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Selective macrophage activation by muramyldipeptide bound to monoclonal antibodies specific for mouse tumor cells

Annie-Claude Roche, Pascal Bailly, Patrick Midoux, and Michel Monsigny

Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, 1, rue Haute, F-45045 Orleans Cedex, France

Summary. *IgM monoclonal antibodies directed against tumor cells which do not mediate antibody-dependent macrophage cytotoxicity (ADMC) even when they are cytotoxic in the presence of complement, have been shown to render macrophages tumoricidal when they carry an immunomodulating agent, i.e., muramyldipeptide (MDP).*

This statement is based on experiments using two IgM monoclonal antibodies selected for their ability to bind L1210 leukemia cells (F_2-10-23-IgM) and 3LL Lewis lung carcinoma cells (6B6-IgM) specifically, as shown by flow cytofluorometry analysis.

The MDP-IgM conjugates, containing 45 MDP molecules per IgM molecule, were prepared by allowing MDP-hydroxysuccinimide ester to react with IgM monoclonal antibodies.

The MDP-IgM conjugates are shown to bind to relevant tumor cells and to induce the activation of thioglycolate-elicited peritoneal mouse macrophages leading to 80% growth inhibition of target cells at optimum concentrations of bound MDP. These concentrations of bound MDP were 10 times lower than the concentration of free MDP, giving a maximum activation that is limited to 20% growth inhibition.

No macrophage activation was evidenced when tumor cells were incubated in the presence of irrelevant MDP-IgM conjugates and macrophages or when macrophages were preincubated in the presence of MDP-IgM conjugates and then incubated in the presence of relevant or irrelevant tumor cells but in the absence of the MDP-IgM conjugates.

The reported results are discussed with reference to the mechanism of activation of macrophage by muramyldipeptide and to the usefulness of such MDP-IgM conjugates as potential antitumor agents in cancer therapy.

Offprint requests to: A.-C. Roche

Abbreviations: ADMC: antibody dependent macrophage mediated cytotoxicity; F-GAM: fluoresceinylthiocarbamyl goat anti mouse antibody; α-Man-BSA: α-mannopyranosyl-phenylthiocarbamyl bovine serum albumin containing some 25 mannose residues (neoglycoprotein); MDP: muramyldipeptide, 2-acetamido-3(2-0-D-lactyl-L-alanyl-D-glutamyl amine) glucopyranose; MDP-F_2-10-23-IgM : Murine monoclonal antibody specific of L1210 leukemia cells and substituted with 45 MDP molecules; MDP–6B6-IgM : Murine monoclonal antibody specific of 3LL Lewis lung carcinoma cells and substituted with 45 MDP molecules; MEM: minimal essential medium; TDM: Trehalose-dimycolate

Introduction

The in vivo antitumor effect of some monoclonal antibodies specific for tumor-associated antigen has already been described elsewhere [7, 9, 22, 24, 25]. In most cases, there is a good correlation between the growth inhibition and the in vitro antibody-dependent macrophage-mediated cytotoxicity (ADMC), suggesting that macrophages may have an important role in the antitumor effect of the antibodies used [25]. Macrophages have been observed to adhere closely to target cells prior to their destruction. But many monoclonal antibodies specific for tumor cells showed no activity; in particular IgM monoclonal antibodies are often devoid of therapeutic effect, despite their high complement-mediated cytotoxic titer, in vitro [22]. Furthermore, macrophages which infiltrate a large number of solid tumors are not efficient in impairing the tumor growth (for review see [10]). Macrophage tumoricidal activation can occur by the action of different agents, such as lymphokines [21] and/or bacterial products [8]. In vitro, tumoricidal activation of macrophages was allowed by muramyldipeptide (MDP), i.e., *N*-acetylmuramyl-L-alanyl-D-isoglutamine, the smallest mycobacterial structure able to induce immunomodulating activities [1]. However, MDP (mol. wt. 492) is very rapidly cleared from the body [20] and so has quite low in vivo efficiency in tumor growth inhibition [3]. When MDP was injected in combination with a suspension of trehalose-dimycolate (TDM), a glycolipid derived from mycobacterial cell wall, MDP was shown to inhibit the growth of a hepatocarcinoma implanted ID in guinea-pigs [15]. The efficacy of MDP is highly improved when it is carried by liposomes [5, 6, 26] or by neoglycoproteins that are selectively recognized and endocytosed by macrophages [19], and it has induced regression of established pulmonary metastases after systemic injection into mice [6]. In both cases, i.e., liposome-encapsulated MDP and MDP-neoglycoproteins, the use of a carrier inducing the internalization of MDP improved its immunostimulant activity. However, the systemic activation of macrophages can have undesirable effects, such as autoimmune diseases. The specific activation of macrophages infiltrating tumor and metastases, instead of the whole population of macrophages, could be efficient in the treatment of tumors and might prevent unwanted immune reactions.

Cells of the mononuclear system have cell surface receptors for immunoglobulins involved in the recognition of immune complexes. Monoclonal antibodies specific for tumor cells and substituted with MDP could be used to trigger the activation of macrophages after adsorptive endocytosis as

soluble immune complexes or as complexes bound to the tumor target cells.

In the present study, we report the activation of thioglycolate-elicited mouse peritoneal macrophages by MDP bound to IgM monoclonal antibodies specific for L1210 leukemic cells (F_2-10-23) [23] and for Lewis lung carcinoma 3LL (6B6) [17].

Material and methods

Animals. C57Bl/6 mice were obtained from Iffa-Credo (Lyon, France).

Cell cultures. Lewis lung carcinoma (3LL) (kindly provided by Dr. F. Lavelle, Rhône-Poulenc Recherches, Vitry-sur-Seine, France) is a malignant metastasizing tumor that develops in C57BL/6 mice, leading to a local tumor and spontaneous pulmonary metastases following IM injection. The 3LL cells (2×10^4 cells/ml) adapted to grow in monolayer culture were grown in tissue culture dishes in minimal essential medium (MEM) with Hank's salts supplemented with 20% heat-inactivated fetal bovine serum (Gibco, Renfrewshire, UK), 2 m*M* L-glutamine (Merck, Darmstadt, W. Germany), 100 U penicillin/ml culture and 100 µg streptomycin/ml (Eurobio, Paris, France) (complete culture medium). The cells were incubated at 37° C in a humidified atmosphere of 5% CO_2 95% air. Cells forming monolayers were grown to confluency (3–4 days) and were harvested after a brief (2 min) incubation with 0.02% EDTA in PBS at 37° C. The cells were washed in MEM and resuspended in complete culture medium.

The DBA/2 lymphoma L1210 cells (kindly given by Dr I. Gresser, Villejuif, France) were adapted to grow in suspension in MEM with Earle's salts supplemented with 10% heat-inactivated horse serum (Pointet Girard, IBF-Réactifs, Villeneuve la Garenne, France) and were collected in the phase of exponential growth.

Preparation and purification of macrophages. Peritoneal exudate macrophages were obtained from the peritoneal cavity of C57BL/6 mice that 4 days earlier had each received 1.5 ml thioglycolate medium IP (Institut Pasteur, Paris, France). Mice were killed by decapitation and peritoneal cells were harvested in Ca^{2+}-, Mg^{2+} -free medium (RPMI 1640, Eurobio) supplemented with 10 m*M* Hepes buffer and antibiotics.

Macrophage suspensions were centrifuged, washed, and resuspended in serum-free medium. Macrophages identified by neutral red uptake were plated into wells of Microtest III plates (Falcon, Sunnyvale, Calif:, Becton-Dickinson). After 1 h incubation at 37° C, nonadherent cells (less than 10%) were removed by washing. The culture medium used throughout was MEM with Hank's salt, 10 m*M* Hepes buffer supplemented with 2 m*M* L-glutamine, 2 m*M* sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum.

Reagent: N-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP, or muramyldipetide) was prepared as described elsewhere [16] or obtained from the Institut Pasteur (Paris, France).

Monoclonal antibodies, $F_2$10-23-IgM specific for L1210 leukemia cells [23], and 6B6-IgM specific for 3LL cells [17] were purified from ascitic fluid by gel chromatography on ACA34 Ultrogel (IBF-Réactifs, Pointet Girard, Villeneuve la Garenne, France).

The covalent attachment of MDP to IgM monoclonal antibodies was performed by carbodiimide-mediated formation of amide linkage between the gamma-glutamyl-carboxyl group of MDP and the primary amino groups of the monoclonal antibodies. In a typical experiment, to a solution of 30 mg (60 µmoles) MDP in 0.6 ml freshly distillated *N*-dimethylformamide, 15 mg (72 µmol) dicyclohexylcarbodiimide and 8.3 mg (72 µmol) *N*-hydroxysuccinimide were added. After 24 h stirring, dicyclohexylurea was eliminated. The organic solution containing the hydroxysuccinimide-MDP ester (3 mg in 60 µl *N*-dimethylformamide) was added to an aqueous solution of monoclonal antibodies (3 mg/ml), adjusted to pH 8.5 with 1 *M* $NaHCO_3$. The reaction mixture was stirred for 24 h at room temperature. The conjugate was purified by gel chromatography on Ultrogel ACA 202 or Ultrogel GF05 (IBF-Réactifs) in phoshate-buffered saline (PBS) pH 8.5. The number of MDP residues per carrier molecule was estimated according to the method of Levvy and McAllan [12] at 45. The absence of LPS contaminants (less than 0.1 ng/ml) was checked using the previously described method [18].

Flow cytofluorometry analysis. Immunofluorescence staining was carried out by incubating 10^6 tumor cells in the presence of appropriate concentrations of monoclonal antibodies for 45 min at 4° C, then with fluorescein-conjugated $(F(ab')_2$ fragment of goat anti-mouse IgM (μ-chain-specific) (F-GAM) (Cappel, Cochranville, USA).

Analysis was performed by using a fluorescence-activated cell analyzer (FACS analyzer, Becton-Dickinson, Mountain View, USA). The size (Coulter system and the fluorescence intensity of each cell were simultaneously recorded at a rate of 250 cells/s (exc: 485 nm ± 20; em: 530 nm ± 30).

In vitro activation of thioglycolate-elicited peritoneal macrophages. Macrophages-mediated cytostasis was assessed by a radioactivity incorporation assay [8]. Target cells (L1210 or 3LL cells) collected from culture during the exponential growth phase were suspended for 1 h with various concentrations of IgM or MDP-bound IgM monoclonal antibodies. Supernatant fluids containing unbound antibodies were discarded and cells were washed with fresh medium. Washed cells were resuspended in complete medium and were added on plated macrophages (4×10^5 macrophages identified by neutral red uptake per well) in a 96-well flat-bottom tissue culture tray. Each well contained 2×10^4 tumor cells in final volume of 200 µl to give an initial macrophage-to-tumor target cell ratio of 20 : 1. The (%) growth inhibition was determined after a further 48-hr, co-cultivation. [^{3}H]-Thymidine (0.5 µCi, 18.5 KBq; specific activity 0.74–1.1 TBq/mmol, CEA, Saclay, France) was added 4 h before harvesting. Cells were collected and washed on Whatman glassfiber filters and radioactivity was measured in aqueous counting scintillant (ACS, Amersham, Bucks. UK), in a β-scintillator counter (Beckman Fullerton, USA). The percentage of cytostatic activity was expressed as growth inhibition: GI (%) = (R-S/R) × 100, where R is the radioactivity incorporated into tumor cells cultivated on nonstimulated macrophages and S is the radioactivity incorporated into tumor cells cultivated on stimulated macrophages. Thioglycolate elicited peritoneal macrophages corresponding to nonstimulated macrophages gave 5%–20% growth inhibition.

Macrophages and target cells were also incubated together in the prescence of MDP, IgM, IgM+MDP, or MDP-IgM

monoclonal antibodies for 48 h before addition of [^{3}H]-thymidine. Control experiments were performed with 'armed' effector cells: macrophages were incubated for 24 h at 37° C in the presence of the putative stimulant and then washed. At this time, tumor target cells were added and co-cultivated for 48 h, after which the analysis was conducted as above.

Results

A monoclonal antibody directed against L1210 leukemia cells (F_2-10-23-IgM) was used to target MDP to activate macrophages, and its efficiency was compared with that of another monclonal antibody (6B6-IgM) specific for Lewis lung carcinoma cells (3LL). F_2-10-23-IgM was highly cytotoxic for L1210 cells in the presence of rabbit complement but not for normal lymphoid cells [23]; 6B6-IgM specifically bound to 3LL cells but was not cytotoxic in the prescence of rabbit complement [17]. The binding activity of monoclonal antibodies, and especially of monoclonal IgM, is very sensitive to chemical modification, so the binding of free and MDP-substituted monoclonal antibodies to their related target cells was analyzed by immunofluorescence. Cytofluorometry analysis showed that 1 μg/ml F_2-10-23-IgM led to saturation of L1210 binding sites, and even 0.1 μg/ml caused significant labeling (Fig. 1); the maximum fluorescence intensity of L1210 cells labeled with F_2-10-23-IgM was 100 times higher than the fluorescence intensity obtained with unrelated tumor cells, i. e., 3LL cells. The binding of F_2-10-23-IgM on L1210 cells was not due to the recognition by the Fc recptors present on these tumor cells, since its F(ab')$_2$ fragments also caused strong labeling (unpublished data).

Forty-five MDP molecules were bound to one IgM molecule. The absence of aggregation and of protein spectroscopic modification was always checked out, after which the binding of the MDP-IgM to L1210 cells was analyzed by flow cytofluorometry (Fig. 1). MDP_{45}-F_2-10-23-IgM was still able to bind L1210 cells, but its affinity was lower than that of free F_2-10-23-IgM; 10 μg/ml F_2-10-23-IgM led to labeling twice as intense as that following MDP_{45}-F_2-10-23-IgM. At higher concentrations (up 100 μg/ml) labeling obtained with the conjugate IgM was close to that obtained with free IgM. The high number of MDP molecules bound to the monoclonal antibody may have partially impaired the binding of the monoclonal antibody.

The spontaneous cytostatic effect of thioglycolate-elicited peritoneal macrophages was very low. The F_2-10-23-IgM monoclonal antibody, despite a high complement-dependent cytotoxic titer, did not mediate ADMC. F_2-10-23-IgM-coated L1210 cells did not induce macrophage tumoricidal activation (Fig. 2). L1210 cells loaded with MDP-F_2-10-23-IgM and incubated with thioglycolate peritoneal macrophages induced their activation to a highly tumoricidal state. The percentage of growth inhibition measured by [^{3}H]thymidine incorporation reached 80% with less than 10 μg/ml bound MDP (Fig. 2A). When similar concentrations of free MDP were used, a 5%–10% growth inhibition was obtained.

MDP-IgM conjugate by itself had no cytotoxicity for tumor target cells. Similar results were obtained with 6B6-IgM monoclonal antibody specific for 3LL cells on relevant cells (Fig. 2B). 3LL cells coated with high concentrations of free 6B6-IgM (200 μg/ml) induced slight activation of thioglycolate

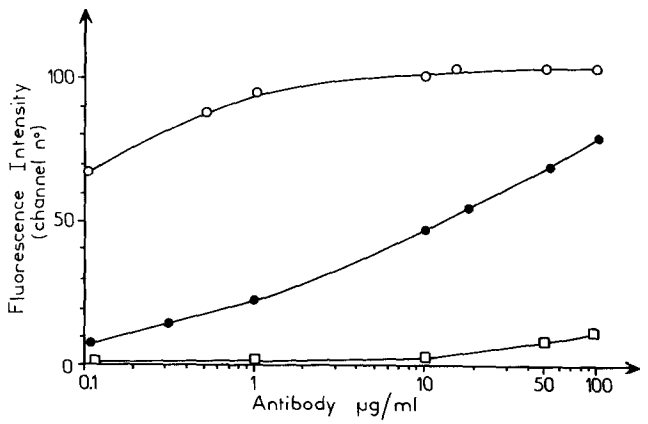

Fig. 1. Flow cytofluorometry analysis of L1210 and 3LL cells stained with F_2-10-23 IgM monoclonal antibody. I_F: Fluorescence intensity of cells labelled with F_2-10-23 IgM – fluorescence intensity of cells incubated with FGAM. I_F is expressed as channel rank. ○———○, F_2-10-23 IgM on L1210; □———□, F_2-10-23 IgM on 3LL; ●———●, MDPF_2-10-23 IgM on L1210

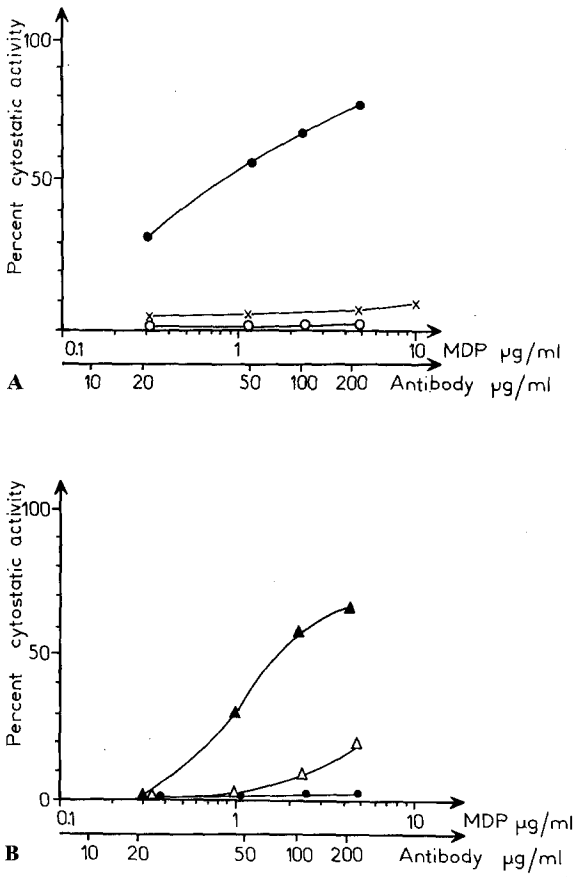

Fig. 2A, B. In vitro cytostatic activity of thioglycolate-elicited peritoneal macrophages stimulated by IgM monoclonal antibody or MDP bound to IgM monoclonal antibody. **A** L1210 cells were sensitized by 1 h treatment with free or F_2-10-23-IgM-bound MDP, washed, and incubated for 48 h with macrophages as described in *Materials and methods*. ○———○, F_2-10-23-IgM; ●———●, MDP – F_2-10-23-IgM; ×———×, MDP; **B** 3LL cells were incubated with free or 6B6-IgM-bound MDP or with F_2-10-23-IgM-bound MDP and treated as above. △———△, 6B6 IgM; ▲———▲, MDP – 6B6-IgM; ●———●, MDP – F_2-10-23-IgM. Spontaneous cytotoxicity of macrophages was less than 5%. MDP alone at the same concentration did not induce any growth inhibition. Experiments in triplicate

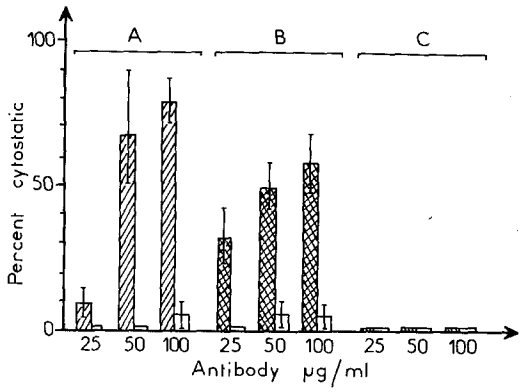

Fig. 3A–C. Titration of peritoneal macrophages cytostasis induced by F_2-10-23-IgM and MDP – F_2-10-23-IgM towards L1210 cells. *Empty bar*, F_2-10-23-IgM; hatched bar, MDP – F_2-10-23-IgM. **A** L1210 target cells were incubated in the presence of antibodies and macrophages; **B** armed target cells (antibody-precoated L1210 cells) were added to plated macrophages; **C** armed macrophages (macrophages preincubated in the presence of antibodies) were incubated with L1210 target cells. Free MDP at a concentration up to that of MDP bound to F_2-10-23-IgM did not induce any cytostatic activity of macrophages against L1210 cells

peritoneal macrophages (20% growth inhibition). However, 3LL cells coated with MDP-6B6-IgM were much more efficient in activating macrophages: 5 µg/ml MDP bound to 200 µg/ml 6B6-IgM induced 70% growth inhibition. 3LL cells incubated in the presence of 50 µg/ml 6B6-IgM carrying about 1 µg/ml MDP were still able to activate macrophages. If 3LL cells were incubated with unrelated MDP-F_2-10-23-IgM no macrophage activation occurred.

To determine whether MDP-IgM-conjugates were recognized by the macrophage Fc recptor, and were acting by a mechanism similar to the direct ADMC in which armed macrophage effector cells killed tumor target cells, thioglycolate-elicited peritoneal macrophage were incubated with various concentrations of MDP-F_2-10-23-IgM (Fig. 3C). After incubation for 24 h macrophages were washed and L1210 target cells were added. We compared the effect of armed macrophages (Fig. 3C) with those of armed L1210 tumor target cells (Fig. 3B). Neither F_2-10-23-IgM nor MDP-F_2-10-23-IgM added directly to macrophages in the absence of L1210 cells was able to trigger antibodymacrophage-mediated-cytotoxicity when L1210 cells were added after macrophage washing. But incubation of MDP-F_2-10-23-IgM-loaded L1210 (Fig. 3B) or macrophages with both the tumor target cells and the specific MDP-IgM-conjugate (Fig. 3A) did lead to tumor cell growth inhibition.

Discussion

In the present study, the experiments clearly indicate that IgM monoclonal antibodies specific for tumor cells and inactive in ADMC are powerful activators of macrophages when they carry muramyldipeptide.

Two monoclonal antibodies, one (F_2-10-23-IgM) specific for L1210 murine leukemia and the other (6B6-IgM) specific for lewis lung carcinoma (3LL) cells were used. Each monoclonal antibodies is specific to its own tumor target cells, only a low cross-reactivity being observed with antibody concentrations higher than 100 µg/ml. An F_2-10-23-IgM concentration as low as 0.1 µg/ml led to significant labeling of L1210 cells. Higher concentrations of 6B6-IgM are necessary to label 3LL cells (10 µg/ml) [17]. If target tumor cells were incubated with the related specific monoclonal antibody (as in classic ADMC) or if macrophages were incubated with the monoclonal antibody (as in direct ADMC) before adding tumor cells, no significant macrophage activation occurred, which is in good agreement with the fact that they are inactive in tumor cell growth inhibition. Thioglycolate elicited peritoneal macrophages, which are not cytostatic by themselves and which are less potent effectors of ADMC than BCG-activated macrophages but can nevertheless be activated by some subclasses of antibodies. Herlyn and Koprowski [7] showed that one IgG_{2a} monoclonal antibody specific for tumor antigen was effective in activating macrophages to a tumoricidal state. Until now, IgM monoclonal antibodies have always been less effective [11] or totally ineffective in ADMC [22], despite their high complement cytotoxic titer. Apparently, attachment of monoclonal antibodies to F_c receptors of macrophages is essential to induce tumor destruction [24]. The presence of IgM receptors on mouse peritoneal macrophages is still controversial [4], but phagocytosis of IgM-immune-complexes mediated from mice treated with glycogen has been reported [13]. IgM monoclonal antibodies specific for tumor cells could be used as carriers of immunomodulators, such as muramyldipeptides, to activate macrophages. Free MDP induced slight macrophage activation in vitro: 50–100 µg/ml MDP induced 10%–20% tumor growth inhibition. MDP bound to α-Man-BSA, a neoglycoprotein specifically endocytosed by macrophages via the membrane lectin, rendered macrophages cytostatic and cytotoxic for several tumor target cells (L1210, 3LL, human melanoma cells) at MDP concentrations lower than 1 µg/ml (unpublished data). The percentage growth inhibition can reach 90% at the optimum concentration of MDP-bound neoglycoprotein. The biological effect of muramyldipeptide depends on an internalization process based upon a fluid-phase-nonspecific pinocytic phenomenon; both neoglycoproteins [19] and liposomes [5, 6, 26], when used as carriers of MDP, allowed active endocytosis and increased the efficacy of MDP. However, the systematic activation of macrophages might induce autoimmune disease; this drawback night perhaps be partially avoid by using specific carriers such as monoclonal antibodies against tumor cells, which would be captured by macrophages only after binding to tumor cells and to tumor antigens. In the present study, MDP was bound to IgM monoclonal antibodies specific for L1210 cells and for 3LL cells, leading to about 45 MDP residues covalently bound per IgM monoclonal antibody molecule. The binding of MDP-IgM was checked by flow cytofluorometric analysis, and it was found that the affinity of MDP-IgM was slightly less than that of free IgM; the optimum concentration of MDP-IgM giving maximal fluorescence may vary from one preparation to another. In a typical experiment 10 µg/ml MDP-bound IgM gave half as much fluorescence as the maximum fluorescence obtained with unsubstituted IgM.

Macrophages co-cultivated with MDP-IgM and the related tumor target cells were activated and induced efficient growth inhibition of tumor cells. The MDP-monoclonal antibodies were effective only after binding to the related target tumor cells. Indeed, when macrophages were incubated for 24 h in the presence of MDP-F_2-10-23-IgM but in the absence of L1210 cells, no growth inhibition occurred upon addition of L1210 cells. Furthermore, L1210 cells loaded with MDP-F_2-10-23-IgM or 3LL cells loaded with MDP-6B6-IgM, and then co-cultivated with macrophages induced the activa-

tion of macrophages and led to a significant inhibition of tumor target cell growth. There was 80% growth inhibition when 5 μg/ml MDP bound to specific monoclonal antibodies was used to arm target cells, and even 0.5 μg/ml bound MDP was much more efficient than 50 μg/ml free MDP. 3LL cells incubated with the unrelated F_2-10-23-IgM was totally ineffective in activating macrophages. In some cases a slight activation of macrophages was observed upon incubation with free IgM, but the effect was always small (less than 20% growth inhibition). Macrophage activation by tumor cells armed with MDP-monoclonal antibodies could be a highly effective way of treating tumors. Several tumor antigen-specific monoclonal antibodies 'homed' specifically to the tumor after IV injection [2]. The macrophage content of tumors appeared to be a limiting factor of in these cases; chemotactic factors linked to monoclonal antibodies could be used to increase the number of macrophages inside or around the tumor to enhance the efficacy of the tumoricidal effect induced by the MDP bound to monoclonal antibody. Furthermore, macrophage activators bound to different tumor-specific monoclonal antibodies could be used simultaneously to overcome antigenic modulation, which could impair the efficacy of the MDP-antibodies. Experiments following this line are currently in progress.

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