Brief Definitive Report

EXPRESSION OF A NEW CELL SURFACE ANTIGEN ON ACTIVATED MURINE MACROPHAGES*

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The critical differences between normal and activated macrophages[1] have not been completely defined. Karnovsky et al. (1) have suggested that the metabolic functions of macrophages differ depending on whether they are activated by immunomodulators such as bacille Calmette-Guérin or pyran, stimulated (elicited) by inoculation with sterile inflammatory agents such as thioglycollate or glycogen, or are normal (unstimulated). Macrophages harvested from animals with chronic intracellular protozoal (2) or bacterial infections (3) have been shown to spread quickly on glass surfaces and they contained greater numbers of lysosomes, mitochondria, and other cellular inclusions than did normal macrophages (4). Moreover, immunologically activated macrophages also exhibited enhanced spreading, adherence, motility, phagocytosis, pinocytosis, and glucose-1-carbon oxidation (4). Several recent reports have indicated that activated and stimulated macrophages responded by increased secretion of lysozyme (5), plasminogen activator (6), collagenase (7), and the second component of complement (8). Similarly, carrier-mediated transport of 2-deoxy-D-glucose and L-leucine was increased in immunologically activated macrophages (9). While some of the metabolic differences between normal vs. stimulated or activated macrophages seem to be clear-cut, metabolically diferentiating the continuum from stimulation to activation has been difficult. Functionally, only activated and not elicited or normal macrophages have been shown to be cytotoxic and cytostatic to tumor cells (10, 11).

Many of the characteristics associated with activation are clearly related to enhanced membrane activity (e.g., membrane ruffling, glucosamine incorporation, pinocytosis, phagocytosis). In this report we describe an antiserum which detects a membrane antigen specifically associated with *Corynebacterium parvum* and pyran-activated macrophages but not detectable on glycogen, or thioglycollate-elicited or normal macrophages. This antiserum provides a simple reproducible tool for differentiating activated macrophages from their normal or stimulated counterparts.

Materials and Methods

Animals. Male and female C57B1/6, A/He, DBA/2, BALB/c, and BDF_1 mice were obtained

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[1] In this manuscript, the term "activated macrophage" is reserved for that population of macrophages that exhibits cytostasis and cytotoxicity for neoplastic cells.

from Jackson Laboratories, Bar Harbor, Maine, Simonsen Laboratories, Gilroy, Calif., or Charles River Breeding Laboratories, Wilmington, Mass.

Reagents. Pyran (lot XA124-177, Hercules Inc., Wilmington, Del.), *C. parvum* (Burroughs Wellcome Co., Research Triangle Park, N. C.), glycogen (type II from oyster, Sigma G-8751, Sigma Chemical Co., St. Louis, Mo.), and thioglycollate (Brewer's thioglycollate, Difco Laboratories, Detroit, Mich.) were used to induce peritoneal exudates as previously described (10, 11).

Cell Preparation. Peritoneal exudate cells (PEC) were obtained 4–7 days after i.p. inoculation of glycogen (0.5 ml/mouse of a 2.5% solution), thioglycollate (1 ml/mouse of a 10% solution), pyran (25 mg/kg), or *C. parvum* (17.5 mg/kg), by washing the peritoneal cavity twice with 4 ml of RPMI 1640. The PEC were collected by centrifugation at 500 *g* for 10 min, washed twice with Hanks' balanced salt solution (HBSS), and resuspended in HBSS with 5% fetal calf serum (FCS) previously absorbed with mouse spleen cells (AFCS). To obtain peritoneal macrophages (PM), PEC were resuspended in RPMI-1640 with 20% FCS and distributed at 4×10^7 PEC per plate into Petri dishes (100×15 mm) and incubated at 37°C in 5% CO_2 for 2 h. Nonadherent cells were washed off twice with RPMI-1640 without FCS, centrifuged at 500 *g* for 10 min, and resuspended in HBSS with 5% AFCS. Adherent cells (PM) were removed from the plates by gently scraping with a rubber policeman. The cells were centrifuged, washed twice with HBSS, and resuspended in HBSS with 5% AFCS. Greater than 95% of the adherent cells phagocytosized latex particles and were esterase positive (12). Single cell suspensions of the thymus were prepared by teasing the tissue in RPMI-1640 and forcing it through a wire screen. Thymus cells were washed twice and resuspended in HBSS with 5% AFCS.

The P388 leukemia was maintained in DBA/2 mice. Before using the P388 cells for absorptions, adherent cells were removed as described for the PEC. The macrophage cell line, P388D1, originally obtained from Dr. H. S. Koren (Duke University, Durham, N. C.) was maintained in spinner flasks in Eagle's Minimal Essential Medium supplemented with 20% FCS, 2 mm glutamine, and containing 100 U of penicillin and 100 μg streptomycin at 37°C in 5% CO_2. This cell line was originally isolated by Dawe and Potter (13) from the P388 DBA/2 mouse leukemia and was recently characterized by Koren et al. (14).

Preparation of Antiserum. Heterologous anti-macrophage serum (AMS) was prepared by injecting rabbits i.v. with P388D1 cells (2×10^7 to 7×10^7 cells per injection). The particular antiserum described in this report was obtained from a rabbit 10 days after the eighth inoculation with P388D1 over a 1-yr period. The Ig from a 33% ammonium sulfate precipitate of the antiserum was rendered specific for macrophages by repeated absorptions at 4°C with DBA/2 thymocytes and P388 leukemia cells depleted of contaminating adherent cells. Absorptions were carried out until no cytotoxicity for thymocytes was detected. Additional absorptions were performed similarly as described in the text.

Cytotoxicity Testing and Immunofluorescence. Cytotoxicity testing was performed by a standard Amos two-stage technique (15). Rabbit serum complement absorbed with mouse spleen and thymus cells and agarose was used. Indirect immunofluorescence was performed using fluorescein isothiocyanate-conjugated goat anti-rabbit IgG absorbed with P388D1 cells. The assay was carried out at 4°C in HBSS with 5% AFCS and 0.1% sodium azide.

Results

The AMS absorbed with thymus and P388 cells was cytotoxic for adherent PM from both *C. parvum* and glycogen-treated mice (Fig. 1). When macrophage-specific AMS was absorbed twice with 10^8 normal PEC per ml of antiserum, the cytotoxic activity against the glycogen-elicited macrophages was completely removed while the cytotoxic activity against the *C. parvum*-activated macrophages was unchanged. Absorption of AMS with P338D1 removed the cytotoxic activity against *C. parvum*-activated macrophages. In a similar experiment, AMS absorbed with normal PEC was cytotoxic for pyran-activated macrophages but not for glycogen, or thioglycollate-elicited macrophages. To rule out the possibility that we were identifying a strain-specific antigen, pyran-activated PEC from five strains of mice (C57B1/6, DBA/2, A, BALB/c, BDF_1) were tested

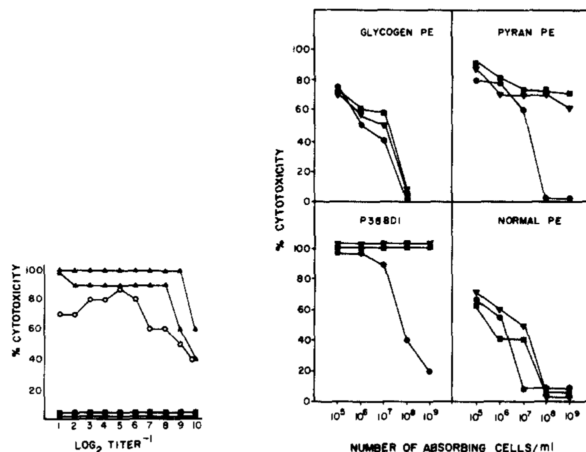

FIG. 1. Cytotoxicity of anti-macrophage serum (AMS) for *C. parvum*-activated and glycogen-elicited peritoneal macrophages: Target *C. parvum*-activated macrophages tested with AMS (△), AMS absorbed with normal PEC (▲), or AMS absorbed with P388D1 (■). Target glycogen-elicited peritoneal macrophages tested with AMS (○), or AMS absorbed with normal PEC (●).

FIG. 2. Quantitative absorption assay of anti-macrophage serum absorbed with varying numbers of PEC from pyran-treated mice (●), glycogen-treated mice (▲), or untreated normal mice (■). AMS absorbed with the specified number of cells/milliliter of AMS was tested at a dilution of 1:2 against glycogen-elicited PEC, pyran-activated PEC, normal PEC, or P388D1 cells. The target cell is shown on each panel.

and all were found equally susceptible to the cytotoxic activity of AMS absorbed with normal C57B1/6 PEC. Furthermore, absorptions with pyran-activated PEC from the C57B1/6 strain removed the cytotoxic reactivity of AMS to all the cell preparations tested (data not presented).

To further characterize the specificity of the antiserum, quantitative absorptions of AMS were done with normal PEC, pyran-activated PEC, or glycogen-elicited PEC; the absorbed antisera were then tested for cytotoxicity against normal PEC, glycogen-elicited PEC, pyran-activated PEC, and P388D1 cells. Absorption of AMS with normal or glycogen-elicited PEC removed the cytotoxic activity to normal and glycogen-elicited macrophages but not to pyran-activated macrophages or P388D1 cells (Fig. 2). About 50% of the cytotoxic activity to normal or elicited PEC was removed by absorption of AMS with 10^7 normal or glycogen-elicited cells/ml and all the reactivity was abolished by absorption with 10^8 normal or glycogen-elicited cells/ml. In contrast, absorption of AMS with normal or glycogen-elicited PEC caused no significant reduction in the cytotoxic activity to P388D1 cells or pyran-activated macrophages. In addition, increasing the cell concentration to 10^9 normal or glycogen-elicited cells/ml did not significantly reduce the AMS reactivity to pyran-activated macrophages or P388D1 cells. However, absorption of the AMS with pyran-activated PEC (10^8 cells/ml) removed cytotoxic activity against pyran-activated macrophages and the P388D1 cell line as well as against normal and glycogen-elicited macrophages (Fig. 2). Both AMS and AMS absorbed with normal PEC were not cytotoxic to thymus cells or nonadherent PEC.

To substantiate these findings in another test system, indirect immunofluo-

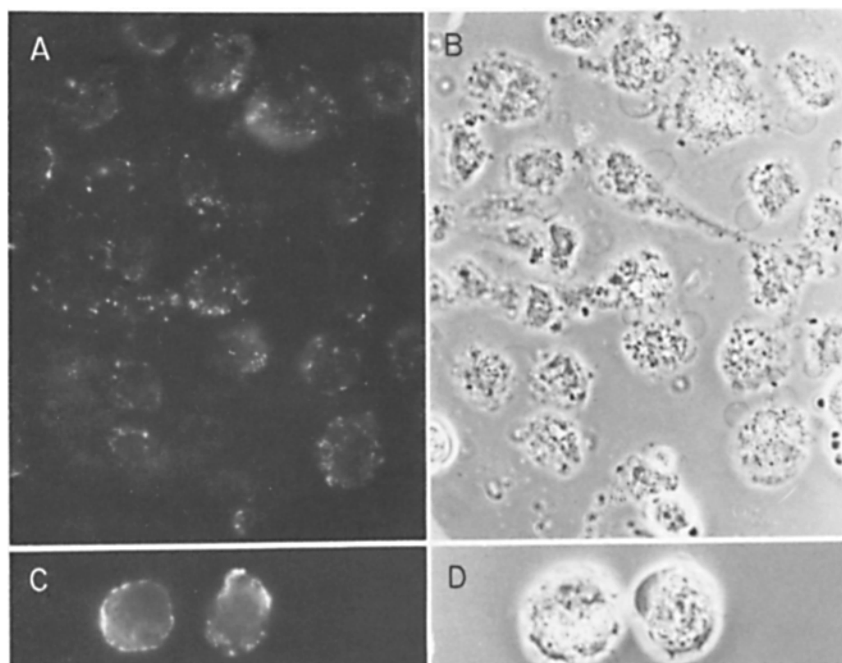

FIG. 3. Immunofluorescent and phase-contrast micrographs of pyran-activated PEC and P388D1 cells. Pyran-activated PEC (A) and P388D1 cells (C) were stained with rabbit AMS absorbed with normal PEC and fluoresceine-labeled goat anti-rabbit IgG. B and D are phase-contrast micrographs of the same microscope fields of pyran-activated PEC and P388D1 cells presented in panels A and C, respectively.

rescence was performed using normal rabbit serum, AMS or AMS absorbed with normal PEC as primary sera, goat anti-rabbit IgG as the fluorescent developing antiserum, and normal PM, pyran-activated PM, and P388D1 as target cells. Fig. 3A and 3C show the patchy fluorescence that was characteristic of pyran-activated macrophages and P388D1 cells respectively. The results in this system corroborated the results obtained with the cytotoxic assay (Table I). Specific AMS stained normal macrophages, P388D1 cells, and pyran-activated macrophages while the AMS absorbed with normal PEC stained only the pyran-activated macrophage and P388D1 cells.

Discussion

The results of this report indicate that macrophages activated by pyran or *C. parvum* which are known to have increased biochemical activity and to be cytotoxic for tumor cells (10, 11) also have a new macrophage cell-surface antigen. This antigen is also present on the P388D1 macrophage cell which functions as an activated macrophage as evidenced by cytostatic and cytotoxic activity for tumor cells (L. G. Baird and A. M. Kaplan, unpublished observations). In contrast, this new macrophage cell-surface antigen was not detected on normal macrophages or macrophages elicited by glycogen or thioglycollate, which are known to have increased biochemical activity but are not cytotoxic for tumor cells (10, 11). Preliminary studies with four bacterial vaccines,

TABLE I
*Determination of an Activated Macrophage-Specific Antigen by Immunofluorescence**

Target cell	Fluorescent cells		
	Normal rabbit serum	Anti-macrophage serum absorbed with normal PEC	Anti-macrophage serum
	%	%	%
Normal PEC	18	29	95
Pyran PEC	21	>99	>99
P388D1	19	>99	>99

* Immunofluorescent activity of normal rabbit serum, anti-macrophage serum, and anti-macrophage serum absorbed with normal PEC for normal PEM, pyran PEM (activated) and the P388D1 macrophage cell line.

Propionibacterium acnes, type I (CN1634, Burroughs Wellcome, *C. parvum*), *P. acnes,* type II (VPI 6637), *P. acnes,* type III (VPI 0204), and *P. granulosum* (VPI 6500) have indicated that only two vaccines, *P. acnes,* type I (CN1634) and *P. acnes,* type II (VPI 6637), could induce: (*a*) regression of the MCA 2182 fibrosarcoma when inoculated intralesionally, (*b*) splenomegaly, and (*c*) peritoneal macrophages cytotoxic to tumor cells in vitro. The new antigen associated with activated macrophages was only detectable on macrophages activated by the two vaccines (CN1634 and VPI 6637) which induced functionally activated cells (Kaplan, Mohanakumar, and Cummins, unpublished observations).

Absorption studies with different cell concentrations suggested that the presence of this antigen was not merely a quantitative phenomena but may be a qualitative one. The presence of this antigen on PM macrophages induced by *C. parvum* and pyran and on P388D1 cells suggested that the antigen is not due to a product of the inducing agent itself. However, further studies are needed to substantiate whether this antigen is a newly synthesized antigen or an expression of an antigen which may be present in extremely low levels or cryptic in normal and elicited macrophages. Moreover, these studies do not address the question of whether this antigen is a single or multiple entity. Studies on the kinetics of antigen appearance indicated that the antigen could be detected on PEC from mice 1 day after inoculation with pyran, was maintained for 25 days, and was no longer detectable at day 30 (unpublished observations). At all of these times when pyran-induced macrophages were tested against activated macrophage-specific AMS, 100% of the peritoneal macrophages was lysed by antiserum and complement. Yet we know that the capacity of pyran-activated peritoneal macrophages to mediate tumor cell cytotoxicity or to inhibit lymphoproliferation is at a peak level from ≅3 to 14 days after i.p. pyran inoculation (10, 11). The function of cells within the antigen-positive population may be related to the amount of antigen present on the macrophage surface or, alternatively, the antigen may be a differentiation antigen which is a marker for, but of itself insufficient to modulate the function of, the macrophages. The relationship of this antigen to the function of activated macrophages and to other macrophage membrane receptors is currently under investigation.

Summary

A macrophage cell-surface antigen associated with pyran and *Corynebacte-*

rium parvum-activated macrophages and P388D1 cells but not detectable on normal or glycogen and thioglycollate-elicited murine macrophages has been described. The antigen was demonstrated both by complement-mediated cytotoxicity and immunofluorescence, using an appropriately absorbed rabbit antiserum to P388D1. This antiserum should enable the characterization of activated macrophage cell populations on an individual cell basis and should be a useful probe to study the interactions of macrophages with tumor cells and the relationship of activation to cell-surface changes.

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