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NOTES

Use of Monoclonal Antibodies to Protein F of *Pseudomonas aeruginosa* as Opsonins for Phagocytosis by Macrophages

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Five protein F-specific monoclonal antibodies were found to opsonize *Pseudomonas aeruginosa* for complement-independent phagocytosis by unelicited mouse peritoneal macrophages, mouse macrophage cell line $P388_{D1}$, and human monocyte-derived macrophages. Immunoglobulin G1 antibodies seemed to be a preferred isotype.

Macrophages are one of the major lines of host defense in the early stages of infections (1). In particular, it has been demonstrated that *Pseudomonas aeruginosa* opsonized by specific immunoglobulins can be taken up and killed by macrophages (7). Our earlier studies indicated that monoclonal antibodies directed against a *Pseudomonas* outer membrane protein (protein F) were protective in mouse infection models, although they failed to enhance complement-mediated killing of the bacteria (2). This implied that the monoclonal antibodies are acting to opsonize *P. aeruginosa* for phagocytosis in the mouse infection model.

In this study, we show that protein F monoclonal antibodies can opsonize *P. aeruginosa* M2 (10) for phagocytosis by human peripheral blood monocytes, mouse peritoneal macrophages, and mouse macrophage cell line $P388_{D1}$. This allowed us to investigate the relative efficiency of different immunoglobulin isotypes, as well as the use of $P388_{D1}$ cells as a model for other macrophage types.

Peritoneal macrophages were obtained from 6- to 8-week-old female BALB/c mice. Cells were washed from the peritoneal cavity with RPMI 1640 medium (Gibco, Burlington, Ontario, Canada) supplemented with 44 mM sodium bicarbonate (Fisher Scientific, Vancouver, British Columbia, Canada), 10% (vol/vol) fetal calf serum (Gibco), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Terochem Laboratories, Vancouver, British Columbia, Canada), 0.04% (vol/vol) 2-mercaptoethanol (Bio-Rad Laboratories, Mississauga, Ontario, Canada), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.), 40 U of penicillin per ml, and 40 mg of streptomycin (Gibco) per ml, pH 7.2. Macrophages were separated from erythrocytes by centrifugation at 192 × *g* for 10 min. Mouse macrophage cell line $P388_{D1}$ was maintained at 37°C, with 10% CO_2 in Nunc flat-bottom flasks (Gibco) with supplemented RPMI 1640 medium. "Day four" cultured human monocyte-derived macrophages were prepared from peripheral blood by the Ficoll-Hypaque separation technique as described previously (12).

To investigate the time course of bacterial uptake in the presence or absence of monoclonal antibody, the visual phagocytosis assay (9) was performed as described in the legend to Fig. 1 and was halted at four time points for assessment. The data obtained from two separate experiments showed that the saline control produced a gradual increase in phagocytosis from 0 to 30 min. After 30 min, no further uptake was observed for the duration of the experiment (data not shown). In the presence of antibody MA2-10 (5), on the other hand, good uptake was observed almost immediately. Uptake kinetics were complex, in that the number of bacteria per macrophage leveled out between 30 and 60 min but increased significantly after 60 min. This produced a biphasic time course of bacterial association. From the results of these experiments, we chose to halt our $P388_{D1}$ phagocytic assays at a time at which the change in the average number of bacteria per phagocyte in the negative controls was static (90 min).

With the negative controls, saline and MA1-3 (a monoclonal antibody directed against an outer membrane epitope that was not surface exposed [3]), most of the macrophages had phagocytosed few or no bacteria after 90 min (Fig. 1). This resulted in a distribution with a large peak at zero and a very low, short shoulder region. When a monoclonal antibody specific for *P. aeruginosa* protein F was added, this major peak shifted over to approximately 5, 7, and 11 bacteria per cell for human peripheral blood monocytes and $P388_{D1}$ and mouse peritoneal macrophages, respectively. The shoulder region also lengthened considerably.

All five anti-F (5) monoclonal antibodies caused significantly increased bacterial association over the negative control with all cell types in most assays (Table 1). Substantial assay-to-assay variability was observed with human peripheral blood monocytes (apparently depending on the donor), as reflected in the high standard deviations. Interestingly, the most-opsonic antibodies were of the immunoglobulin G1 (IgG1) subclass in all cases.

Certain trends could be seen in the ability of specific monoclonal antibodies to mediate phagocytosis by all three macrophage cell types. For example, MA4-10 always resulted in the highest phagocytic index, MA5-8 always scored in the middle-to-high region, and MA4-4 was consistently one of the two weakest opsonins. It is thus apparent that the mouse macrophage cell line $P388_{D1}$ can be used as a model for unelicited mouse peritoneal macrophages and cultured

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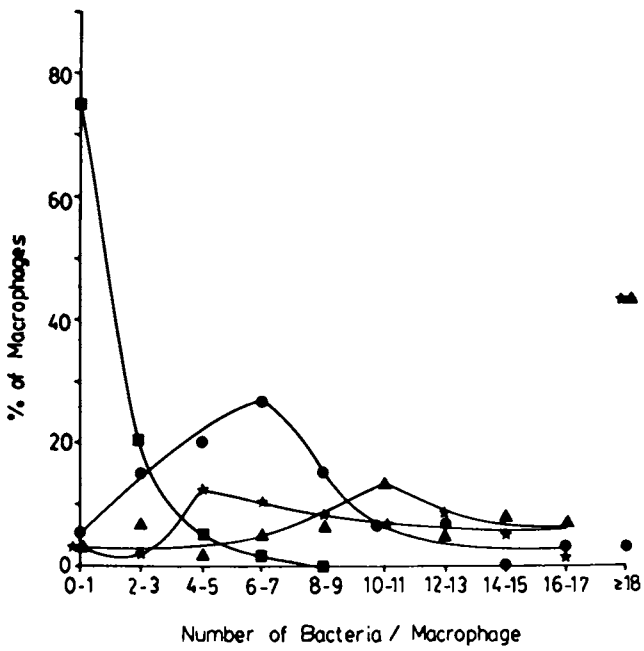

FIG. 1. Percentage of macrophages associated with specific numbers of bacteria after 90 min. The visual inspection phagocytic assay used was modified from that published previously (9). One milliliter of buffer without fetal calf serum was added to a washed cultured macrophage monolayer to give a final concentration of 1×10^6 cells per ml. Bacteria (grown to log phase in BM2 glucose [6]) were added to give a bacteria-to-macrophage ratio of 20:1, and monoclonal antibody (titer, 10^8) was used at 10% of assay volume. After incubation at 37°C in 10% CO_2 for 90 min (peritoneal macrophages or $P388_{D1}$) or 60 min (human peripheral blood monocytes), cells were scraped from the dish with a rubber policeman and resuspended with gentle pipetting. Portions of this suspension were cytocentrifuged onto a glass slide and stained with Diff-Quik (Canlab, Vancouver, British Columbia, Canada) for viewing under oil at 1,000×. The number of bacteria in each of 60 cells was counted. Negative control MA1-3 (■) distribution was approximately the same for all macrophage cell types. Addition of monoclonal antibody MA4-10 shifted the major peak for mouse peritoneal macrophages (▲), $P388_{D1}$ cells (●), and human peripheral blood monocytes (★).

human peripheral blood monocytes in assessment of phagocytosis of *P. aeruginosa*.

Previous data have suggested that there are at least two distinct highly conserved epitopes on porin protein F (5). Monoclonal antibodies MA4-4, MA2-10, MA4-10, and MA5-10 were all hypothesized to react against one epitope, while MA5-8 was specific for the other. Of the four monoclonal antibodies directed against a similar epitope, the three IgG1 monoclonal antibodies were substantially more opsonic than was the one IgG2a isotype. In the past, there has been some dispute as to the opsonic potential of IgG1. This isotype has been reported to have a low affinity for intact macrophage cells (11) and a high affinity for the isolated Fc receptors in column systems (8). It is important to note that while macrophages bind monomeric IgG1 with a relatively low affinity, high-molecular-weight aggregates of IgG1 bind at least as well as do IgG2a and IgG2b (4). This suggests that the IgG1-bacteria complex may be seen as an aggregate of immunoglobulin by the macrophages in our system. Of the three IgG1 antibodies hypothesized to be directed against a common epitope of protein F, one, MA4-10, produced substantially better opsonophagocytosis in all cell types

TABLE 1. Enhancement of the association of *P. aeruginosa* M2 with mouse peritoneal macrophages, $P388_{D1}$ cells, and human peripheral blood monocytes using monoclonal antibodies to protein F

Opsonin treatment (isotype)	Specific antigen	Bacteria associated/phagocyte (mean ± SD)[a]		
		Mouse peritoneal macrophages[b]	$P388_{D1}$[c]	Human peripheral blood monocytes[d]
Control (saline)		ND[e]	3.0 ± 1.9	2.3 ± 1.9
MA1-3 (IgG1)	H2/I	1.6 ± 1.2	3.1 ± 2.9	3.8 ± 2.0
MA4-4 (IgG2a)	F	4.6 ± 3.4	4.3 ± 2.2	5.2 ± 3.0
MA5-8 (IgG2b)	F	5.3 ± 2.7	5.8 ± 3.4	6.4 ± 5.2
MA5-10 (IgG1)	F	5.9 ± 3.6	4.7 ± 1.7	6.1 ± 2.7
MA2-10 (IgG1)	F	5.1 ± 2.3	5.7 ± 2.1	9.9 ± 6.0
MA4-10 (IgG1)	F	14.6 ± 9.0	7.3 ± 2.2	10.0 ± 7.5

[a] Values from three independent experiments for mouse peritoneal macrophages and for $P388_{D1}$ cells and from six independent experiments for human peripheral blood monocytes.

[b] $P < 0.01$ (by Student's t test) for all assays when compared with the MA1-3 control.

[c] $P < 0.01$ in all three assays (MA5-8, MA2-10, and MA4-10) or two of three assays (MA4-4 and MA5-10) when compared with the negative controls (MA1-3 or saline).

[d] $P < 0.01$ in three to five of six assays when compared with the negative controls (saline or MA1-3).

[e] ND, Not done.

tested. This may have been due to a better geometric complementarity between the antigen-binding pocket of MA4-10 and its corresponding antigenic determinant. Additionally, microheterogeneity in the protein structure or glycosylation patterns of the Fc portion of MA4-10 may have produced a better binding affinity for the macrophage Fc receptor. In either case, increased association of bacteria with the macrophage would be the expected result. Our data regarding the opsonic potential of IgG1 subclass antibodies were confirmed in another set of experiments using *P. aeruginosa* H103, $P388_{D1}$, and monoclonal antibody MA1-8 (an IgG1 antibody directed against lipopolysaccharide O antigen). This antibody displayed a significant ability to opsonize H103 for phagocytosis (J. Sawyer and R. E. W. Hancock, unpublished observations), thereby demonstrating that the phenomena we observed are not restricted to the protein F antigen.

In conclusion, we have demonstrated that monoclonal antibodies directed against protein F are capable of opsonizing *P. aeruginosa* for phagocytosis by all three macrophage cell types tested. This suggests that the mechanism of protection afforded by monoclonal antibodies in vivo (2) is through opsonization for phagocytosis. The specificity and effectiveness of these anti-F monoclonal antibodies provides them with substantial potential as immunotherapeutic agents.

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