Effect of Pyran Copolymer on Activation of Murine Macrophages: Evidence for Incomplete Activation by Use of Functional Markers

Dolph O. Adams, William J. Johnson, Philip A. Marino, and Jack H. Dean

ABSTRACT

The degree of activation of peritoneal macrophages elicited by pyran copolymer (MVE-2) was studied in C57BL/6J mice. When cytotoxicity was examined under endotoxin-free culture conditions, the pyran-elicited macrophages could not complete cytolytic peritoneal mononuclear phagocytes elicited by sterile inflammatory agents such as fetal calf serum. The responsive macrophages, although not resident peritoneal macrophages, can respond to lymphokines by developing into a further stage, macrophages which are termed primed. Primed macrophages, in contrast to inflammatory macrophages, could effect cytostasis of tumor cells; their cytostatic potential was also augmented by addition of endotoxin. Taken together, the results indicated that pyran copolymer elicits primed but not fully activated murine macrophages.

INTRODUCTION

The activation of murine macrophages for tumor cytotoxicity proceeds via a cascade of stages, which culminates in the fully activated macrophage (19, 30, 37). The first step in this chain is the responsive macrophage, young mononuclear phagocytes elicited by sterile inflammatory agents such as fetal calf serum. The responsive macrophages, although not resident peritoneal macrophages, can respond to lymphokines by developing into a further stage, macrophages which are termed primed. Primed macrophages, although not responsive macrophages, can respond in turn to traces of endotoxin by becoming activated macrophages, fully capable of cytosis. The second signal may also be provided by lymphokines, products from bacteria, and supernatants of tumor cells (30). Of particular significance, this general sequence of development pertains to activation for microbicidal function and for tumoricidal function in vivo (9, 37).

Numerous immunomodulators are thought to produce tumor rejection by activating mononuclear phagocytes (for reviews, see Refs. 17 and 18). Defining precisely the effect of various immunopotentiators on macrophage development would obviously facilitate use of such biological response modifiers. Analyzing the state of activation of a population of mononuclear phagocytes, however, has been hampered by several problems: (a) the widespread contamination of media, sera, tissue culture constituents, and reagents with endotoxin can provide artifactual second signals in vitro, and thus make distinction of activated versus primed macrophages difficult (44, 45); (b) the stages of activation are currently defined operationally by the inductive signal or signals which must be added to push a given set of macrophages to full cytolytic competence, making analyses indirect and cumbersome; (c) quantification of either microbicidal or tumoricidal function requires several days for completion, producing long intervals between obtaining the macrophages and completion of the analysis. The ability to obtain tissue culture reagents virtually free of endotoxin (44), and the development of a panel of objective markers that characterize the stages of murine activation (21), have obviated these problems and made analysis of the stages of activation quite feasible.

Homogeneous, low-molecular-weight fractions of maleic anhydride-vinyl ether copolymer (2:1) (MVE or pyran copolymer) have been found in numerous laboratories, including ours, to activate murine macrophages for cytotoxicity (12, 16, 24, 32, 33). By use of endotoxin-free culture conditions and use of objective markers characterizing the stages of activation, we here report that pyran does not fully activate murine macrophages, but rather induces primed macrophages.

MATERIALS AND METHODS

Mice. Inbred female C57BL/6J mice were obtained from Harlan Sprague-Dawley, Madison, Wis. Mice of at least 8 to 16 weeks of age, but less than 30 weeks, were used in all experiments. The animals were housed in a vivarium and were fed mouse chow and water ad libitum.

Reagents. BCG,5 Phipps strain 1029, was purchased from the Trudeau Institute, Saranac Lake, N. Y. The PCS used for i.p. injection was obtained from Sterile Systems, Logan, Utah, and was supplemented with lipopolysaccharide (0.1 ng/ml) (35). Pyran copolymer (MVE-2) was purchased from Hercules, Inc., Wilmington, Del. Endotoxin prepared by Westphal phenolic extraction from Escherichia coli 026:B6 (Catalogue No. 3121; Difco Laboratories, Detroit, Mich.) was dissolved in phosphate-buffered saline and boiled for 1 hr. Reagents were screened for endotoxin by the Limulus amoebocyte lysate assay, and only those free of endotoxin, except the lipopolysaccharide and PCS used for injections, were used in all assays save that for cytostasis. Na251CrO4 (specific activity, 50 to 400 μCi/ml) and [3H]dThd (2 or 6.7 Ci/mmol) were purchased from New England Nuclear, Boston, Mass.

Macrophages. Peritoneal macrophages elicited by PCS were obtained 24 hr after the i.p. injection of 1 ml of PCS containing 0.1 ng of endotoxin per ml, which was used because of a recent report indicating that ng of endotoxin may be necessary to induce responsive macrophages (35).

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5 The abbreviations used are: BCG, Bacillus Calmette-Guerin; PCS, fetal calf serum; [3H]dThd, [3H]thymidine; PEC, peritoneal exudate cells; CP, cytolytic proteinase.
This procedure consistently yielded 4 to 6 x 10⁶ PEC, of which 50 to 65% were macrophages. Pyran-elicited macrophages were generally obtained 5 days after the injection of 100 μg of MVE-2. In some experiments, this procedure was altered (see "Results"). BCG-activated macrophages were obtained as previously described (4). Resident macrophages were obtained by peritoneal lavage from untreated mice. The PEC after lavage were plated as described previously (29), and were judged to consist principally of macrophages (greater than 98%), as demonstrated by microscopy, staining for nonspecific esterase, and uptake of IgG-coated particles (28).

Target Cells. The tumor cell lines, P815 (a mastocytoma from DBA/2 mice), MCA-V (a methylcholangiathrene-induced sarcoma from C57BL/6J mice), and MBL-2 (a Maloney virus-induced leukemia of C57BL/6J mice) were maintained in vitro as described previously (1, 13). The P815 tumor target cells were labeled with Na₂[³⁵Cr]O₄ as described previously (29), and the MCA and MBL-2 tumor cells were labeled with [³H]dThd as described previously (3, 13). All cell lines were maintained in vitro in Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 2 mm glutamine, 125 units penicillin per ml, 62.5 μg streptomycin per ml, and 10% heat-inactivated FCS (Sterile Systems, Inc.). All washes were performed using Hanks' balanced salt solution (Microbiological Associates, Walkersville, Md.) containing 5% FCS. All tissue culture media, sera, and reagents used, with 2 exceptions (see "Reagents" and "Quantification of Cytostasis"), were endotoxin-free. All experiments were performed without the addition of endotoxin; at a lower BCG macrophages:target cells ratio (i.e., 10:1), endotoxin enhanced cytostasis of tumor cells.

Quantification of Cytostasis. A microculture assay using MBL-2 target cells, modified from the visual counting method of Schultz et al. (41), has been described in detail previously (13). In brief, MBL-2 leukemia target cells from 48-hr cultures were added in 0.1 ml of complete medium to wells (20,000 targets/well) of a flat-bottomed microtiter plate (Catalogue no. 76-003-05, Linbro Plastics). This assay was not performed for 48 hr, and the resultant degree of cytolysis was monitored and quantified as described in "Materials and Methods." The cultures were conducted in the absence or presence of 10 ng of endotoxin per ml, as quantified by the Limulus amoebocyte lysate assay (Cape Cod Associates, Woods Hole, Mass.).

Quantification of Binding, Macrophage-mediated Cytotoxicity, and CP. These assays have been described in detail elsewhere. Binding of target cells to the macrophage monolayers was quantified as described previously (29). Cytolysis of the P815 tumor target cells was quantified as described previously (4). Cytolysis was also determined in some experiments by quantifying release of [³H]dThd from MCA-V-adherent sarcoma targets, as described previously (1). The preparation and quantification of CP have been described in detail (1, 22). CP is defined as the lytic activity contained in supernatants of activated macrophages, when these supernatants are obtained, prepared, and tested. CP activity in supernatants of activated macrophages, when these supernatants are obtained, prepared, and tested according to the protocol described (1, 22).

Quantification of Cytostasis. A microculture assay using MBL-2 target cells, modified from the visual counting method of Schultz et al. (41), has been described in detail previously (13). In brief, MBL-2 leukemia target cells from 48-hr cultures were added to wells (20,000 targets/well) of a flat-bottomed microtiter plate (Catalogue no. 76-003-05, Linbro Plastics). This assay was not performed under endotoxin-free conditions. Macrophages were obtained from the PEC of nontreated or treated mice by placing the PEC on microexudate-coated flasks, washing off the nonadherent cells, and then removing the adherent macrophages. The resultant purified macrophages were added (4 x 10⁶ or 2 x 10⁶ macrophages) in 0.1 ml of medium to the wells (20,000 targets/well) of a flat-bottomed microtiter plate (Catalogue no. 76-003-05, Linbro Plastics). This assay was not performed for 48 hr, and the resultant degree of cytolysis was monitored and quantified as described in "Materials and Methods." The cultures were conducted in the absence or presence of 10 ng of endotoxin per ml, as quantified by the Limulus amoebocyte lysate assay (Cape Cod Associates, Woods Hole, Mass.).

RESULTS

Effects of Pyran Copolymer on Macrophage Cytotoxic Function. We have previously reported that macrophages elicited by pyran copolymer are activated for cytolytic activity in vitro (12). When we examined these macrophages under endotoxin-free culture conditions (defined as <0.25 ng of endotoxin per ml, the limit of sensitivity of our assay), we obtained strikingly different results. Under the endotoxin-free conditions, the pyran-elicited macrophages were not cytolytic but did become so when pulsed with endotoxin (Chart 1A). BCG-activated macrophages, by contrast, were directly cytolytic (Chart 1B). Their cytolytic capacity was, however, enhanced by the addition of endotoxin to the assays (Chart 1B). The data indicate that pyran copolymer elicits a population of macrophages which are primed, while BCG elicits a population comprising activated and primed macrophages.

We next examined another form of macrophage-mediated cytotoxicity to tumor cells, cytostasis. At both E:T ratios tested, pyran-elicited macrophages were not fully cytostatic, but became so when exposed to traces of endotoxin (Table 1). BCG-activated macrophages, at a high E:T ratio (20:1), were cytostatic without the addition of endotoxin; at a lower BCG macrophages:target cells ratio (i.e., 10:1), endotoxin enhanced cytostatic activity (Table 1). Responsive macrophages elicited by FCS (21) were not cytostatic with or without addition of endotoxin (Table 1). The data suggest that macrophages elicited by pyran copolymer require endotoxin for effecting maximum cytostasis of tumor cells.

Effect of Various Injection Schedules on Induction of Macrophage Cytotoxic Function. To determine if pyran could acti-
vate macrophages under other circumstances, we varied both the interval between injection of pyran and harvest of the macrophages and the dose of pyran. Varying the interval from 6 days to either 4 or 8 days (Chart 2, A and B), or the dose of pyran from 100 μg/mouse to either 200 or 500 μg/mouse (Chart 2, C and D), still produced only primed macrophages. In no case was full activation observed (Chart 2).

Characterization of Pyran-elicited Macrophages by Use of Objective Markers. We have previously reported that objective markers can be used to characterize the stages of activation of murine macrophages (21). In brief, responsive, primed, and activated macrophages can be distinguished from resident peritoneal macrophages, because the former 3 macrophages display 5 markers characteristic of inflammatory macrophages (11). Specifically, the markers displayed by the responsive macrophages are: increased spreading, increased secretion of plasminogen activator, increased phagocytosis via the Fc receptor, ingestion via the C3 receptor, and decreased content of the ectoenzyme 5'-nucleotidase (21). Primed macrophages can be distinguished from responsive macrophages by their capacity for augmented binding of tumor cells and by their potential to secrete CP when pulsed with traces of endotoxin (21). Finally, activated macrophages can be distinguished from primed macrophages by their capacity for spontaneous secretion of CP (21).

The pyran-elicited macrophages displayed 5 markers of inflammatory macrophages (Table 2), thus distinguishing the pyran-elicited macrophages from resident peritoneal macrophages. The pyran-elicited macrophages bound tumor cells in a manner comparable to that of BCG-activated macrophages, and much better than responsive macrophages elicited by FCS (Chart 3), marking the pyran-elicited macrophages as either primed or activated. Finally, the pyran-activated macrophages did not secrete CP spontaneously, but did so when pulsed with traces of endotoxin (Chart 4), marking the pyran macrophages as primed. Taken together, the expression of these several markers clearly indicates that populations of pyran-elicited macrophages can be typed as primed macrophages.

Table 1

<table>
<thead>
<tr>
<th>Eliciting agent</th>
<th>Macrophage:MBL-2 target cell ratio</th>
<th>Lipopolysaccharide added</th>
<th>[%H]dThd incorporation (cpm x 10^-4)</th>
<th>% of cytostasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20:1</td>
<td>-</td>
<td>19.9 ± 0.5</td>
<td>47.7</td>
</tr>
<tr>
<td>None</td>
<td>10:1</td>
<td>-</td>
<td>27.7 ± 0.7</td>
<td>42.2</td>
</tr>
<tr>
<td>None</td>
<td>20:1</td>
<td>+</td>
<td>24.7 ± 1.1</td>
<td>98.9</td>
</tr>
<tr>
<td>None</td>
<td>10:1</td>
<td>+</td>
<td>31.3 ± 1.3</td>
<td>93.6</td>
</tr>
<tr>
<td>Pyran 20:1</td>
<td>10:1</td>
<td>-</td>
<td>10.4 ± 6</td>
<td>84.9</td>
</tr>
<tr>
<td>Pyran 10:1</td>
<td>10:1</td>
<td>-</td>
<td>16.0 ± 0.8</td>
<td>50.1</td>
</tr>
<tr>
<td>Pyran 20:1</td>
<td>10:1</td>
<td>+</td>
<td>0.3 ± 0.04</td>
<td>100.0</td>
</tr>
<tr>
<td>Pyran 10:1</td>
<td>10:1</td>
<td>+</td>
<td>1.6 ± 0.2</td>
<td>42.2</td>
</tr>
<tr>
<td>BCG 20:1</td>
<td>10:1</td>
<td>-</td>
<td>3.0 ± 0.2</td>
<td>50.1</td>
</tr>
<tr>
<td>BCG 10:1</td>
<td>10:1</td>
<td>-</td>
<td>13.8 ± 1.2</td>
<td>84.9</td>
</tr>
<tr>
<td>BCG 20:1</td>
<td>10:1</td>
<td>+</td>
<td>0.7 ± 0.1</td>
<td>97.1</td>
</tr>
<tr>
<td>BCG 10:1</td>
<td>10:1</td>
<td>+</td>
<td>0.6 ± 0.1</td>
<td>98.0</td>
</tr>
<tr>
<td>FCS 20:1</td>
<td>10:1</td>
<td>-</td>
<td>17.6 ± 0.8</td>
<td>11.8</td>
</tr>
<tr>
<td>FCS 10:1</td>
<td>10:1</td>
<td>-</td>
<td>24.8 ± 1.9</td>
<td>10.5</td>
</tr>
<tr>
<td>FCS 20:1</td>
<td>10:1</td>
<td>+</td>
<td>22.4 ± 1.2</td>
<td>12.3</td>
</tr>
<tr>
<td>FCS 10:1</td>
<td>10:1</td>
<td>+</td>
<td>24.3 ± 0.9</td>
<td>11.9</td>
</tr>
</tbody>
</table>

\[ ^{a} \text{Microculture (1 μg) of E. coli lipopolysaccharide B 0127:B8. This concentration did not significantly alter spontaneous [%H]dThd incorporation in MBL-2 target cells in the absence of macrophages (data not shown).} \]

\[ ^{b} \text{Mean ± S.E. of 3 separate experiments.} \]

\[ ^{c} \text{Significantly different from appropriate control at } p < 0.001 \text{ by Student's } t \text{ test.} \]

\[ ^{d} \text{Significantly different from appropriate control at } p < 0.0001 \text{ by Student's } t \text{ test.} \]

DISCUSSION

The activation of mononuclear phagocytes is central to host resistance against many tumors, so that modulation of macrophage activation is an important immunotherapeutic strategy (17, 18). Developing a clear understanding of the cellular physiology of activation would doubtless facilitate rational pharmacological manipulation of this defense system. Activation of murine mononuclear phagocytes, however, is complex, proceeding via a sequential cascade of interacting signals and appropriately responsive macrophages (19, 30, 37). The stages by which macrophages develop in this cascade are defined by the signals required (see "Introduction"). The operational nature of these definitions hampers, to some degree, establishment of the degree of activation of a given population of mononuclear phagocytes. A panel of objective markers, including markers previously reported to characterize inflammatory macrophages (11), distinguishes macrophages in these stages (21). The markers characterize populations of mononuclear phagocytes, among which may be considerable heterogeneity (43).

Pyran copolymer represents an excellent probe to examine this question critically, because it is a biological response modifier of defined composition. Pyran copolymer, which was used here in the purified MVE-2 form, is known to induce tumor rejection in vivo (13, 15, 23, 31, 34, 36, 38-42). One effect, as reported by several laboratories including ours, is to induce macrophages activated for tumor cytotoxicity (12, 16, 24, 32, 33). In fact, pyran-elicited macrophages, when examined under endotoxin-free culture conditions, are not fully activated for tumor cytotoxicity, but become so when pulsed with ng of endotoxin (Chart 1). Varying the interval between injection of pyran and harvest of the macrophages, or increasing the dose of pyran, did not alter this result (Chart 2). Thus, pyran-elicited macrophages appear to be in the primed stage when assessed by operational criteria. This result, in contrast to previous reports of pyran

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Table 2
Expression of inflammatory markers by pyran-elicited macrophages

Macrophages were obtained from the peritoneal cavity of C57BL/6J mice given 100 μg of pyran i.p. 6 days before harvest.

<table>
<thead>
<tr>
<th>Macrophages</th>
<th>Spreading (% of macrophage spread)</th>
<th>Phagocytosis via Fc (ingested cpm of 51Cr- E-1gG)</th>
<th>Phagocytosis via C3 (ingested E-lgM-complement/ 100 macrophages)</th>
<th>5'-Nucleotidase (mil.-units/mg macrophage protein)</th>
<th>Secretion of plasminogen activator (units secreted/ 10^6 macrophages/ 48 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resident</td>
<td>4 ± 2^a</td>
<td>496 ± 25</td>
<td>18 ± 5</td>
<td>105.0 ± 4.1</td>
<td>38 ± 11.4</td>
</tr>
<tr>
<td>Pyran elicited</td>
<td>76 ± 3</td>
<td>27,585 ± 1.351</td>
<td>282 ± 4</td>
<td>3.2 ± 11.7</td>
<td>554 ± 44</td>
</tr>
<tr>
<td>BCG activated</td>
<td>73 ± 2</td>
<td>30,352 ± 1,511</td>
<td>312 ± 7</td>
<td>1.2 ± 1.5</td>
<td>451 ± 14</td>
</tr>
</tbody>
</table>

^a E. erythrocyte.
^b Mean S.E.
^c Similar results were obtained in 2 additional experiments.

inducing fully activated macrophages, is doubtless attributable to performing the cytolytic assays in the virtual absence of endotoxin (<0.25 ng/ml). The widespread contamination of sera with endotoxin in amounts ranging from 10 to 1000 ng/ml (43) could readily account for this difference.

The cytostatic potential of pyran-elicited macrophages also appeared to depend upon the presence of endotoxin (Table 1). The cytostatic ability of activated macrophages has been consistently reported to be greater than that of resident peritoneal macrophages (for example, see Refs. 10, 25, and 27). The precise point in the activation cascade at which competence for mediating cytostasis develops, however, has not yet been defined. The present data imply capacity for mediating cytostasis, and the capacity for mediating cytosis may develop in tandem. Although the assay used here actually detects a combination of target stasis and lysis, the observation that pyran-elicited macrophages reduced incorporation of [3H]dThd in the absence of target stasis and lysis, the observation that pyran-elicited macrophages as primed in vivo (20). Taken together, analysis of functional marker data clearly indicates that pyran-elicited macrophages are primed. The finding that pyran copolymer primes rather than activates macrophages is not contradictory to reports of successful immunotherapy of tumors in vivo with pyran, since a variety of substances can push primed macrophages to full cytolytic competence (30).

The utility of this panel of objective markers for assessing the degree of macrophage activation produced in vivo has now been validated for one biological response modifier. We are currently examining the effects of other biological response modifiers. As our understanding of the immunopharmacology of macrophage activation develops, it will be critical to apply these observations to models of immunotherapy in vivo.

REFERENCES

**Induction of Primed Macrophages by Pyran**


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