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Stimulation of Polymorphonuclear Leukocyte Bactericidal Activity by Supernatants of Activated Human Mononuclear Cells

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Supernatants of phytohemagglutinin-activated human mononuclear cells stimulated polymorphonuclear leukocyte (PMN) activity against the gram-negative organism *Serratia marcescens*. In the absence of serum opsonins, when control PMN could not impede bacterial growth, stimulated PMN averaged more than 0.6-log kill of the original bacterial inoculum. In the presence of optimal amounts of serum opsonins, when control PMN were significantly bactericidal, stimulated PMN killed, on the average, at least 0.6 log more of bacteria. Stimulation was not found when PMN were preincubated with supernatants for 1 h or less. The data strongly suggested that the action of the PMN stimulating factor was independent of and different from classically described serum opsonins. PMN stimulating activity may be an additional lymphokine-mediated immune defense mechanism enabling hosts to kill invading microorganisms.

Over the past 11 years, numerous biologically active substances, called lymphokines, have been demonstrated in the supernatants of in vitro cultures of antigen- or lectin-activated lymphocytes. These lymphokines have been considered in vitro correlates of cell-mediated immunity and are thought to play a role in host resistance to infectious microorganisms (4, 15). Specifically, chemotactic factors and migration inhibition factors have been described which affect the motility of either polymorphonuclear leukocytes (PMN) or cells of the monocyte-macrophage system (4, 15, 16). Lymphokine-mediated activation of macrophages and monocytes, as measured by enhancement of their metabolic, phagocytic, and bacteriostatic capabilities, has also been reported (6, 7, 12, 17).

Recently, Lomnitzer et al. (10) reported that supernatants of phytohemagglutinin (PHA)-activated mononuclear cells can stimulate PMN metabolism and phagocytosis. Nevertheless, by using a standard assay, they could not detect correspondingly increased PMN bactericidal activity against *Staphylococcus aureus*. Since PMN bactericidal activity has been shown to be important to host immune defense against gram-negative bacteria (20), we studied the ability of mononuclear cell supernatants to stimulate PMN killing of a gram-negative organism, *Serratia marcescens*. Our data indicate that supernatants of lectin-activated mononuclear cells can stimulate PMN bactericidal activity. Furthermore, the PMN stimulating factor (PSF) present in these supernatants does not appear to have the functional characteristics of a serum opsonin.

MATERIALS AND METHODS

Preparation of cells. Mononuclear cells, separated from normal human adult peripheral blood by dextran sedimentation followed by Ficoll-Hypaque density centrifugation (3), were washed three times with warm Ca^{2+} and Mg^{2+}-free Hanks balanced salt solution and then resuspended in RPMI 1640 containing 10% heat-inactivated and virus-screened fetal calf serum, 2% HEPES buffer (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), and 1% glutamine. Similarly isolated PMN were cleared of erythrocytes by hypotonic saline lysis and then washed once with normal saline. These cells consisted of >98% viable PMN.

Production of a PSF. To produce a PSF, mononuclear cells were cultured at a concentration of 2×10^6 to 3×10^6 cells per ml in a final volume of 2 ml in loosely-capped plastic tubes (16 × 125 mm) lying nearly horizontal for 3 to 7 days at 37°C in 5% CO_2-humidified atmosphere. E-PHA, prepared according to the method of Weber et al. (19) (a gift of R. P. MacDermott), was added at concentrations ranging from 1 to 20 μg/ml to experimental tubes at the start of the culture and to control supernatants after they had been separated from the cells. After mononuclear cell culture, all tubes were centrifuged at 250 × *g* for 15 min. The supernatants were decanted into vials and then either used directly or frozen at −20°C for subsequent use. Control cultures, run simultaneously, were identical to the experimental cultures with the

exception that they lacked PHA. After supernatant collection, however, PHA was added to control supernatants in amounts equivalent to those used to stimulate lymphokine production in the experimental tubes. This insured that any stimulated PMN bactericidal activity induced by experimental supernatants was not due to the direct effect of PHA on either PMN or bacteria.

Stimulation of PMN. After the final wash in normal saline, the isolated PMN were resuspended in supernatants from cultures of either PHA-activated or control mononuclear cells in volumes of 0.2 to 1.0 ml at a concentration of 20×10^6 per ml. The PMN were then preincubated for 3 h at 37°C in 5% CO_2-humidified atmosphere in 15-ml plastic conical centrifuge tubes lying nearly horizontal. PMN preincubated with supernatants of PHA-activated mononuclear cells are hereafter referred to as stimulated PMN, whereas PMN preincubated with control mononuclear cell supernatants are referred to as control PMN.

Bacteria. The bacteria used, *S. marcescens*, were of a strain isolated from a bacteremic patient and could be killed by PMN in cooperation with heat-inactivated serum from normal adults. This PMN bactericidal activity did not require heat-labile components of complement. Also, the bacteria used were not killed by serum and complement in the absence of PMN.

PMN bactericidal assay. The bactericidal capability of PMN was tested in a modified form of the assay of Hirsch and Strauss (8). Use of a microtiter plate in measuring PMN bactericidal activity was developed by J. C. Sadoff (personal communication, manuscript in preparation). We used a modification of this microtiter plate system which permitted bacterial dilution and dispersion in test tubes. The technique required a total volume of only 0.1 ml per well and allowed us to efficiently test numerous variables by using small amounts of sera, PMN, and supernatants. *S. marcescens*, taken during log-phase growth in Trypticase soy broth, were washed and appropriately diluted in Eagle medium. A 10-μl amount (containing 1×10^6 bacteria) was then added to round-bottom microtiter wells containing 70 μl of a constant number (0.5×10^6 to 2×10^6) of a preincubated (stimulated or control) PMN. The remaining 20 μl in the reaction mixture consisted of various concentrations of heat-inactivated normal human serum and/or complement and/or Eagle medium. Our source of complement was freshly frozen human agammaglobulinemic serum which was routinely tested for functionally active complement.

To determine PMN bactericidal activity, 10-μl samples were taken from each well before and after 1 and 2 h of incubation at 37°C under constant vigorous shaking of the sealed microtiter plate. Before plating onto Trypticase soy agar, samples were appropriately diluted in test tubes (12 by 17 mm; containing 0.1% bovine serum albumin in distilled water), which were then vigorously blended in a Vortex mixer. This procedure insured that PMN were selectively lysed and that bacteria were adequately dispersed. In selected experiments, sonic oscillation was also used for this purpose.

Calculations. PMN bactericidal activity expressed as $\log_{10}$ was calculated by subtracting the $\log_{10}$ of the total number of viable bacteria remaining after 60 or 120 min of test incubation from the $\log_{10}$ of the number of bacteria viable in the initial inoculum; negative logarithmic numbers represent bacterial growth during the bactericidal assay. PMN bactericidal activity expressed in terms of percent was calculated by the formula 100 − [100 × (number of bacteria at 60 or 120 min)/(number of bacteria in the initial inoculum)]; 0% represents bacterial growth. Statistical analysis was performed by calculating the standard error of the bactericidal activity using propagation of error formulas based on a Poisson distribution of the bacterial counts (1). *P* values were determined from the normal theory test of the difference in bactericidal activity of stimulated PMN as compared to that of control PMN assayed simultaneously.

RESULTS

The growth curves of representative experiments depicted in Fig. 1 through 3 demonstrate that PMN exposed to supernatants of activated mononuclear cells (stimulated PMN) had a greater bactericidal capability than PMN preincubated with supernatants of nonactivated mononuclear cells (control PMN). In preliminary experiments, activated mononuclear cell

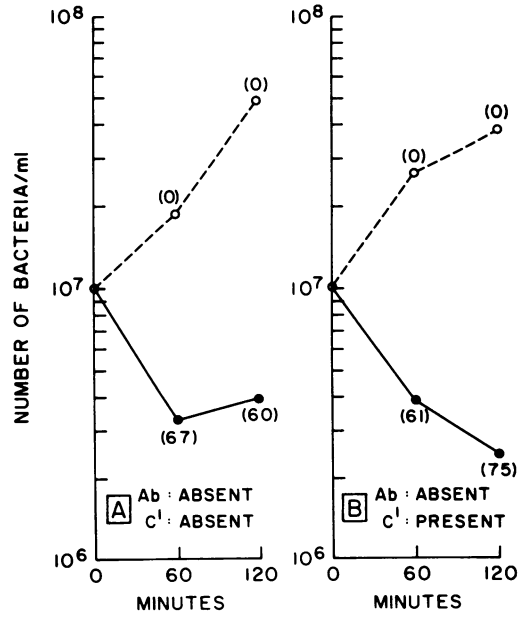

FIG. 1. *Stimulation of PMN bactericidal activity in the absence of normal human serum (Ab) (representative experiments). (A) Both Ab and complement (C') absent. (B) Ab absent but complement present. In both (A) and (B) stimulated PMN (●) are compared with control PMN (○). Numbers in parentheses represent percent PMN bactericidal activity calculated according to the formula described in the text.*

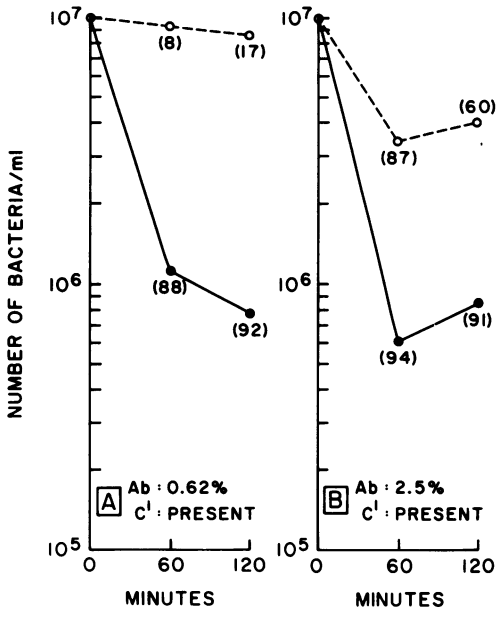

FIG. 2. *Stimulation of PMN bactericidal activity in the presence of both normal human serum (Ab) and complement (C') (representative experiments). (A) 0.62% serum. (B) 2.5% serum. In both (A) and (B) stimulated PMN (●) are compared with control PMN (○). Numbers in parentheses represent percent bactericidal activity calculated by the formula in the text.*

supernatants, in the absence of PMN, were not directly bactericidal. Furthermore, PMN preincubated for 3 h with control mononuclear cell supernatants were able to kill bacteria in cooperation with serum opsonins as effectively as PMN that were not preincubated. This confirmed that the increased PMN bactericidal activity seen in our system was indeed owing to increased activity of stimulated PMN and not decreased activity of control PMN.

As shown in Table 1 (experiments 1 through 4) and in the representative experiment graphed in Fig. 1A, in the absence of serum antibody and complement, control PMN were unable to impede bacterial growth during the 2-h bactericidal assay. Under these same conditions, stimulated PMN, tested simultaneously, killed a mean of 0.61 log (73%) and 0.68 log (76%) of the initial inoculum at 1 and 2 h, respectively. In experiments performed early in our investigations, activated mononuclear cell supernatants could not induce (or increase) PMN bactericidal activity when preincubated with PMN for only 1 h; the supernatants were also not effective when merely added to PMN in the bactericidal assay (i.e., without preincubation). The mechanism of action of PSF, therefore, is decidedly different from that of serum opsonins which effectively promote PMN bactericidal activity without preincubation with PMN (e.g., Table 1, experiments 14 through 17, using control PMN). For this reason we prefer to call the biologically active factor in activated mononuclear cells supernatants a PMN stimulant rather than an opsonin.

We wanted to determine if the PSF could be demonstrated even in the presence of serum opsonins. Accordingly, we compared the bactericidal activity of control PMN to that of stimulated PMN when complement and/or various concentrations of serum (containing heat-stable opsonic antibodies) were present in the PMN bactericidal assay mixture. When no serum antibody was present, control PMN could not impede bacterial growth even in the presence of complement. Stimulated PMN, however, were bactericidal; after 1 h of test incubation, they killed a mean of 0.64 log (75%) of the bacteria present in the initial inoculum, and after 2 h, a mean of 0.66 log (78%) of the bacteria were killed (Table 1, experiments 5 through 7 and Fig. 1B). When serum antibody and complement were both present, control PMN caused either bacteriostasis (Table 1, experiments 8 through 10

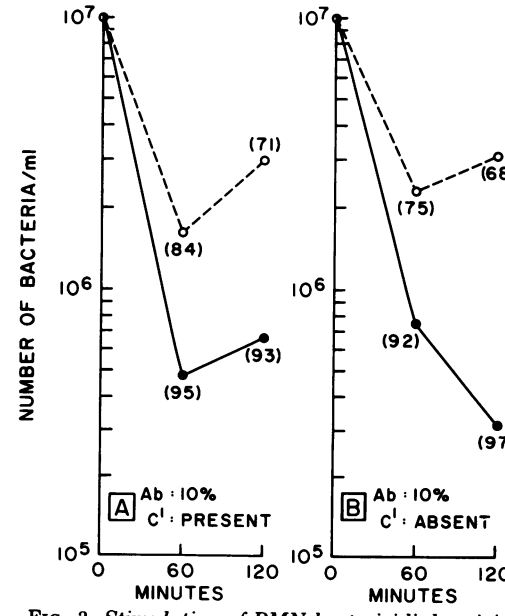

FIG. 3. *Stimulation of PMN bactericidial activity in the presence of 10% normal human serum (representative experiments) (A) complement (C') present. (B) Complement absent. In both (A) and (B) stimulated PMN (●) are compared with control PMN (○). Numbers in parentheses represent percent bactericidal activity calculated by the formula shown in the text.*

TABLE 1. *Effect of mononuclear cell supernatants on PMN bactericidal activity*

Expt	Opsonins present		PMN bactericidal activity[b]			
			60 min		120 min	
	% Serum	C'[a]	Control	Stimulated	Control	Stimulated
			Log_{10} (%)	Log_{10} (%)	Log_{10} (%)	Log_{10} (%)
1	0	0	−0.27 (0)	0.54 (71)[c]	−0.69 (0)	0.49 (68)[c]
2	0	0	−0.75 (0)	0.43 (64)[c]	−1.04 (0)	1.01 (90)[c]
3	0	0	−0.14 (0)	0.99 (90)[c]	−0.32 (0)	0.83 (85)[c]
4	0	0	−0.27 (0)	0.48 (67)[c]	−0.69 (0)	0.40 (60)[c]
5	0	+	−0.26 (0)	0.88 (87)[c]	−0.11 (0)	0.79 (84)[c]
6	0	+	−0.58 (0)	0.64 (77)[c]	−0.92 (0)	0.59 (74)[c]
7	0	+	−0.42 (0)	0.41 (61)[c]	0.59 (0)	0.61 (76)[c]
8	0.62	+	0.00 (0)	0.65 (78)[c]	−0.47 (0)	0.48 (67)[c]
9	0.62	+	−0.34 (0)	1.17 (93)[c]	0.02 (1)	1.02 (91)[c]
10	0.62	+	0.04 (8)	0.91 (88)[c]	0.08 (17)	1.12 (92)[c]
11	2.5	+	0.33 (53)	0.74 (82)[d]	0.13 (27)	0.57 (73)[c]
12	2.5	+	0.87 (87)	1.25 (94)[c]	0.39 (60)	1.03 (91)[c]
13	2.5	+	0.59 (74)	0.72 (81)[e]	0.75 (82)	1.40 (96)[c]
14	10	+	0.74 (82)	1.59 (98)[c]	0.98 (90)	1.65 (98)[c]
15	10	+	0.59 (74)	0.93 (88)[d]	0.70 (80)	1.10 (92)[f]
16	10	+	0.80 (84)	1.31 (95)[c]	0.53 (71)	1.12 (93)[c]
17	10	+	0.63 (76)	1.31 (95)[c]	1.07 (92)	2.02 (99)[c]
18	10	0	0.62 (75)	1.12 (92)[c]	0.50 (68)	1.51 (97)[c]
19	10	0	0.36 (56)	0.82 (85)[c]	0.49 (68)	0.95 (89)[c]
20	10	0	0.47 (66)	0.87 (87)[d]	0.42 (62)	0.88 (87)[f]

[a] C', The presence (+) or absence (0) of complement.
[b] Calculation of PMN bactericidal activity is described in the text.
[c] $P < 0.0002$.
[d] $P < 0.002$.
[e] $P < 0.09$.
[f] $P < 0.008$.

and Fig. 2A) or varying degrees of bactericidal activity depending on the amount of serum antibody added (Table 1, experiments 11 through 17 and Fig. 2B and 3A). Under each of these experimental conditions, stimulated PMN exhibited significantly increased bactericidal activity compared with that of control PMN tested simultaneously (see *P* values, Table 1). Even in the presence of both complement and 10% serum, after 60 min of test incubation, stimulated PMN killed an average of 0.60 log more of bacteria than simultaneously tested control PMN; after 120 min, an average 0.65 log more of bacteria were killed by stimulated PMN. Experiments performed in the presence of 10% serum but in the absence of complement also demonstrated significant PSF activity (Table 1, experiments 18 through 20 and Figure 3B). Considering all of the above data, the effect of activated mononuclear cell supernatants appears to be independent of the effects of antibody and complement.

DISCUSSION

The precise nature of the PSF we have described is presently unknown. Since stimulating activity could not be demonstrated when PMN were incubated with activated mononuclear cell supernatants for 1 h or less, PSF does not appear to have the functional characteristics of a serum opsonin. Furthermore, PHA has been shown to inhibit antibody production in vitro (18). It is therefore highly unlikely that the biologically active factor in our PHA-stimulated supernatants is specific opsonic antibody. Stimulated mononuclear cells have been shown to secrete complement components (9). Unlike PSF, however, when serum complement (known to be physiologically active) was preincubated with PMN for 3 h, it could not stimulate bactericidal activity in our system (data not shown). This suggests that PSF is not identical to serum complement.

The lack of increased PMN bactericidal activ-

ity found when PMN were not preincubated with activated mononuclear cells supernatants is consistent with the results of Lomnitzer et al. (10). They have reported increased glucose oxidative metabolism, Nitro Blue Tetrazolium dye reduction, and phagocytosis of *Candida albicans* in the absence of increased bactericidal activity against staphylococci when PMN were exposed (without preincubation) to PHA-activated mononuclear cell supernatants for 45 min. Although they used a different organism than we did in testing for PMN bactericidal activity, preliminary data from our laboratory indicate that PSF activity can also be demonstrated against group B type III streptococci. This possible dissociation between onset of stimulation of PMN phagocytosis and bactericidal activity suggests mediation of PSF activity via an activatable enzyme or product within PMN that is distinct from that measured by Nitro Blue Tetrazolium dye reduction or glucose oxidation. Of interest in this regard is the demonstration by Musson and Becker (11) that PMN phagocytosis required activity of a serine esterase which may be present either in an active or "activatable" form. Furthermore, PMN lysozomal release induced by chemotactic factor apears to be mediated primarily via an activatable esterase (2). We speculate that such activatable enzymes may be important to the preincubation that is necessary for PSF activity.

Biochemical and physical characteristics of PSF need to be performed to distinguish it from other lymphokines which may affect PMN such as chemotactic factor and leukocyte inhibition factor. Alternatively, stimulation of PMN bactericidal activity may be an additional function of these previously described lymphokines. It would not be unusual for one factor to have more than one biological function. Nathan et al. (13) clearly demonstrated that the lymphokine in stimulated lymphocyte supernatants responsible for macrophage activation (as measured by adherence, phagocytosis, and glucose oxidation) could not be distinguished physiochemically from the migration inhibitory factor. It is also possible that the active moiety in our stimulated mononuclear supernatants is tuftsin, the phagocytosis-stimulating tetrapeptide isolated from normal sera (5). Although to our knowledge, in vitro lymphocyte production of tuftsin has not been described previously, it is likely that tuftsin is a mononuclear cell product since it is deficient in the sera of splenectomized patients (5).

Since both lymphocytes and monocytes are present in our mononuclear cell cultures, and since both cell types are capable of producing a variety of biologically active substances (17), it would be premature at present to assume that the PSF described in this paper is lymphocyte derived. Preliminary results from our laboratories, however, indicate that PSF activity is present in supernatants of a nonphagocytic long-term cultured lymphoid cell line (G. H. Lowell and A. S. Cross, Fed. Proc. **37**:6, 1978).

Whatever the nature and source of the factor, it would be interesting to determine whether purified bacterial antigens could also cause lymphocytes to secrete a PSF. If this were to occur in vitro (e.g., after infection or immunization), lymphokine-mediated stimulation of PMN bactericidal activity might then be considered an additional immune defense mechanism whereby hosts clear invading microorganisms.

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LITERATURE CITED

1. **Armitage, P.** 1973. Statistical methods in medical research, 2nd edition. John Wiley & Sons, New York.
2. **Becker, E. L., and H. J. Showell.** 1974. The ability of chemotactic factors to induce lysozomal enzyme release. II. The mechanism of release. J. Immunol. **112**:2055-2062.
3. **Boyum, A.** 1968. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):77-88.
4. **Campbell, P. A.** 1976. Immunocompetent cells in resistance to bacterial infections. Bacteriol. Rev. **40**:284-313.
5. **Constantopoulos, M. D., U. A. Najjar, and J. W. Smith.** 1972. Tuftsin deficiency: a new syndrome with defective phagocytosis. J. Pediatr. **80**:564-572.
6. **Fowles, R. E., I. M. Fajardo, J. L. Leibowitch, and J. R. David.** 1973. The enhancement of macrophage bacteriostasis by products of activated lymphocytes. J. Exp. Med. **138**:952-964.
7. **Godal, T., R. J. W. Rees, and J. O. Lamvik.** 1971. Lymphocyte-mediated modification of blood-derived macrophage function in vitro; inhibition of growth of intracellular mycobacteria with lymphokines. Clin. Exp. Immunol. **8**:625-637.
8. **Hirsch, J. G., and B. Strauss.** 1964. Studies on heat-labile opsonin in rabbit serum. J. Immunol. **29**:145-154.
9. **Littman, B. H., and S. Ruddy.** 1977. Production of the second component of complement by human monocytes: stimulation of antigen activated lymphocytes or lymphokines. J. Exp. Med. **145**:1344-1352.
10. **Lomnitzer, R., A. Glover, and A. R. Rabson.** 1977. The effect of PHA-activated mononuclear cell supernatants on polymorphonuclear leukocyte function. Clin. Exp. Immunol. **29**:501-508.
11. **Musson, R. A., and E. L. Becker.** 1977. The role of an activatable esterase in immune-dependent phagocytosis by human neutrophils. J. Immunol. **118**:1354-1365.
12. **Nathan, C. F., M. L. Karnovsky, and J. R. David.** 1971. Alternations of macrophage functions by mediators from lymphocytes. J. Exp. Med. **133**:1356-1376.
13. **Nathan, C. F., H. G. Remold, and J. R. David.** 1973. Characterization of a lymphocyte factor which alters macrophage function. J. Exp. Med. **137**:275-290.

14. **Remold, H. G., and J. R. David.** 1974. Migration inhibition factor and other mediators of cell-mediated immunity, p. 25-42. *In* R. T. McClusky and S. Cohen, (ed.), Mechanisms of cell-mediated immunity. John Wiley & Sons, New York.
15. **Rocklin, R. E.** 1974. Products of activated lymphocytes: leukocyte inhibitor factor (LIF) distinct from migration inhibitory factor (MIF). J. Immunol. **112**:1461-1466.
16. **Rocklin, R. E., C. T. Winston, and J. R. David.** 1974. Activation of human blood monocytes by products of sensitized lymphocytes. J. Clin. Invest. **53**:559-564.
17. **Waksman, B. H., and Y. Namba.** 1976. On soluble mediators of immunologic regulation. Cell. Immunol. **21**:161-175.
18. **Waldman, T. S., S. Broder, M. Durm, B. Meade, R. Krakauer, M. Blackman, and C. Goldman.** 1976. T cells suppression of pokeweed mitogen induced immunoglobulin production, p. 509-521. *In* J. J. Oppenheim and D. L. Rosenstreich (ed.), Mitogens in immunobiology. Academic Press, Inc., New York.
19. **Weber, T., C. T. Nordman, and R. Grasbeck.** 1967. Separation of lymphocyte-stimulating and agglutinating activities in phytohemagglutinin (PHA) from *Phaseolus vulgaris*. Scand. J. Haematol. **4**:77-80.
20. **Young, L. S., M. J. Martin, R. D. Meyer, R. J. Weinstein, and E. T. Anderson.** 1977. Gram-negative rod bacteremia: microbiologic, immunologic, and therapeutic considerations. Ann. Intern. Med. **86**:456-471.