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Plasma-Dependent Chemotaxis of Macrophages Toward BCG Cell Walls and the Mycobacterial Glycolipid P3

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BCG cell walls, associated with oil droplets in the form of emulsions in saline, generate macrophage chemotactic activity from fresh guinea pig plasma. Serum and heat-inactivated plasma were inactive, suggesting involvement of complement or fibrinogen-derived chemotactic factors. Suspensions of cell walls and oil droplets each generated chemotactic activity from plasma, and the activity of the cell wall vaccine was due to the additive effects of these two components. A mycobacterial glycolipid (P3), which is a constituent of BCG cell walls, also had plasma-dependent chemotactic activity. The results suggest that macrophage chemotaxis may be an important part of the immunopotentiating activity of these mycobacterial products.

Recently, products derived from *Mycobacterium bovis* BCG have been shown to have many of the immunopotentiating properties of the viable organism. BCG cell walls, associated with oil droplets, induce protection against airborne infection with virulent tubercle bacilli (9), and they bring about regression of established tumors (16). Injection of the cell wall vaccine leads to granulomatous inflammation with the accumulation of large numbers of macrophages (8), and macrophages are thought to be important in the immunopotentiating activity of microbial agents (3, 5, 12). This communication describes the chemotactic activity of BCG cell walls and a glycolipid derived from them. The results suggest that chemotactic attraction of macrophages may be one aspect of the immunopotentiating activity of these mycobacterial products.

MATERIALS AND METHODS

Guinea pigs. Inbred strain 2 guinea pigs were obtained from the Frederick Cancer Research Center (Frederick, Md.) or from the Rocky Mountain Laboratory colony. Female guinea pigs weighing 400 to 500 g were used in all experiments.

BCG cell walls and component P3. BCG cell walls were prepared in a Ribi press using the Pasteur strain as described previously (8, 9). The glycolipid P3 was prepared by elution from microparticulate silica gel columns according to published methods (1, 10).

Oil droplet suspensions. These were prepared in tissue homogenizers by grinding the microbial products in light mineral oil and emulsifying the resulting paste in saline containing 0.2% Tween 80 (8, 9). Suspensions containing oil droplets alone or cell walls alone were prepared by emulsifying each component separately in Tween-saline. The final concentration of oil was about 1%, and the concentration of microbial products in the resulting emulsions was 750 μg/ml. These BCG cell wall-oil droplet emulsions were originally developed as antituberculosis vaccines (9). For uniformity and convenience, these emulsions will be referred to as vaccines.

Chemotaxis. A modification of the method of Wilkinson (15) was used to measure macrophage chemotaxis. Pairs of guinea pigs were injected intraperitoneally with 15 ml of 1% oil droplets in Tween-saline, and the resulting exudates were harvested after 4 or 5 days. The peritoneal exudate cells were washed in Eagle minimal essential medium and suspended in Gey solution at a concentration of 7×10^6 trypan blue-excluding cells/ml. These suspensions contained greater than 95% mononuclear cells as judged by Giemsa and supravital staining. Chemotaxis was carried out in modified 24-well Linbro tissue culture plates. Holes were drilled in a culture plate lid to accept tuberculin syringe barrels. The syringe barrels were cut to extend to a short distance from the bottoms of the tissue culture wells. A total volume of 1.25 ml of test fluid was used in each well, and these fluids contained 10% (vol/vol) concentrations of test agents with or without 10% plasma or serum in Gey solution. Membrane filters (5-μm pore size, Millipore Corp.) were glued to the ends of the syringe barrels. The syringe barrels were then loaded with 0.1 ml of the peritoneal cell suspensions, lowered into the test fluids, and incubated for 6 h at 37°C in an atmosphere of 5% CO_2 in air. The filters were removed by immersion in propanol and stained according to the method of Wilkinson (15). Each test agent was assayed in duplicate, and only the cells that had migrated through the filter were counted. Four random fields were counted on each filter under ×312.5 magnification. Positive controls, consisting of 0.6% crude casein in Gey solution, and

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negative controls (Gey solution) were incorporated in each experiment to insure the proper function of the system.

RESULTS

Chemotactic activity of the BCG cell wall vaccine. The ability of the BCG cell wall-oil droplet vaccine to attract guinea pig macrophages was tested in the presence or absence of normal plasma or serum. The cell wall vaccine was not directly chemotactic, but in the presence of 10% plasma 78 cells per high-power field (HPF) were attracted (Table 1). This represents chemotactic activity more than fourfold greater than that of the plasma control. However, little activity was generated from serum by the cell wall vaccine, and chemotaxis in the serum control was greater than in the plasma control, as reported by others (13). Plasma, inactivated at 56°C for 30 min, also failed to provide a source of chemotactic activity when incubated with the cell wall vaccine. These results suggest that the BCG cell wall-oil droplet vaccine generates chemotactic activity from heat-labile constituents of normal plasma.

Other experiments were done to determine whether the results observed were due to chemotaxis or enhanced random migration (17). Mixtures of BCG cell wall-oil droplet suspensions and plasma were included in the lower compartment, the upper compartment, or both compartments of chemotaxis chambers, and the number of cells migrating through the filters was determined. The presence of the BCG cell wall vaccine plus plasma in the lower compartment resulted in migration of an average of 135 cells/HPF. Incubation of these components in the upper compartment resulted in the migration of only 2 cells/HPF, and incorporation of these components in both compartments brought about the migration of 20 cells/HPF. Therefore, appreciable migration of the macrophages was demonstrated only in the presence of a positive gradient of chemotactic substances, which indicates that the cell wall vaccine induces true chemotaxis rather then enhanced random migration.

Chemotactic activity of the cell wall and oil droplet components of the BCG cell wall vaccine. The BCG cell wall vaccine consists of cell walls and oil droplets suspended in saline containing Tween 80; the contribution of each of these components to the chemotactic activity of the vaccine was examined. Preliminary experiments indicated that Tween 80 in saline did not generate chemotactic activity from plasma. However, the cell wall and oil droplet components did have activity (Table 2). The complete vaccine attracted 100 cells/HPF in these experiments. Suspensions of cell wall without oil attracted an average of 46 cells/HPF in the presence of plasma, and emulsions containing only oil droplets brought about the migration of 56 cells/HPF. Therefore, the chemotactic activity of the BCG cell wall vaccine appears to be due to the additive effects of the cell wall and oil droplet components of the vaccine.

Chemotactic activity of P3 cell wall component. The glycolipid P3 is a component of BCG cell walls that is essential for their immunopotentiating activity (1). In addition, P3 itself is a highly active immunopotentiating agent (11). Accordingly, the chemotactic activity of P3 was examined. Oil droplet emulsions containing P3 were not chemotactic in the absence of plasma (Table 3), but in the presence of 10% plasma 93 macrophages/HPF were attracted. These results suggest that P3 may contribute to the chemotactic activity of BCG cell walls and that macrophage chemotaxis may be one aspect of the immunopotentiating activity of this mycobacterial glycolipid.

DISCUSSION

Macrophages are a prominent feature of the host response to mycobacteria, and chemotaxis may be involved in the accumulation of these cells. Indeed, Symon et al., found that whole cells of *M. tuberculosis* bring about the production of a macrophage chemotactic factor from plasma or serum (13). The major activity was associated with protein fractions of *M. tuberculosis*, and the cell walls had relatively little

TABLE 1. *Chemotactic activity of the BCG cell wall vaccine in the presence of plasma*

Test agent[a]	Chemotactic activity (cells/HPF)[b]			
	Gey	Normal plasma	Normal serum	Heated plasma
Cell walls[c] on oil droplets	3.1 ± 0.8	78.0 ± 6.6	36.7 ± 3.4	12.8 ± 1.5
None (control)	1.6 ± 0.3	17.7 ± 3.3	27.7 ± 2.7	5.9 ± 0.9
Casein	82.2 ± 5.2	–	–	–

[a] Test agents were incubated, as indicated, in Gey solution with or without normal plasma, normal serum, or inactivated plasma (56°C for 30 min), and tested for chemotactic activity.

[b] Values are the mean ± standard error of the mean for three experiments.

[c] BCG cell walls (lot no. 214) associated with oil droplets in a stable saline emulsion.

TABLE 2. *Role of cell walls and oil droplets in the chemotactic activity of the BCG cell wall vaccine*

Test agent[a]	Chemotactic activity (cells/HPF)[b]	
	Gey	Plasma
Cell walls[c] on oil droplets	1.4 ± 0.4	100.1 ± 6.7
Cell walls	1.2 ± 0.3	45.7 ± 3.7
Oil droplets	2.0 ± 0.5	56.0 ± 3.9
None (control)	3.9 ± 0.8	23.0 ± 1.6
Casein	77.2 ± 9.5	–

[a] Test agents were incubated in Gey solution with or without plasma, as indicated, and tested for chemotactic activity.

[b] Values are the mean ± standard error of the mean for two experiments.

[c] BCG cell walls (lot no. 234) associated with oil droplets in a stable saline emulsion.

TABLE 3. *Plasma-dependent chemotactic activity of the glycolipid P3 from BCG cell walls*

Test agent[a]	Chemotactic activity (cells/HPF)[b]	
	Gey	Plasma
P3 on oil droplets	1.6 ± 1.0	92.6 ± 8.1
Oil droplets	0.2 ± 0.1	38.0 ± 5.4
None (control)	1.6 ± 1.0	18.1 ± 1.5
Casein	99.5 ± 18.6	–

[a] Test agents were incubated in Gey solution with or without plasma, as indicated, and tested for chemotactic activity.

[b] Values are mean ± standard error of the mean for two experiments.

activity. The present studies indicate that BCG cell walls also are moderately effective in generating chemotactic activity from plasma. This activity is considerably enhanced when the cell walls are associated with oil droplets in saline emulsions, and the cell wall-oil droplet vaccines generate appreciable chemotactic activity from plasma. The BCG cell wall vaccine appears to induce a true chemotactic response because appreciable macrophage migration occurred only in the presence of a positive gradient of chemotactic substances.

The enhancement of the chemotactic activity of BCG cell walls by oil is due to the production of chemotactic activity by the mineral oil itself. Injection of mineral oil into the peritoneal cavities of animals is a standard method for obtaining macrophages. The ability of oil to generate chemotactic activity from plasma may in part account for its ability to induce exudates containing macrophages. In addition, BCG cell walls require association with oil for their immunopotentiating activity (9). The enhanced chemotactic activity of oil-associated BCG cell walls may help to explain this observation.

The BCG cell wall vaccine generated chemotactic activity from fresh plasma, but activity was not produced from heat-inactivated plasma. This suggests involvement of complement-derived chemotactic factors (4). However, fresh serum also failed to serve as a source of chemotactic activity, and this finding indicates possible involvement of components of the clotting system (6). Fibrinogen has recently been shown to be a source of chemotactic peptides (6), and it is both heat labile and absent from serum. Therefore, one possibility is that the BCG cell wall vaccine may interact with plasma fibrinogen to generate chemotactic activity. Recent studies have also demonstrated the production of chemotactic activity from serum components by platelet enzymes (14). It is possible that the oil droplet suspensions could activate platelets in plasma to generate chemotactic activity. However, removal of platelets from plasma by differential centrifugation did not reduce the chemotactic activity. The results available at present are most consistent with plasma fibrinogen as the source of chemotactic activity, but additional studies will have to be done to clarify the relative roles of platelets, complement, and fibrinogen in the chemotactic activity generated by oil-associated BCG cell walls.

The host response to the BCG cell wall-oil droplet vaccine is characterized by granulomatous inflammation with the accumulation of large numbers of macrophages (8). The present studies demonstrate that cell wall-oil droplet emulsions generate macrophage chemotactic activity from plasma. The production of this activity may be one mechanism for the attraction of macrophages to sites of injection of this vaccine.

The BCG cell wall component, P3, is found in cord factor and wax D preparations from mycobacteria, and it is a complex glycolipid composed of trehalose and several different mycolic acids (10). It enhances the granulomagenic activity of various other microbial products (1, 7, 11). The mechanism of action of P3 is not understood, and the only activity that has been clearly established is its ability to facilitate the binding of molecules to oil droplets (2). The results of the present studies suggest that another function of P3 may be to attract macrophages through the production of chemotactic factors.

Morphological (3, 12) and in vitro (5) studies have suggested that macrophages may serve as effector cells in the antitumor activities of microbial agents. Therefore, the ability to attract macrophages may be an important feature of microbial immunostimulants. The BCG cell wall vaccine has immunopotentiating activity

as exemplified by induction of tumor regression (16), and P3 is also a potent antitumor agent when coupled with other microbial products (1, 7, 11). Both of these agents can elicit the accumulation of macrophages by generating chemotactic activity from plasma. This suggests that macrophage chemotaxis may be an important part of the host response to these immunopotentiating agents.

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