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Antibacterial Product of Peritoneal Exudate Cell Cultures from Guinea Pigs Infected with Mycobacteria, Listeriae, and Rickettsiae

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In an in vitro model of cellular immunity, the antibacterial product of immunologically mediated mononuclear cell activation was studied from guinea pigs infected with listeriae and rickettsiae and compared with the product previously described from animals infected with mycobacteria. We found that this product, active against gram-positive bacilli, appeared to be identical in the three different infections with regard to its heat stability, its chromatographic adsorption and elution pattern, its susceptibility to inactivation by proteolytic enzymes, and its antibacterial spectrum.

The importance of macrophages as effectors of cell-mediated immunity to a variety of microorganisms is undisputed. Under certain experimental conditions, acquired cellular resistance to infection lacks specificity in its expression, and it has been emphasized that this involves a heightened nonspecific microbicidal power of macrophages (19). It has been shown in experiments in vivo that this nonspecific activity of macrophages is due to the activation of macrophages as a result of lymphocyte-mediated immunological processes (12, 16, 17, 20, 21). The mediators are specifically sensitized, replicating, short-lived, thymus-derived lymphocytes, i.e., T-cell mediators. Simon and Sheagren (27) reported the inhibition of growth of *Listeria monocytogenes* in cultures of peritoneal exudate (PE) cells obtained from guinea pigs injected with protein antigens in complete Freund adjuvant when they were cultured in the presence of specific antigen. Using a similar system, Bast et al. (1) reported the release of a soluble listericidal factor(s) in cell cultures from BCG-infected guinea pigs. In vitro antilisterial activity of soluble products released by listeria-immune murine peritoneal macrophages has also been described by Sethi et al. (24). Middlebrook et al. (22) have described an in vitro model of cellular immunity with separation and recombination of cell types and kinetic studies of assay of an antibacterial cell product(s), in further elucidation of the mechanisms of the immunologically mediated macrophage bactericidal activity. It was seen that a doubly nonspecific mechanism of macrophage stimulation was effected by phytohemagglutinin (PHA) provided the nonadherent cells (lymphocytes) of the cell culture population were obtained from a guinea pig previously infected with *Mycobacterium bovis* BCG. It was suggested that perhaps guinea pigs infected with other agents might also yield such cell cultures.

The purpose of this study was to determine whether the PE cells from guinea pigs infected with other microorganisms, namely *Rickettsia mooseri* and *L. monocytogenes*, release a factor(s) that leads to extracellular killing of *L. monocytogenes* as well as other bacterial target cells. The properties of the factor produced in culture as a result of these infections are compared with those of the material obtained from BCG-infected guinea pigs (25, 26).

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MATERIALS AND METHODS

Animals. Hartley and Wright strain 2 guinea pigs weighing 400 to 500 g were used.

Infection. Guinea pigs were infected with 10^5 viable listeriae intraperitoneally or with 8.2×10^3 plaque-forming units of *R. mooseri* (Wilmington seed) per 0.1 ml intradermally into the outer thigh of the right hind leg.

Preparation of listeria antigen. A 12-h culture of *L. monocytogenes* in 3% tryptic soy broth was treated with phenol (Fisher Scientific Co., Pittsburgh, Pa.) at a final concentration of 0.1% for 3 h at room temperature with intermittent shaking. Killed bacterial cells were washed twice with saline, centrifuged at $280 \times g$ for 20 min, and resuspended to give a final concentration of 1 mg/ml.

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Preparation of *R. mooseri* antigen. *R. mooseri* antigen was prepared by the method described by Fiset and Silberman (8).

PE cells. PE cells from listeria- and rickettsia-infected guinea pigs were elicited with sodium caseinate (Difco Laboratories, Detroit, Mich.) and prepared for culture as previously described for cells from BCG-infected guinea pigs (26). Viability of cells collected was greater than 90%, as judged by trypan blue exclusion. The PE cell count was 10^8 cells from each animal, of which 40 to 55% were macrophages and 10 to 20% were lymphocytes.

Preparation of macrophage cultures. The PE cells were cultured as previously described (26). Antigens, prepared as described above, or mitogens (Phytohemagglutinin-P and *Salmonella typhosa* endotoxin; Difco) were added after the standard 90-min preliminary incubation period at specified final concentrations. The plates were then incubated for 40 to 72 h under 5% CO_2 in air.

Assay of listeria-sterilizing activity (LSA). LSA was titrated in cell-free culture supernates as described before (26). One unit of LSA was defined (25) as the reciprocal of the highest dilution at which definite antibacterial activity for *L. monocytogenes* was observed.

Uptake of LSA by killed organisms and effects of ribonuclease (RNase) and Pronase on this uptake. This was done as previously described (25).

Purification of LSA produced by PE cells from listeria- and rickettsia-infected guinea pigs. LSA from cultures of PE cells from listeria- and rickettsia-infected guinea pigs was purified by the method described previously (26). Briefly, cellulose nitrate membrane fragments were washed several times in deionized water and packed into an acrylic column (0.9 by 15 cm; Pharmacia, Uppsala, Sweden). A portion of the pool of active culture supernatants pretreated with penicillinase was then applied to the column. After the column was loaded, it was washed with 20 ml of 0.15 M NaCl followed by 20 to 100 ml of deionized water. LSA was then eluted with 0.02 N HCl at a flow rate of 6 ml/h. Fractions (2 ml) were collected in polystyrene tubes and assayed for their antilisteria activity. The protein content of each tube was determined by the method of Lowry et al. (14).

Susceptibility of other organisms to column-purified LSA (PLSA). Overnight tryptic soy broth cultures of other gram-positive organisms were tested for inhibition of growth by LSA preparations under experimental conditions identical to those described previously (26).

Effects of pH and temperature on LSA. Purified LSA preparations prepared from culture supernatants of PE cells from listeria- and rickettsia-infected guinea pigs were tested for their stability at different pH values and temperatures by methods previously described (26).

Enzyme treatments. The effect of various enzymes on PLSA preparations from PE cell culture supernatants from listeria- and rickettsia-infected animals was tested in a manner similar to that used for PLSA preparations from culture supernatants from BCG-infected guinea pigs (26).

Only plastic tubes, pipettes, etc., were used in handling LSA preparations because of its strong adsorbability to glass (26).

RESULTS

The release of LSA in the presence of antigens and mitogens was studied in the guinea pigs infected with *R. mooseri* and *L. monocytogenes* 5 to 6 weeks after infection. No LSA could be detected in cultures from guinea pigs sacrificed 8 weeks after infection unless specific antigen or nonspecific mitogen was used. LSA was observed in the absence of an antigen or mitogen; however, the yield was higher (two- to fourfold) in the presence of 2.5 μg of antigen/ml (Table 1). No LSA was present in culture supernatants of PE cells incubated with 12.5 μg of antigen/ml. PHA, a T-cell-stimulating mitogen, stimulated the highest titers of LSA. Endotoxin, a B-cell mitogen, was without effect. These results are similar to those observed in culture supernatants of BCG-infected guinea pigs except that in the latter instance LSA was rarely found in the absence of PHA or the specific antigen purified protein derivative (25).

One property of LSA from BCG-infected guinea pig cell cultures was its adsorption by phenol-killed listeria, corynebacteria, and BCG, and not by other organisms. When concentrated active culture supernatants from listeria- and rickettsia-infected guinea pigs were incubated with 0.1% phenol-killed bacterial cell suspensions for 60 min at room temperature, the centrifuged supernatants lost their antibacterial activity against listeria. Therefore, it seems that the material(s) produced as a result of these infections is similar to those released in culture by PE cells from *M. bovis*-infected guinea pigs.

Treatment of killed listeria with RNase at a final concentration of 50 μg/ml resulted in considerable loss in their ability to adsorb PLSA from cultures of PE cells from BCG-infected animals; Pronase, under the same conditions, was inactive (26). A similar loss in adsorption of PLSA by RNase-treated killed bacterial cells was also observed for LSA produced by guinea pigs infected with rickettsiae and listeriae, and Pronase treatment was ineffective.

As with LSA from cultures of cells from BCG-infected guinea pigs, only corynebacteria and *Bacillus subtilis* among gram-positive organisms were found to be susceptible (in addition to the listeria).

Before attempting any further characterization, this LSA was partially purified. Since it was observed (26) that LSA released by PE cells from BCG-infected animals is adsorbed by Nal-

TABLE 1. *LSA in culture supernatants from guinea pigs infected with different agents*

Infecting agent	Antigen	LSA (U/ml)	SS[a]
Rickettsia mooseri[b]	None	8	
	R. mooseri		
	(i) 2.5 μg/ml	16	<0.05
	(ii) 12.5 μg/ml	<2	<0.05
	PHA (10 μg/ml)	24	<0.05
	Endotoxin (10 μg/ml)	8	>0.05
Listeria monocytogenes[c]	None	6	
	L. monocytogenes		
	(i) 2.5 μg/ml	12	<0.05
	(ii) 12.5 μg/ml	<2	<0.05
	PHA	48	<0.05
	Endotoxin	6	>0.05
BCG[d]	None	<2	
	PPD[e] (10 μg/ml)	96	<0.05
	PHA	40	<0.05
	Endotoxin	<2	>0.05

[a] Statistical significance of difference from control: P value by Student's t test.
[b] Average of triplicate cultures from three of four guinea pigs sacrificed 5 to 6 weeks after infection; one animal was negative.
[c] Average of triplicate cultures from five of eight guinea pigs sacrificed 6 weeks after infection. Three animals yielded inactive cultures at 3 weeks after infection.
[d] Average of triplicate cultures from three different experiments.
[e] PPD, purified protein derivative.

gene filters (Sartorius cellulose nitrate membrane), the behavior of LSA obtained in the present study was similarly studied on a column of cellulose nitrate membrane (Millipore) fragments. The elution pattern of LSA from supernatants of cell cultures from guinea pigs infected with the listeriae and rickettsiae was essentially the same as that seen with cultures from BCG-infected guinea pigs. As previously observed, LSA was eluted in a rather sharp peak, although the highest LSA titer did not correspond to the highest protein concentration (26).

The PLSA prepared in these studies was found to be nondialyzable against deionized water for longer than 48 h and in this respect also behaved like the LSA found in culture supernatants of PE cells from BCG-infected guinea pigs.

PLSA from guinea pigs infected with listeriae and rickettsiae was stable from pH 4 to 8 at 56°C for 30 min, but lost more than 50% of its sterilizing activity at pH 2 and 10 during the same period. These results are similar to those observed for PLSA obtained from BCG-infected guinea pigs. A complete loss in activity was observed after a 5- or 10-min incubation at 100°C at neutrality.

The LSA released into culture supernatants of PE cells from listeria- and rickettsia-infected guinea pigs and purified by the cellulose nitrate membrane column was treated with the same enzymes studied in the experiments with BCG PLSA in order to see whether the biochemical nature of all these materials is the same. The decrease in titer was calculated by comparison with the sterilizing activity of the untreated factors.

Deoxyribonuclease and RNase had no effect on the activity of the material to sterilize *L. monocytogenes*. Protease, trypsin, and papain reduced LSA titers only slightly after 30 min of incubation at 37°C. However, a complete loss in activity was observed after a 3-h incubation at the same temperature. Penicillinase had no effect on PLSA, and pepsin decreased PLSA titers only slightly. These results are in complete agreement with those obtained with BCG PLSA.

DISCUSSION

Macrophages are the cells responsible for disposing of most foreign material. They are also host cells for a wide variety of obligatory and facultative intracellular parasites. The macrophage response to intracellular bacterial infections has been studied in animal systems both in vivo and in vitro (18, 20, 27), and it has been shown that the cellular immune response mediating the killing of bacteria in such systems involves both lymphocytes and macrophages. In previous studies of Middlebrook et al. (22), LSA was detected in supernatants of PHA-stimulated macrophage-lymphocyte cell cultures from one out of eight "normal" guinea pigs studied, and the possibility was raised that

this sterilization activity could be a generalized property associated with infection. To confirm this hypothesis and to see whether antilisterial factors produced by BCG infection in animals are similar to products released by cell cultures from guinea pigs infected with other unrelated organisms, this present study was undertaken.

Hartley and Wright strain 2 guinea pigs were infected with *R. mooseri*, an obligate intracellular parasite that multiplies in macrophages (10) and is not related to BCG, and with *L. monocytogenes*. PE cells elicited with sodium caseinate were cultured in vitro in the presence of specific antigens as well as PHA and endotoxin.

The release of a listericidal factor(s) in cultures was detected if the infected animals were sacrificed within 5 to 6 weeks after infection. LSA was often found in supernatants of unstimulated PE cells from guinea pigs infected with listeriae and rickettsiae but not from cells from BCG-infected animals in the absence of an antigen or mitogen. This could be due to the fact that macrophages were already sufficiently stimulated in vivo to continue elaboration of LSA in culture in vitro. It is interesting to note that guinea pigs killed 8 weeks after infection with listeriae or rickettsiae did show some LSA, but then only in the presence of specific antigen or PHA. The titers after this time period were severalfold lower as compared with 5- to 6-week culture supernatants. No LSA was present when the antigen concentration was 12.5 μg/ml; however, this loss in activity could be due to adsorption of LSA by particulate antigens since it is already known that LSA produced by BCG-infected animals is taken up by the killed listeria cells used as antigen (25).

PHA, a T-cell mitogen, stimulates normal animals to produce various lymphokines such as macrophage migration inhibitory factor, skin reactive factor, lymphotoxin, blastogenic factor, and others (5). In experiments with mice infected with *L. monocytogenes*, North and Spitalny (23) found an accumulation of immunity-mediating T-cells in casein-induced PE cells. In view of these findings, it appears that macrophages in the unstimulated peritoneal cavity are not, or not sufficiently, stimulated in order to inhibit intracellular multiplication even if the host is vaccinated with a potent vaccine. Only when a local inflammation is elicited, permitting influx of T-cells, does stimulation of macrophages take place. In the experiments of Middlebrook et al. (22), the addition of PHA to mixed populations of "normal" lymphocytes and macrophages induced the morphological manifestations of macrophage "stimulation." However, LSA was detected in that study only when PHA-stimulated lymphocytes were obtained from BCG-infected guinea pigs. The present study extends these observations because PHA used in culture increased the titers of LSA when PE cells were obtained from animals infected with three unrelated infectious agents. Endotoxin, a B-cell stimulator, failed to stimulate the production of LSA. Therefore, it seems clear that the release of this antilisterial material involves an interaction between macrophages and T-lymphocytes.

Fowles et al. (9) have shown that macrophages incubated for 3 days in fractions rich in macrophage migration inhibitory factor exhibited enhanced bacteriostasis (2- to 10-fold) compared with controls. Recently Cole (4) has reported that the soluble products of immune lymphocyte cultures can activate murine peritoneal macrophages to kill *L. monocytogenes* if the antigen used in both the immunization and lymphocyte stimulation is also present on the target intracellular organism. Lymphocytes themselves, however, have a greater influence on macrophages than do their mediators (6), and it has been proposed that lymphocytes may produce essential factors that are more labile than mediators such as macrophage migration inhibitory factor, or a direct contact between the lymphocyte and macrophage may be required for actual bactericidal activity. The enhanced bactericidal activity of macrophages towards *L. monocytogenes* as a result of incubation with antigen and lymphocytes was first reported by Simon and Sheagren (27). In subsequent reports, immune lymphocytes from lymph nodes (13) or PE cells (28), when added to normal macrophages in the presence of antigen, enhanced the bactericidal capacity of macrophages for listeria. These results are in agreement with the results of Middlebrook et al. (22) except that in the latter studies no intracellular killing or extracellular release of listericidal factor(s) was observed when supernatants from lymphocyte cultures preincubated with antigen or PHA were added to normal macrophages. This could, however, be due to concentration differences in the mediators as compared with the studies of Fowles et al. (9).

Acquired cellular resistance is believed to be responsible for antibacterial immunity in tuberculosis (15), brucellosis (11), and listeriosis (18). The increased microbicidal capacity of macrophages from animals infected with these bacteria is not specifically directed against the inducing organism (19), with the result that cross-resistance has been reported to exist between these infections (7, 19). Blanden et al. (3) have reported that macrophages from mice infected with *L. monocytogenes* possessed en-

hanced microbicidal effects against *Salmonella typhimurium* and that salmonella-infected mice were totally resistant to intravenous challenge with *L. monocytogenes*. As reported earlier (22), LSA from BCG-infected guinea pigs was not found to be active against *Salmonella typhosa* or *Salmonella typhimurium*. PLSA produced by listeria- and rickettsia-infected guinea pigs had the same spectrum of activity as BCG PLSA. These preparations did not inhibit the growth of other organisms tested. These results agree in some respects with the studies of Behin and co-workers (2). They have reported the limited selective ability of activated macrophages to kill listeriae in experimental cutaneous leishmaniasis of the guinea pig. Alternatively, it is possible that these soluble products may have other important biological properties, as yet unrecognized, or the mechanism(s) in vivo may be quite different than in vitro, e.g., requiring phagocytosis.

LSA produced by PE cells from BCG-infected guinea pigs was strongly adsorbed onto some killed bacterial cells. The material under study was also taken up by killed listeriae and corynebacteria and not by *Diplococcus pneumoniae* and *Streptococcus salivarius*. The uptake of LSA under study was likewise considerably reduced by treatment of killed bacterial cells with RNase before incubation with active preparations.

LSA in purified protein derivative-stimulated culture supernatants was purified by use of a cellulose nitrate membrane column (26). The results shown here confirm that the same or very similar material is produced by cultured PE cells from guinea pigs infected with BCG, *R. mooseri*, or *L. monocytogenes*.

The pH and temperature stability of LSAs produced by immune macrophage-lymphocyte cultures in the present study also proved to be the same as those produced by cultures from BCG-infected guinea pigs.

The effect of proteolytic enzymes on the purified preparations of LSAs from listeria- and rickettsia-infected animals was similar to that on BCG PLSA. It therefore seems reasonable to conclude that the same antibacterial cationic protein(s) is produced by appropriately stimulated PE cell cultures from guinea pigs with different bacterial infections without specificity for the infecting agents.

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