from each of 3 mice were counted (n = 3).

increase in macrophages was observed after administration of pyran and *C. parvum*. On Day 7 macrophages made up to 82 and 65% of the PEC populations, respectively. On the other hand, test polymer-elicited PEC remained close to resident cells in terms of cell composition. Lymphocytes were the major cell type observed in normal and polymer-elicited PEC populations with the exception of S-MA elicited PEC populations where monocytes comprised 71% of the cells on Day 5.

Peroxidase Activity in Test Polymer-Elicited and Control Macrophage Populations

The cells responsible for the cytotoxic activity observed in pyran and *C. parvum*-elicited PEC population are thought to come from peripheral blood monocytes which migrate into the peritoneal cavity in an inflammatory response to i.p. administration of these agents. This idea is based on the studies of Ruco et al. who described the enhanced capacity of peroxidase-positive inflammatory macrophages to become activated under the influence of lymphokines [24]. The quantification of these newly arrived tissue macrophages may be accomplished by determining cellular lysosomal peroxidase activity, which is found in promocytes, monocytes, and in tissue macrophages which have recently migrated from the peripheral blood [25]. The lysosomal peroxidase activity of PMA after administration of selected test polymers were determined as shown in Table 5.

On Day 1 after pyran, *C. parvum* and IAS administration of the adherent PEC population consisted of 72, 59, and 37% peroxidase-positive cells, respectively. On Day 3, the percent peroxidase-positive adherent cells fell to 43% for pyran and to 25% for *C. parvum*. Thioglycollate-elicited adherent PEC consisted of 31% peroxidase-positive cells on Day 1. This value decreased with time after thioglycollate administration. In contrast, the peroxidase activity in adherent PEC populations elicited with CDA-MA, MP-MA, and S-MA remained at normal levels at all time points.

DISCUSSION

A series of polyanionic compounds were synthesized and evaluated in mice for their ability to elicit tumoricidal peritoneal macrophages. The cellular responses to these compounds were also determined.

Murine peritoneal macrophages (PMϕ) were activated to varying degrees by the polymers. CDA-MA, MP-MA, and IAS-UM2 elicited the highest degree

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TABLE 5. Peroxidase Activity in Adherent Peritoneal Exudate Cells after Administration of Eliciting Agents[a,b]

Agent	Day 1	Day 3	Day 5
CDA-MA	10 ± 0	5 ± 0.9	7 ± 0.9
MP-MA	6 ± 0.4	7 ± 0.4	5 ± 0.9
IAS	37 ± 1.8	3 ± 0.4	5 ± 0.4
LS	ND	10 ± 0.9	3 ± 0.4
Pyran	72 ± 2.7	43 ± 0.9	5 ± 0.4
C. parvum	59 ± 1.8	25 ± 1.3	6 ± 0.4
Thio	31 ± 2.2	19 ± 0.9	9 ± 1.3
None		7 ± 0.9	

[a]PEC were obtained from mice that received 50 mg/kg of CDA, MP, IAS, LS, or pyran i.p., 17.25 mg/kg *C. parvum* i.p., 1 mL of a 10% solution of thioglycollate, i.p. or nothing 1, 3, and 5 d prior to collection. Cells were cultured in 8-chamber Lab-Tek tissue culture chamgers, in complete media, for 1 h under standard conditions. Nonadherent cells were washed off, and the adherent monolayers were stained with Sigma leukocyte peroxidase Kit #90.A, according to manufacturer's directions. The data depict a representative experiment of two performed in which percent peroxidase-positive adherent cells were determined in 5 mice/group; 200 cells/mouse.

[b]Data represent the mean ± SEM of 5 mice (n = 5).

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of cytotoxic activity among the polymers tested (Figs. 2 and 3). ISA-PM10, MAVA-PM10, and EA elicited moderate cytotoxic activity, while S-MA, AV-MA, and E-MA did not elicit significant cytotoxic activity.

The PMϕ populations elicited with the polymers were evaluated for 5'N, LAP, and APD ectoenzyme activities. This profile has previously been used to discriminate between resident/normal, thioglycollate/inflammatory, and tumoricidal/activated PMO populations [21]. Results indicated that polymer-elicited PMO did not possess the elevated alkaline phosphodiesterase phenotype exhibited by thioglycollate/inflammatory Mϕ (Fig. 4). Leucine aminopeptidase (LAP) activity was not helpful in discriminating macrophage activation states although it has been previously reported that it was elevated in activated macrophage populations [22]. More recently, it has been suggested that LAP is an ubiquitous ectoenzyme whose activity more closely corresponds

to cell size than activation state [26]. The leucine amino peptidase levels consistently increased with time in culture (data not shown) which could be a reflection of the increase in cell surface area of PMO upon adherance and spreading in culture. All of the polymer-elicited PMO exhibited elevated levels of 5'N activity compared to pyran, *C. parvum*, and thioglycollate-elicited PMϕ (Fig. 4). Indeed, MAVA-PM10, CDA-MA, and MP-MA elicited PMϕ, which showed good tumoricidal activity, exhibited levels of 5'N activity which approached that of normal/resident PMϕ. Elevated levels of 5'N had been previously associated only with nontumoricidal, resident PMϕ.

The magnitude of the whole peritoneal exudate cell response to the polymers was slightly elevated but remained comparable to normal untreated mice at all time points (Table 3). In contrast, the whole PEC number gradually increased after administration of pyran and *C. parvum*. The composition of PEC after administration of the polymers also mimicked that of resident PEC, while a gradual and marked increase in the percentage of macrophages was observed after administrations of pyran and *C. parvum* (Table 4).

The peripheral blood leukocyte (PBL) counts of mice which received eliciting agents were generally increased compared to normal mice (Table 1). A marked increase in the percentage of monocytes was observed in the blood of mice that received pyran and *C. parvum* when compared to mice that received test polymers, thioglycollate, or nothing (Table 2).

Lysosomal peroxidase activity, which is found in promonocytes, monocytes, and newly arrived tissue macrophages, has been associated with macrophages inflammatory exudates and the ability of PMϕ to become activated to tumoricidal capacity [24]. Ruco and Meltzer suggested that newly arrived peroxidase-positive PMϕ could be more effectively activated to tumoricidal activity by lymphokines than could resident PMϕ [24]. In a different system, Lee et al. found that the PMϕ which responded best to a lipopolysaccharide (LPS) activation signal resided in the peroxidase-negative population [27]. Our results showed that PMϕ activation by pyran and *C. parvum* was associated with a peroxidase-positive PMϕ population which was presumably drawn from the peripheral blood pool. This idea is supported by the observation that an increase in peripheral blood monocytes was observed with time after administration of pyran and *C. parvum* (Table 2) and that these agents have previously been shown to cause stimulation of macrophage colony formation in the bone marrow [28, 29]. The PMϕ activated by CDA-MA and MP-MA, like resident cells, were predominantly peroxidase negative and were not associated with an increase in peripheral blood monocytes (Tables 2 and 5). IAS elicited PMϕ were 37% peroxidase positive on Day 1 and declined to normal values by Day 3. We are particularly interested in the observation that, in

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addition to demonstrating negligible levels of peroxidase activity, CDA-MA and MP-MA elicited PMϕ, like resident PMϕ and in contrast to pyran and *C. parvum* elicited PMϕ, were 5'N positive, consisted primarily of lymphocytes, and had normal total PEC counts. These data suggest that CDA-MA and MPA-MA activated PMϕ *in situ.*

The studies cited do not rule out the possibility that the polymers may be stimulating other cell types, particularly lymphocytes, which may be involved in macrophage activation directly or through their products. Certainly, soluble lymphocyte products, such as lymphokine, macrophage activating factor, λ-interferon, and β-interferon, have been shown to participate in the priming and/or triggering of the macrophages to tumoricidal activity [30-33]. The possibility that the polymers may, themselves, deliver the trigger signal for macrophage activation must also be considered. While the priming signal for macrophage activation appears to be dependent on environmental and/or lymphocyte-derived factors, many substances which can deliver the trigger signal for macrophage activation are lipophilic in character. These include phorbal myristate acetate, dimethylsulfoxide, lipopolysaccharide, lipid A, nystatin, amphoteracin B, and ethanol [34]. Lipophilicity is conferred on the polymers by virtue of either their six-membered ring structures or by virtue of their carbon side chains (Fig. 1). It has been suggested that reorganization of the macrophage plasma membrane lipid composition is crucial to macrophage activation, and that this reorganization renders the macrophage more susceptible to the reception of trigger signals for activation [35]. The fact that membrane composition is directly involved in LPS-responsiveness to B cells has been established [36]. These observations suggest the possibility that, due to their lipophilic character, lipophilic polymers may deliver the trigger signal for macrophage activation. Indeed, the lipophilicity of the polymers, as determined by the previously mentioned criteria, correlated with their ability to elicit tumoricidial PMϕ.

While toxicity studies have not been carried out, the polymers in general did not appear to induce the marked hepatomegaly and splenomegaly which is routinely observed with administration of pyran and *C. parvum.* Furthermore, no death attributable to drug toxicity was observed with any of the three drug dosages used in this study. These results are, however, preliminary, and detailed toxicology must be carried out as well as dosage optimization, analysis of effects in other tumor systems, and an analysis of other potential antitumor mechanisms such as interferon induction and natural killer cell activation.

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ACKNOWLEDGMENTS

The authors wish to acknowledge the support in part of this research by National Institutes of Health Grant A1-15612-02 as well as the Massey Cancer Center at Virginia Commonwealth University.

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