JOURNAL OF BACTERIOLOGY, June 1969, p. 1030-1035

Vol. 98, No. 3
Printed in U.S.A.

Inhibition of Leukocyte Migration by a Staphylococcal Factor

BABETTE. B. WEKSLER[1] AND M. J. HILL
Wright-Fleming Institute, St. Mary's Hospital Medical School, London, W.2, England

Received for publication 13 January 1969

Cell wall mucopeptide isolated from virulent strains of *Staphylococcus aureus* has previously been found to potentiate subcutaneous staphylococcal lesions in mice. This cell wall fraction was found to inhibit the migration of polymorphonuclear leukocytes toward a chemotactic stimulus, as tested by the micropore filter chamber technique. A close correlation was shown to exist between in vivo "mouse virulence" of staphylococcal strains and the in vitro inhibition of leukocyte migration by the cell wall factor.

The nature of the initial host reaction after bacteria enter tissues largely determines the ultimate extent of a bacterial lesion (15). Factors that inhibit, delay, or alter this initial response, such as neutropenia, increased tissue pressure, administration of adrenaline, endotoxin, or anti-inflammatory drugs, result in more severe infections (4-7). Such factors all reduce the number of leukocytes appearing at the bacterial entry point.

It has been suggested that bacterial factors other than toxins might also delay the initial host inflammatory reaction and thus contribute to bacterial virulence. Cohn (6) observed that the entry of polymorphonuclear leukocytes (PMN) into the peritoneal cavity of mice was consistently delayed after injection of *Staphylococcus aureus*, but not after the injection of *S. albus*; this difference was not related to exotoxin release. In evaluating differences in virulence among strains of *S. aureus*, Agarwal (1) noted that strains which were successful in establishing subcutaneous lesions in mice initially inhibited leukocyte influx and edema at the lesion site for several hours, whereas strains unable to produce necropurulent lesions elicited an immediate inflammatory response. Hill (10) extracted the cell wall mucopeptide from such "mouse virulent" strains of *S. aureus*, and demonstrated that this material inhibited early edema formation and permitted lesion formation by a dose of *S. aureus* that, alone, is insufficient to establish a lesion. Mucopeptide from "mouse nonvirulent" strains has no such effect.

We have studied the effect of staphylococcal cell wall mucopeptide on the cellular component of the acute inflammatory response by evaluating its effect on the migration of PMN in vitro.

[1] Present address: Department of Medicine, The New York Hospital-Cornell Medical Center, New York, N.Y. 10021.

MATERIALS AND METHODS

The strains of *S. aureus* used were grouped as "virulent" and "nonvirulent" on the basis of their capacity to form necropurulent lesions in mice after subcutaneous injection of 10^3 colony-forming units on cotton dust by the technique of Agarwal (1). Virulent strains produced large, necrotic lesions at 24 hr after inoculation and bacterial viable counts of greater than 10^7, whereas nonvirulent strains produced only erythematous nodules without necrosis at 24 hr and viable counts of less than 10^6.

Virulent strains, with their sources, included: NCTC 9789 (the propagating strain for phage type 80) from the National Collection of Type Cultures, Colindale, England; AHF 1, AHF 2, CF 1, CF 2, and SF 1, strains isolated from outbreaks of bovine mastitis, from M. E. Sharpe, National Institute for Research in Dairying, Shinfield, Reading, England; strain 1531, isolated post-mortem from a human meningeal infection, originally obtained from S. Fischer, Melbourne (8); 66/7, isolated from a case of tropical myositis in Uganda; Coulson, a *Staphylococcus* but coagulase-negative, isolated from a patient with fatal septicemia at St. Mary's Hospital, London, England; strains Orbach, 7641, 7634, 7581, and Ward 52 A, coagulase-positive strains isolated from clinical specimens at the Wright-Fleming Institute, London. Nonvirulent strains were NCTC 7121, a very good producer of α-hemolysin, NCTC 7291, and NCTC 10039 (the propagating strain for phage type 83A) from the National Collection of Type Cultures, Colindale; all remaining strains were isolated on routine cultures at the Wright-Fleming Institute.

All cultures were grown at 37 C with gentle shaking in a medium containing 10 g of Oxoid nutrient broth no. 2 and 10 g of Oxoid yeast extract per liter. The period of cultivation was 3 hr unless otherwise noted.

Mucopeptide. Mucopeptide was isolated from cell walls of mechanically disintegrated staphylococci by

treatment with 1% sodium deoxycholate for 6 hr at 0 C. Full details of this method have been published (9). As has been reported, the deoxycholate-insoluble residue contains less than 1% teichoic acid, no detectable phosphorus or lipid, and very little polysaccharide The residue consists of mucopeptide and a small amount of protein (10). Treatment of the residue with trypsin, formyl blocking of amino groups, and heating do not affect lesion-enhancing activity, strongly suggesting that the mucopeptide is the component responsible for this activity.

In these experiments, the deoxycholate-insoluble residue was rendered soluble by treatment with crystalline egg white lysozyme (Koch Light Ltd.), 50 IU per mg of residue, in phosphate buffer at *p*H 7.0 for 4 hr at room temperature. After removal of insoluble material by centrifugation, the mucopeptide solution was heated to 100 C for 15 min to inactivate the lysozyme and was then lyophilized. The heat treatment is known to have no effect on the lesion-enhancing activity of the mucopeptide.

PMN were obtained from normal adult white mice and guinea pigs by peritoneal washout 4 to 16 hr after intraperitoneal injection of sterile beef broth. Human peritoneal PMN were obtained from the effluent fluid from patients undergoing peritoneal dialysis for renal failure; over 90% of cells in such fluids are PMN. The PMN were washed and resuspended in medium 199 containing either 0.5% ovalbumin or 5 to 10% fresh serum at a final concentration of 5×10^6 to 6×10^6 PMN/ml.

Chambers. Migration of PMN toward a known chemotactic stimulus was measured in modified Boyden chambers (12) fitted with membrane filters (Millipore Corp., Bedford, Mass.) having a pore size of 3 μm. Each chamber contained 10^6 to 1.2×10^6 PMN. The chemotactic stimulus was a suspension of 10^7, three-times washed micrococci in medium 199 plus 5 to 10% fresh serum. Redissolved lyophilized mucopeptide, 0.4 to 5.0 mg/10^6 PMN, either was added to the cell suspension or was used to pretreat the membrane filter (Millipore Corp.). The chambers were incubated at 37 C for periods as long as 3 hr; the filters were then removed, fixed, and stained. All tests were run in duplicate.

Leukocyte migration in chambers. The PMN settle in a single layer on top of the membrane filter (Millipore Corp.) and migrate through the filter toward the chemotactic stimulus in an evenly dispersed front. The depth of migration was determined by measuring the distance from the cell front to the starting surface, with the micrometer on the fine focusing adjustment. Five random fields were measured on each filter; agreement was ± 5 μm. Positive controls (containing a chemotactic stimulus but no mucopeptide) and negative controls (lacking the chemotactic stimulus) were run simultaneously in each experiment.

In positive control chambers, the PMN migrated rapidly through the filter, reaching the lower side after 1 to 1.5 hr. When no chemotactic stimulus was present (bacteria without serum or TC 199 alone), the PMN did not migrate more than 40 to 50 μm.

RESULTS

Effects of mucopeptide from virulent and from nonvirulent strains. Mucopeptide from a virulent strain of *Staphylococcus* markedly inhibited the rate of migration of PMN toward a chemotactic stimulus when the PMN were mixed with a solution of mucopeptide. Exposure of PMN to mucopeptide prepared from nonvirulent strains did not retard the rate of migration. The results of a typical experiment appear in Fig. 1 and illustrate the migration rate of normal, untreated cells, the lack of effect of exposure to nonvirulent mucopeptide (NCTC 7121), and the inhibited migration of cells treated with virulent mucopeptide (NCTC 9789).

Washing the PMN after exposure to mucopeptide solution did not restore normal migration. When the solution of mucopeptide was mixed with the chemotactic material in the lower compartment of the chamber, instead of being mixed directly with the PMN in the upper compartment, little inhibition of migration occurred (Table 1). Mucopeptide appeared to act directly on the leukocytes; however, the effect did not seem to be due to death of cells. A test for cell viability by trypan blue exclusion indicated that greater than 90% of the PMN, treated with either virulent or nonvirulent mucopeptide, were viable.

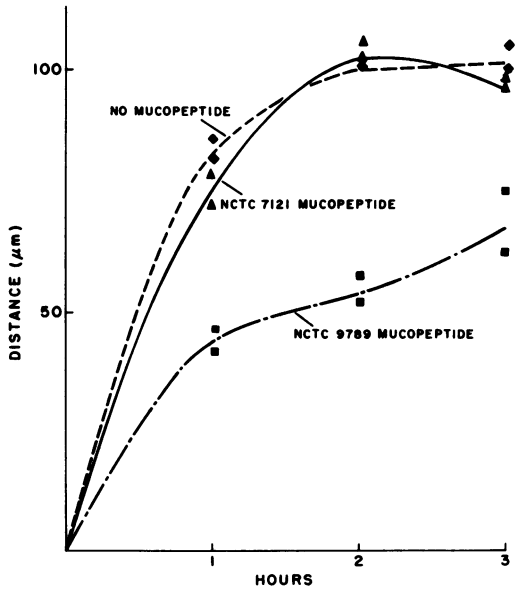

FIG. 1. *Inhibition of PMN leukocyte migration toward a chemotactic stimulus after exposure to mucopeptide from a virulent strain of S. aureus (NCTC 9789) but not after exposure to mucopeptide from a nonvirulent strain (NCTC 7121). Key: ◆, control; ■, NCTC 9789; ▲, NCTC 7121.*

TABLE 1. *Leukocyte migration after addition of virulent mucopeptide to top and bottom compartments of chamber*

Prepn	Leukocyte migration (μm)	
	NCTC 9789	AHF 1
Mucopeptide in top (mixed with PMN).....	41.6 ± 6.5	60.6 ± 3.1
Mucopeptide in bottom (mixed with chemotactic factor)..	94.6 ± 12.3	103.7 ± 9.2
No mucopeptide.	107.3 ± 14.4	107.3 ± 14.4

TABLE 2. *Inhibition of leukocyte migration by different doses of mucopeptide*

Amt of mucopeptide (mg/10^6 PMN)	Migration (per cent of control)
Strain NCTC 9789	
0.8	83
1.6	81
3.0	74
3.3	65
5.0	70
Strain SF 1	
0.4	94
0.8	76.5
1.6	63

Increasing the concentration of virulent mucopeptide produced increased the inhibition of PMN chemotaxis (Table 2). In this range, nonvirulent mucopeptide lacked inhibitory effect.

Effect of age of bacterial culture. The lesion-promoting effect of virulent mucopeptide in vivo depends on the age of the bacteria from which the cell wall mucopeptide is prepared. Maximal lesion potentiation is obtained from log-phase cultures at 3 hr of growth, whereas mucopeptide from cultures in the stationary phase (24 hr) does not potentiate lesions. Edema inhibition in vivo follows a similar time pattern, except that mucopeptide from 24-hr cultures actually stimulates rapid edema formation instead of inhibiting it (10).

However, leukocyte migration in vitro was inhibited equally well by mucopeptide prepared at all stages in the staphylococcal growth cycle up to 24 hr; only after 40 hr of growth was there diminution in the inhibitory effect (Table 3).

Effect of lysozyme treatment. Treatment of mucopeptide with high doses of lysozyme for a prolonged period (for example, 1,000 units per mg of cell wall residue for 12 hr) destroyed the lesion-potentiating and edema-inhibiting effects. This treatment did not diminish the capacity to inhibit chemotaxis in vitro and sometimes even enhanced it (Fig. 2). Prolonged lysozyme treatment did not confer any inhibitory effect on nonvirulent mucopeptide (Table 4). PMN migrated normally after exposure to 1,000 units of inactivated lysozyme per ml alone.

TABLE 3. *Inhibition of leukocyte migration by mucopeptide from bacterial cultures of different ages*

Strain	Age (hr)	Migration (per cent of control)
Virulent		
NCTC 9789	3	70
	7	68
	12	62
	24	77
Orbach	3	67
	24	69
	40	93
Nonvirulent		
1531a	7	100
	16	100
	24	100

Correlation between in vivo and in vitro effects of mucopeptide. Mucopeptide from 26 strains of *S. aureus* and one strain of micrococci was tested in vitro for inhibition of leukocyte migration (Table 5).

The results show that the strains may be divided into four groups: (I) virulent strains the mucopeptide of which potentiates lesions and inhibits leukocyte migration and edema formation (e.g., strain NCTC 9789); (II) nonvirulent strains the mucopeptide of which does not potentiate lesions, inhibit leukotaxis, or inhibit edema (e.g., strain NCTC 7121); (III) nonvirulent strains the mucopeptide of which does not potentiate lesions or inhibit edema formation, but which inhibits leukocyte migration (e.g., strain 8331); (IV) nonvirulent strains the mucopeptide of which fails to potentiate lesions or inhibit leukocyte migration but which inhibits edema accumulation (e.g., strain 66/7).

DISCUSSION

These results demonstrate that cell wall mucopeptide from strains of *S. aureus* that are virulent when injected subcutaneously into mice inhibits PMN migration in vitro as in vivo. Mucopeptide from all mouse-virulent strains tested inhibited chemotaxis, and no strain that yielded a noninhibitory mucopeptide was virulent.

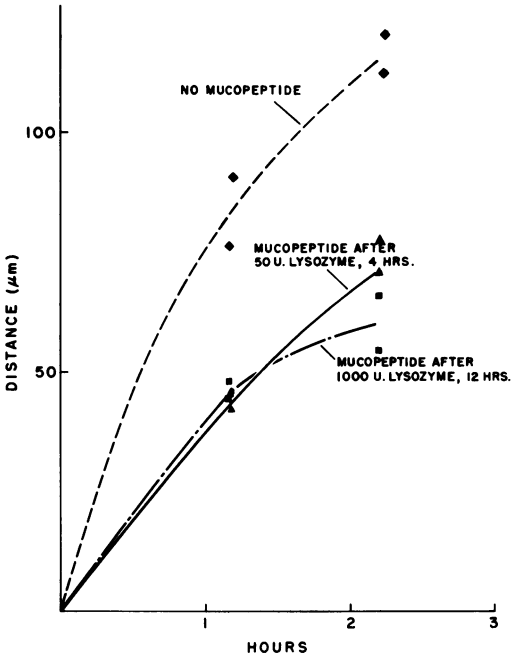

FIG. 2. *Chemotaxis of PMN leukocytes after exposure to virulent mucopeptide prepared by different treatments with lysozyme. Inhibition of migration compared to control. Key: ◆, control; ▲, mucopeptide from strain Orbach, 50 units of lysozyme for 4 hr; ■, mucopeptide from same strain, 1,000 units of lysozyme for 12 hr.*

TABLE 4. *Effect of lysozyme treatment of mucopeptide on leukocyte migration*

Mucopeptide source	Migration (per cent of control)	
	Lysozyme (50 units/mg)	Lysozyme (1,000 units/mg
Virulent strains		
NCTC 9789	67	65
Orbach	57	53.5
Nonvirulent strains		
Wood 46	97	95
8475	95	95.5
8504	100	100
Lysozyme alone		100

Mucopeptide from a number of strains that are nonvirulent in vivo inhibited chemotaxis in the Boyden chamber; none of these, however, was able to inhibit early edema formation in vivo. The four groups of strains listed in Table 5 are those found by Agarwal (2). Our use of in vitro techniques has allowed us to study many more strains, and our results are consistent with Agarwal's conclusion that a virulent strain must inhibit both the fluid and the cellular components of the initial inflammatory response in order to establish a lesion (2).

The mucopeptide component responsible for inhibition of leukocyte migration is resistant to lysozyme and does not vary in activity during the growth cycle; in contrast, lysozyme treatment of mucopeptide destroys its lesion-potentiating (aggressin) activity and its effect on fluid exudation (10). It could be postulated that aggressin activity is a property of a specific high-molecular-weight component of the cell wall mucopeptide and that this fraction inhibits both leukocyte migration and fluid accumulation at the lesion site. On digestion with lysozyme, the resultant low-molecular-weight fractions lose their effect on fluid accumulation and therefore lose their aggressin activity, even though they still retain the ability to inhibit leukotaxis.

The mechanism of inhibition of leukotaxis by these mucopeptide preparations is not known. Ward (17) showed that anti-inflammatory drugs such as hydrocortisone and chloroquine inhibit PMN chemotaxis in vitro by a direct irreversible effect on the PMN. Since these are lysosome stabilizing agents, it was suggested that such agents may prevent the release of leukocytic enzymes necessary for the chemotactic response. However, preliminary results (Hill, *unpublished data*) indicate that mucopeptide from virulent strains of *S. aureus* increases lysosomal lability.

Other known inhibitors of chemotaxis, organophosphorous esters, ethyl acetate, acetylated aromatic amino acid derivatives, and the complement chemotactic factor C′(5–7) itself, act by inhibiting PMN serine esterases (17). The precise location of these enzymes in or on the PMN has not been determined.

The differences between mucopeptide of virulent and nonvirulent strains of *S. aureus* are not known, but the role of small structural differences in cell wall components in bacterial virulence is well established. In *Streptococcus pyogenes*, the surface protein of M^+ and M^- strains has the same composition (15) and probably differs only in the degree of cross-linking of the protein (11). Further, Medearis et al. (13) reported that strains of *E. coli* B 011 capable of synthesizing a complete cell wall polysaccharide were less susceptible to early phagocytosis and killing by macrophages in vivo and PMN in vitro than were mutant strains deficient in certain cell wall polysaccharide-synthetic enzymes. The differences in susceptibility to phagocytosis could be abolished by growing the mutants in media containing the specific missing sugars. Since mutant and parent

TABLE 5. *Relationship between strain virulence and inhibition of edema in vivo and leukocyte migration in vitro*

Group[a]	Strain	Edema inhibition	Migration of control PMN (μm)	Migration of treated PMN (μm)	Per cent migration of treated PMN untreated PMN	Significance (P)[b]
I	NCTC 9789	+	100.9 ± 10.6	68.2 ± 11.8	68	.001
	1531	+	98.0 ± 9.9	80.7 ± 13.1	82	.005
	Orbach	+	98.0 ± 9.9	71.0 ± 6.9	72	.001
	7641	+	96.2 ± 14.0	59.6 ± 2.4	62	.001
	7634	+	96.2 ± 14.0	63.9 ± 3.6	66	.001
	7581	+	127 ± 7.3	112.0 ± 6.1	88	.02
	Ward 52A	+	127 ± 7.3	90.0 ± 5.6	71	.001
	SF 1	+	105.8 ± 5.0	74.8 ± 13.6	70	.001
	Coulson	+	121.6 ± 5.8	68.8 ± 7.6	70	.001
	AHF 1[c]	+	101.3 ± 9.2	47.2 ± 8.1	46	.001
	AHF 2	+	92.4 ± 3.1	66.2 ± 9.2	72	.001
	CF 1[c]	+	101.3 ± 9.2	34.1 ± 5.1	34	.001
	CF 2[c]	+	105.0 ± 14.3	61.4 ± 17.1	58	.001
II	NCTC 7121	0	100.9 ± 10.6	103.1 ± 7.9	103	NS
	NCTC 7291	0	92.4 ± 3.1	93.4 ± 4.4	101	NS
	7666	0	121.0 ± 5.9	123.6 ± 3.0	101	NS
	8679	0	107.4 ± 11.4	103.6 ± 5.3	97	NS
	NCTC 10039	0	86.6 ± 6.3	81.3 ± 3.6	94	NS
	8475	0	126.4 ± 6.6	120.9 ± 8.7	96	NS
	8504	0	126.4 ± 6.6	125.5 ± 10.4	99	NS
	8515	0	128.5 ± 2.5	131.7 ± 4.3	98	NS
III	8662	0	86.5 ± 6.3	63.6 ± 7.8	74	.001
	8331	0	116.5 ± 5.8	83.9 ± 6.9	72	.001
	8636	0	105.0 ± 14.3	59.8 ± 13.2	57	.001
	8617	0	107.4 ± 11.4	86.9 ± 9.2	81	.001
	8356	0	107.4 ± 11.4	58.4 ± 11.3	55	.001
	1340/4[c]	0	63.5 ± 11.9	34.4 ± 8.6	54	.001
	8318	0	107.4 ± 11.4	80.1 ± 11.8	75	.001
IV	66/7	+	119.2 ± 1.5	91.1 ± 9.4	76	.001

[a] Strains of group I are virulent; strains of groups II to IV are nonvirulent.
[b] All values are less than the decimals indicated.
[c] Mucopeptide dose was 1.3 mg/10^6 PMN. Otherwise dose was 0.6 mg/11^6 PMN.

strains all produced endotoxin of equal potency, the differences in virulence were considered independent of endotoxin.

Similarly, differences in the virulence of staphylococci, as measured by inhibition of early inflammatory response, appear independent of toxin production. Mice immunized with α-hemolysin remain susceptible to subsequent infection with the same bacterial strain, although the lesions show less dermonecrosis (2). However, mice immunized with mucopeptide of virulent strains are protected against subsequent infection with whole virulent staphylococci of the same or other strains (Hill, *in press*). Such protection is not conferred by prior infection with live organisms or by immunization with mucopeptide of nonvirulent strains. In protected mice, the edema-inhibiting activity of mucopeptide from virulent stains was abolished. We are now making tests to discover whether serum from immunized mice will neutralize the antichemotactic effect of mucopeptide on PMN.

ACKNOWLEDGMENT

Rosalind Steward gave excellent technical assistance. The helpful guidance of R. E. O. Williams is deeply appreciated.

B. W. was the recipient of Public Health Service Postdoctoral Fellowship 1-F2-A1-35,701-01 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Agarwal, D. S. 1967. Subcutaneous staphylococcal infection in mice. I. The role of cotton dust in enhancing infection. Brit. J. Exp. Pathol. 48:436–467.
2. Agarwal, D. S. 1967. Subcutaneous staphylococcal infection in mice. II. The inflammatory response to different strains of staphylococci and micrococci. Brit. J. Exp. Pathol. 48:468–482.
3. Agarwal, D. S. 1967. Subcutaneous staphylococcal infection in mice. III. Effect of active and passive immunization and anti-inflammatory drugs. Brit. J. Exp. Pathol. 48:483–500.

4. Andriole, V. T., and B. Lytton. 1965. The effect and critical duration of increased tissue pressure on susceptibility to bacterial infection. Brit. J. Exp. Pathol. 46:308–317.
5. Burke, J., and A. A. Miles. 1958. The sequence of vascular events in early infective inflammation. J. Pathol. Bacteriol. 76:1–19.
6. Cohn, Z. 1962. Determinants of infection in the peritoneal cavity. I. Response to and fate of *Staphylococcus aureus* in the mouse. Yale J. Biol. Med. 35:21–28.
7. Cohn, Z. 1962. Determinants of infection in the peritoneal cavity. II. Factors influencing the fate of *Staphylococcus* in the mouse. Yale J. Biol. Med. 35:29–47.
8. Fisher, S. 1965. Experimental staphylococcal infection of the subcutaneous tissue of the mouse. J. Infec. Dis. 115:285–292.
9. Hill, M. J. 1967. Action of bile salts on bacterial cell walls. Nature 214:1152–1153.
10. Hill, M. J. 1968. A staphylococcal aggressin. J. Med. Microbiol. 1:33–43.
11. Hill, M. J., A. M. James, and W. R. Maxted. 1963. Some physical investigations of the behavior surfaces. IX. Studies on the streptococcal cell wall. Biochim. Biophys. Acta 75:402–413.
12. Hurley, J. V. 1963. Incubation of serum with tissue extracts as a cause of chemotaxis of granulocytes. Nature 198:1212–1213.
13. Medearis, D. N., B. Camitta, and E. Heath. 1968. Cell wall composition and virulence in *E. coli*. J. Exp. Med. 128: 399–414.
14. Miles, A. A., E. Miles, and J. Burke. 1957. The value and duration of defense reactions of the skin to the primary lodgment of bacteria. Brit. J. Exp. Pathol. 38:79–96.
15. Tepper, B. S., J. A. Hayashi, and S. S. Barkulis. 1960. Studies of streptococcal cell walls. V. Amino acid composition of cell walls of virulent and avirulent group A hemolytic streptococci. J. Bacteriol. 79:33–38.
16. Ward, P. 1966. The chemosuppression of chemotaxis. J. Exp. Med. 127:693–709.
17. Ward, P., and E. L. Becker. 1967. Mechanisms of the inhibition of chemotaxis by phosphonate esters. J. Exp. Med. 125:1001–1020.
18. Ward, P., I. Lepow, and L. Newman. 1968. Bacterial factors chemotactic for polymorphonuclear leukocytes. Amer. J. Pathol. 52:725–736.