Brief Definitive Report

ESTABLISHMENT OF A HYBRIDOMA SECRETING A MONOCLONAL ANTIBODY SPECIFIC FOR ACTIVATED TUMORICIDAL MACROPHAGES*

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It is now well accepted that macrophages are implicated as important cells not only in resistance to tumor cells and infection but also in the immune system as effector and regulatory cells (1). Metabolic functions of macrophages differ depending on whether they are activated by Bacillus Calmette-Guérin (BCG), pyran copolymer, or *Corynebacterium parvum*, or elicited by injection with thioglycollate medium (TGC), proteose-peptone (PP), or mineral oil (2). Thus, activated or elicited macrophages were shown to secrete lysozomal enzymes (3), plasminogen activator (4), collagenase (5), and hydrogen peroxide (6).

In contrast to the extensive studies on their functions, little is known about membrane characteristics of activated, elicited, or resident macrophages. Although several studies have indicated that Mac-1,2,3 (7, 8), F4/80 (9, 10), and 2.6 (11) monoclonal antibodies defined macrophage cell surface markers, none of these monoclonal antibodies have been able to discriminate activated macrophages from elicited or resident macrophages. The only report of such a selective antibody was from Kaplan and his associates (12, 13), who produced a heterologous rabbit antiserum against the $P388D_1$ mouse macrophage cell line that, after very extensive absorptions with the proper cells, detected a cell surface antigen (AMϕCSA) associated with activated macrophages induced by pyran or *C. parvum*.

To understand precise mechanisms by which macrophages become activated, we attempted to produce a series of monoclonal antibodies specific for activated macrophages. In this report, we describe successful production of such a monoclonal antibody specific for activated tumoricidal macrophages. This monoclonal antibody should provide a useful tool for the analysis of activation or differentiation of macrophages.

Materials and Methods

Animals. C3H/HeN mice and LOU/CN rats were kindly provided by Dr. R. B. Herberman, Biological Development Branch, National Cancer Institute Frederick Cancer Research Facility, and bled in our facility. BALB/c mice were purchased from Shizuoka Animal Laboratory Center, Shizuoka, Japan.

Reagents. Pyran copolymer (NCS 46015, Hercules Research Center, Wilmington, DE) and *C. parvum* (lot CA771/B, Wellcome Research Laboratories, Beckengham, England) were kindly obtained from Dr. H. T. Holden, Biological Development Branch, NCI-FCRF. Pyran was dissolved in sterile 0.9% NaCl solution, and the pH was adjusted to 7.2 with 1 N NaOH. PP

* Supported in part by grants from the Ministry of Education, Science and Culture, Japan.

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 J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/10/1286/06 $1.00
Volume 156 October 1982 1286–1291

and TGC were purchased from Difco Laboratories, Detroit, MI. Mineral oil was obtained from Aldrich Chemical Co., Milwaukee, WI.

Cell Preparation. Peritoneal exudate cells (PEC) were obtained from the peritoneal cavity of mice treated with TGC or PP (1 ml/mouse of a 10% solution, day −4), pyran (25 mg/kg of body weight, day −8) or *C. parvum* (2.1 mg/mouse, day −8). To obtain purified macrophages, PEC were incubated in petri dishes (100 × 15 mm) for 1–3 h at 37°C, and adherent cells (macrophages) were obtained from the plates by gently scraping with a rubber policeman after removing nonadherent cells. For the preparation of neutrophils, mice were treated intraperitoneally with TGC (−18 h) and peritoneal exudate cells were harvested. Blood monocytes were purified from peripheral blood by adherence.

Cell Fusion. Immune spleen cells from LOU/CN rats primed with two injections of pyran-activated PEC from C3H/HeN or BALB/c mice were fused with the P3 × 63Ag8U1 (P3U1) mouse myeloma cells as described previously (14, 15). Screening for a hybridoma secreting a monoclonal antibody specific for activated macrophages was by a ^{125}I-labeled protein, a binding assay on pyran-activated macrophages, or TGC-elicited macrophages. Hybridoma with appropriate characteristics was cloned twice by the limiting dilution method and then used for the present study.

Binding Assay. The cells (2–5 × 10^5 cells in 50 μl of phosphate-buffered saline [PBS] containing 2% bovine serum albumin [BSA] and 10 mM NaN_3) were first incubated for 2–3 h at 4°C with 50 μl of hybridoma supernatant in microtest plates, washed in PBS containing 2% BSA and 10 mM NaN_3, followed by incubation for 4–6 h with ^{125}I-labeled protein A (30,000–100,000 cpm/well) at 4°C. After incubation, the cells were washed twice, and the radioactivity of ^{125}I-labeled protein A bound to cell surfaces was counted by a gamma counter.

Purification of AcM.1 Monoclonal Antibody and Preparation of Biotinyl-AcM.1 Antibody. Supernatant from AcM.1 tissue culture was concentrated 10-fold with an Amicon concentrator under the nitrogen pressure. Then, concentrated supernatants were applied on an affinity column of protein A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Biotinylation of purified AcM.1 antibody was carried out by the method of Bayes and Wilchek (16).

Fluorescence Study. Aliquots of 1–3 × 10^6 cells to be stained were pelleted by centrifugation and incubated with 10–20 μl of biotinylated AcM.1 antibody (1 mg/ml) for 30 min on ice. After washing with 10 ml of cold PBS containing 0.5% BSA and 10 mM NaN_3, the cells were resuspended in 10–20 μl of fluorescein isothiocyanate (FITC)-conjugated avidin (FITC-avidin, 1 mg/ml, E. Y. Laboratories, Inc., San Mateo, CA), and the mixture was further incubated for 20 min on ice. After washings, the cells were examined with a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York). Cytofluorometry was performed with the EPICS V cell sorter (Coulter Electronics Inc., Hialeah, FL), using appropriate scatter windows to screen out cellular debris, dead cells, and cell aggregates (laser power, 800 mW; fluorescence gain 20; and photomultiplier, 750–800 V).

Treatment of Cells with Antibodies and Complement. Cells were treated with AcM.1 monoclonal antibody (1:1) or with anti-Thy 1 monoclonal antibody (1:400, clone F7D5, Olac 1976 Ltd, Shawa Farm, Blackthorn Bicester Oxon, OX60 OTP, England) for 30 min on ice. After centrifugation, the cells were suspended in rabbit complement (C) diluted 1:8, and the mixture was incubated for another 30 min at 37°C.

Macrophage-mediated Cytotoxicity. Macrophage-mediated cytotoxicity was assessed as described previously (17). Target cells were labeled with 100–200 μCi of sodium ^{51}chromate (New England Nuclear, Boston, MA) for 60 min at 37°C, washed three times, and then added to effector cells at 5 × 10^3 cells/well. After 18 h of incubation, the plates were centrifuged for 10 min at 350 *g*, 0.1 ml of supernatant was harvested, and the radioactivity was counted in a gamma counter. The percent of specific cytotoxicity was calculated as: ([cpm in experimental group − cpm with thymocyte control]/[total cpm incorporated into target cells − cpm with thymocyte control]) × 100. The baseline control release of the target cells cultured with thymocytes for 18 h varied between 20.3 and 31.6% of the total radioactivity incorporated.

Results and Discussion

From approximately 600 hybrids obtained from four separate experiments, in which immune spleen cells from LOU/CN rats treated with multiple injections of pyran-activated PEC of C3H/HeN or BALB/c mice were fused with the P3U1 mouse

TABLE I

Reactivity of a Monoclonal Antibody (AcM.1) with Mouse Primary Cells

Cell preparations	Percent of cell types in preparations			Percent AcM.1 positive ± SE	Percent killed ± SE
	Macrophages	Lymphocytes	Neutrophils		
Resident PEC	65	30	5	<5	<5
TGC-PEC (day 4)	80	10	10	<5	<5
Proteose-peptone-PEC (day 4)	74	18	8	<5	<5
Pyran-PEC (day 8)	75	18	7	73 ± 4	68 ± 3
C. parvum-PEC (day 8)	77	9	14	62 ± 5	60 ± 5
TGC-PEC (18 h)	25	10	65	<5	ND‖
Blood monocytes	95	5	0	<5	ND
Spleen cells	10	90	0	<5	<5
Bone marrow cells	—‖	—	—	<5	ND
Thymocytes	5	95	0	<5	<5

* Stained by Wright-Giemsa method.
‡ Determined by fluorescence microscopy and cytofluorometry.
§ Cytotoxicity at 1:2 dilution of AcM.1 antibody plus C was determined by a dye exclusion test.
‖ Not done.

myeloma cells, we obtained only one stable clone secreting a monoclonal antibody, termed AcM.1, specific for pyran-activated macrophages but not TGC-elicited macrophages (mϕ), cloned twice and used for the present study. The isotype of the antibody was IgG_{2c} and it had the capacity to fix C.

The specificity of the AcM.1 monoclonal antibody was determined by fluorescence microscopy, ^{125}I-labeled protein A-binding assay, and C-dependent cytotoxicity. As shown in Table I, the AcM.1 monoclonal antibody reacted only with pyran- or *C. parvum*-activated PEC, but not with TGC-, or PP-elicited PEC, resident PEC, blood monocytes, neutrophils, spleen cells, bone marrow cells, or thymocytes. Although these results suggested that this AcM.1 antibody was specific for activated macrophages, we further tested the reactivity of the AcM.1 antibody by using purified macrophages (>95% nonspecific esterase-positive and phagocytic cells) from various PEC_s by plastic adherence. The results in Fig. 1 indicate that positive binding by the AcM.1 monoclonal antibody was noted on activated macrophages induced by pyran or *C. parvum*, whereas TGC-, PP-, or mineral oil-elicited macrophages were found to be negative. In addition, resident macrophages were also shown to be negative (data not shown). Thus, the results indicated that this AcM.1 monoclonal antibody was specific for activated macrophages.

Previous studies indicated that pyran and *C. parvum* are potent immunostimulants that render macrophages tumoricidal (18). As shown in Fig. 2, it is evident that tumoricidal capacity of macrophages was only associated with pyran- or *C. parvum*-activated cells but not with TGC-elicited macrophages. To directly demonstrate that tumoricidal macrophages bear the AcM.1 antigen, we treated pyran-activated PEC with the AcM.1 monoclonal antibody or anti-Thy-1 monoclonal antibody plus C and then tested for cytotoxicity against tumor cells. Fig. 3 shows that pretreatment of pyran-activated PEC with the AcM.1 antibody plus C abolished ~80% cytotoxicity against tumor cells. However, the AcM.1 antibody only or antibody plus heat-inactivated (56°C for 30 min) C and anti-Thy-1 monoclonal antibody, as expected, did not affect cytotoxicity of macrophages. Thus, to our knowledge, this monoclonal antibody (AcM.1) is the first unique marker of tumoricidal macrophages.

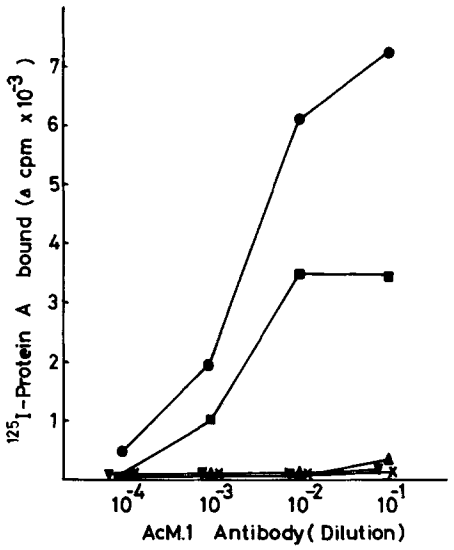

FIG. 1. Expression of AcM.1 antigen on pyran and *C. parvum*-activated macrophages or thioglycollate medium (TGC)-, proteose-peptone (PP)-, and mineral oil-elicited macrophages. Peritoneal macrophages purified from PEC of C3H/HeN mice treated at day −8 with pyran (25 mg/kg of body weight) or *C. parvum* (2.1 mg/mouse), or at day −4 with 10% TGC (1 ml/mouse), 10% PP (1 ml/mouse), or mineral oil (1 ml/mouse) were mixed with 50 μl of various dilutions of AcM.1 antibody in 96-well microtest plates, and then incubated for 1–2 h on ice. After washing twice with 100 μl of cold PBS containing 2% BSA and 10 mM NaN_3, the cells were further treated with 50 μl of ^{125}I-labeled protein A (34,000 cpm/well) for 2–3 h on ice. After vigorous washing with cold PBS plus 2% BSA and 10 mM NaN_3, each well was cut by scissors and counted in a gamma counter. Each point represents the mean of triplicate wells. ●, Pyran Mφ; ■, *C. parvum* Mφ; ▲, TGC Mφ; ▼, PP Mφ; ×, mineral oil-Mφ.

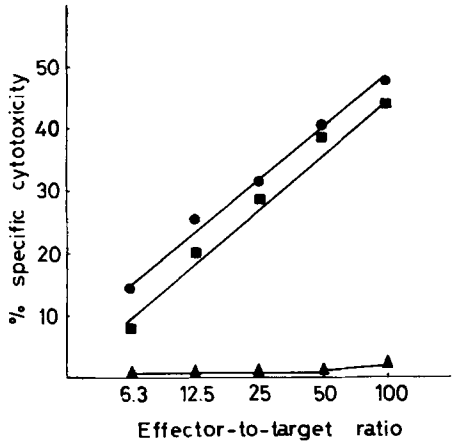

FIG. 2. Macrophage-mediated cytotoxicity from mice treated with pyran, *C. parvum*, or TGC. Peritoneal exudate cells from mice treated at −8 d with pyran (25 mg/kg of body weight) or *C. parvum* (2.1 mg/mouse), or at −4 d with 10% TGC (1 ml/mouse), were harvested and adhered to plastic dishes. After removing nonadherent cells with vigorous washings, adherent cells (>95% macrophages by morphological test) were recovered by rubber policeman and then tested for macrophage-mediated cytotoxicity against ^{51}Cr-labeled L1210 target cells (5×10^3 cells/well). ●, *C. parvum* Mφ; ■, Pyran Mφ; ▲, TGC Mφ.

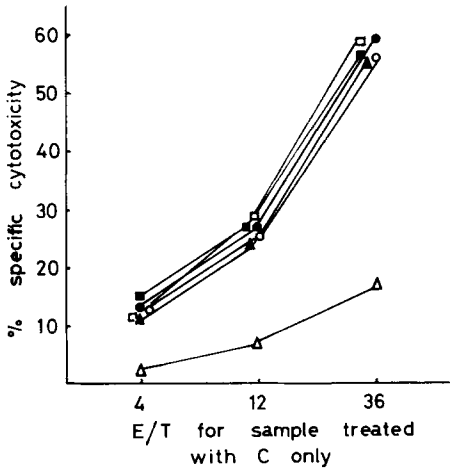

FIG. 3. Effect of pretreatment of pyran-activated peritoneal exudate cells with AcM.1 antibody and complement (C) on cytotoxicity against tumor cells. Pyran-activated PEC were treated with AcM.1 antibody (1:1) and C (△), monoclonal anti-Thy 1 antibody (1:400) and C (▲), AcM.1 antibody (1:1) and medium (□), AcM.1 antibody (1:1) and heat-inactivated C (■), medium and C (●), or medium for both incubation (○), and then tested for tumor cytotoxicity against ^{51}Cr-labeled L1210 target cells that are insensitive to natural killer (NK) lysis. Similar results were obtained by using *C. parvum*-activated PEC as effector cells.

A few years ago, Kaplan et al. (12) reported new cell surface antigens (AMϕCSA) on activated macrophages, as determined by a heterologous antiserum adsorbed properly. The present study confirms the previous study and extends it by describing a monoclonal antibody (AcM.1) that can discriminate activated macrophages from elicited or resident macrophages. However, relationship between AcM.1 and AMϕCSA has not been established yet. Our preliminary experiment indicates that two polypeptides (67,000 and 44,000 mol wt) are identified as the AcM.1 antigen (unpublished data). Thus, it is suggested that the AcM.1 antigen is different from Mac-1,2,3 (7, 8), F4/80 (9, 10), and 2.6 (9, 10), detected by monoclonal antibodies and membrane-associated factor B (19). However, further investigation is necessary to determine whether the AcM.1 antigen is an acquired product (such as complement component) or an endogenous macrophage product.

In conclusion, all these data on the pattern of reactivity strongly indicate that the AcM.1 antigen is a new cell surface marker for activated tumoricidal macrophages and should provide a useful probe to investigate the mechanisms of activation of macrophages. The relationship of this AcM.1 antigen to the function of activated macrophages and to other macrophage antigens is now under investigation.

Summary

New activated macrophage-specific antigen (AcM.1) detected by a monoclonal antibody has been described. The AcM.1 antigen was only detectable on activated macrophages induced by pyran and *Corynebactrium parvum* but not on resident or thioglycollate medium (TGC), proteose-peptone, and mineral oil-elicited macrophages, and not on blood monocytes and neutrophils.

Activated macrophages induced by pyran and *C. parvum*, as expected, exhibited tumor cytotoxicity, whereas TGC-elicited macrophages did not show any cytotoxicity. Moreover, pretreatment of pyran-activated peritoneal exudate cells with AcM.1 antibody plus complement abolished ~80% cytotoxicity.

Thus, this AcM.1 antigen detected by a monoclonal antibody is the first unique marker of activated tumoricidal macrophages and should provide a useful probe for investigating the mechanisms of activation or differentiation of macrophages.

We are grateful to Dr. R. B. Herberman, NCI-FCRF, for his thorough review of the manuscript, and to Miss Yumiko Otonari for her excellent technical assistance.

Received for publication 29 June 1982 and in revised form 28 July 1982.

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