cyte is extremely easy and accurate. The ability to distinguish between the liver epithelial cell (Chang's) and HeLa is more difficult but can be accomplished if one scans a number of cells. It is not proposed that all tissue culture cell lines can be distinguished in this manner but knowledge of their surface features may be of some help.

Some of the features of cell surfaces described here can be seen and have been photographed in the light microscope. The undulating membrane and the patchy distribution of the microvilli in the chicken monocyte and the folding back of the HeLa cell edge correspond to similar structures seen in light micrographs and reported by various investigators(7,8). It would appear, then, that the present technics have not introduced an inordinate amount of cell distortion.

Summary. An improved method for the electron microscopic study of intact tissue culture cells is based on the use of carbon-collodion coated stainless steel grids upon which the cells are grown. Cell preparations are fixed with buffered osmium and metal shadowed. This technic has been applied to the study of 5 cell lines including mouse fibroblasts strain "L" (Earle), HeLa (Gey), liver epithelial (Chang), chicken fibroblasts and chicken monocytes. Distinctive surface features of these cell lines are described.

1. Porter, K. R., Claude, A., Fullam, E. F., *J. Exp. Med.*, 1945, v81, 233.

2. Selby, C. C., *Texas Rep. Biol. and Med.*, 1953, v11, 728.

3. Selby, C. C., Berger, R. E., *Cancer*, 1952, v5, 770.

4. Bang, F. B., Gey, G. O., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 78.

5. Palade, G. E., *J. Exp. Med.*, 1952, v95, 285.

6. Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1931, v49, 17.

7. Gey, G. O., Shapros, P., Borysko, E., *Ann. N. Y. Acad. Sci.*, 1954, v58, 1089.

8. Pomerat, C. M., *U. S. Armed Forces Med. J.*, 1953, v1, 11.

Received May 19, 1961. P.S.E.B.M., 1961, v107.

The Tumor Necrotizing Effect of Lipoid A Component of *Escherichia coli* Endotoxin.* (26765)

E. MIHICH, O. WESTPHAL, O. LÜDERITZ AND E. NETER

Department of Experimental Therapeutics, Roswell Park Memorial Institute, Depts. of Bacteriology and Pediatrics, University of Buffalo Medical School, Buffalo, N. Y. and Wanderforschungsinstitut, Freiburg, Germany

The endotoxins of gram-negative bacteria cause a large variety of primary and secondary reactions in susceptible hosts, including fever, the local and generalized Shwartzman reactions, hemorrhagic necrosis in experimental tumors, as well as alterations in non-specific resistance, immunological response, properdin levels, and dermal reactivity to epinephrine(1-5). Chemically, the endotoxins are complexes made up of polysaccharide, protein or peptide, and lipoid. It is the polysaccharide moiety that is responsible for serologic specificity. Recent studies suggest that the lipoid A component of this complex is pyrogenic, alters nonspecific resistance to experimental infection, and is responsible for enhanced reactivity of the rabbit to epinephrine(6,7).

A trichloracetic acid extract from *Staphylococcus aureus* strain D was found to alter dermal reactivity of rabbits to epinephrine (8) in a fashion comparable to that of endotoxins from gram-negative bacteria and the lipoid A component of *E. coli* endotoxin(7). The same staphylococcal extract caused extensive hemorrhagic necrosis of mouse sarcoma 180(9). The present study was undertaken, therefore, to determine whether *E. coli* lipopolysaccharide and the corresponding lipoid A component also produce hemorrhagic necrosis of sarcoma 180 of mice, and to com-

* This investigation was supported in part by research grants from Nat. Cancer Inst. and from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

TABLE I. Comparative Necrotizing Effect of *E. coli* Lipopolysaccharide (LPS) and Lipoid A on Sarcoma 180.

7th day after tumor implantation		8th day after tumor implantation			
		Route of treatment			
		Intraper.		Intrav.	
Treatment			%		%
Extract	μg/mouse	No. of animals	positive	No. of animals	positive
None	0*	59	12	40	10
LPS	5	—	—	15	40
"	10	20	20	15	47
"	20	50	54	30	70
"	40	20	70	20	95
Lipoid A	25	—	—	15	40
"	50	10	50	25	64
"	100	30	70	19	68
"	200	10	90	10	100

* Control animals were inj. with equivalent amounts of saline solution.

pare the effects of the *E. coli* lipopolysaccharide with that of the same staphylococcal extract.

Materials and methods. Sarcoma 180 was implanted subcutaneously in female Ha ICR Swiss mice weighing between 18 and 23 g. The trocar transplantation technic and caliper measurements of tumor diameters were previously described(10). Lipoid A was prepared from the highly purified lipopolysaccharide obtained from *E. coli* 0111:B4 by hydrolysis and fractionation with organic solvents(6). This preparation, analyzed at the Freiburg laboratory, was composed of D-glucosamine, phosphoric acid (ester), and the 4 long-chain fatty acids, lauric, myristic, palmitic, and beta-hydroxy-myristic acid. It contained less than 0.01% of polysaccharide. The lipoid A material was dissolved in 0.5% dextran of low molecular weight. The lipopolysaccharide and lipoid A preparations used were the same as those used in the previous study(7). Each preparation was injected either intraperitoneally or intravenously into tumor bearing mice on the 7th day after implantation; control animals received saline. At time of treatment the average diameter of the tumors ranged from 11 to 13 mm. The effects were observed on the following day by gross inspection of the tumor and of the overlying skin, both in the intact animal and after exposure of the tumor, as described previously(9). Histologic examination of the lesions of several animals confirmed the gross findings in each case.

Results. The data on the tumor-necrotizing efficacy of *E. coli* lipopolysaccharide and its corresponding lipoid A fraction are shown in Table I.

Lipoid A, like the original lipopolysaccharide, produces hemorrhagic necrosis in 90 to 100% of the tumors. Approximately 4 to 5 times larger amounts of lipoid A than of the lipopolysaccharide were required to obtain the effect in a similar percentage of animals. Intravenous administration of lipopolysaccharide produced hemorrhagic necrosis in a larger percentage of mice than intraperitoneal injection; lipoid A was equally effective when given by these 2 routes. From these results it is concluded that the polysaccharide-free lipoid A component of *E. coli* 0111:B4 endotoxin produces hemorrhagic necrosis in sarcoma 180, similar to the original lipopolysaccharide, but that it is less effective on a weight basis.

The results of the evaluation of the *E. coli* lipopolysaccharide and of the staphylococcal extract are summarized in Table II. Both bacterial preparations caused tumor necrosis at doses as low as 10 μg per mouse; the staphylococcal extract, however, was slightly more potent than the *E. coli* lipopolysaccharide. Different rates of absorption of the 2 preparations from the peritoneal cavity may be responsible for this difference, for in one experiment both extracts were equally active when administered intravenously.

TABLE II. Comparative Necrotizing Effect of *E. coli* Lipopolysaccharide and Staphylococcal Extract on Sarcoma 180.

Treatment		Hemorrhagic necrosis	
Extract	μg/mouse	No./Treated	% positive
None	0*	6/50	12
E. coli lipopolysaccharide	10	2/10	20
	20	23/40	58
	40	7/10	70
S. aureus D extract	10	6/10	60
	20	18/20	90
	40	9/10	90

* Control animals were given equivalent amounts of saline solution.

Discussion. Endotoxins of gram-negative bacteria have attracted renewed interest during the past few years. The extraordinary number of biologic effects that they exert in susceptible animals have prompted further studies on their chemical composition in relation to their biologic activity. The lipopolysaccharide moiety exerts most, if not all, of the biologic effects. The lipoid A component of this complex was found to be pyrogenic, to alter nonspecific resistance to experimental infection, and to enhance reactivity of the rabbit to epinephrine(6,7). The present study describes an additional activity of the lipoid A component, namely, that this fraction can cause hemorrhagic necrosis of mouse sarcoma 180. In the present experiments, lipoid A was approximately 5 times less effective than the lipopolysaccharide complex from which it was derived. The tumor necrotizing activity of lipoid A cannot be attributed to contamination with intact lipopolysaccharide, for careful examination for sugar constituents showed that the lipoid A preparation used contained less than 0.01% of polysaccharide. It is conceivable that only a portion of lipoid A is responsible for the biologic effect described here. It is important to point out in this connection that by aqueous ether extraction Ribi and associates obtained highly effective endotoxic products with a low lipoid content(11). Products containing 1 to 4% bound lipoid were as toxic as endotoxins containing as much as 15 to 30%. The identity of the chemical determinant(s) responsible for the numerous biologic activities of endotoxins requires further study.

Recently, Schaedler and Dubos(12) observed that albino mice (Rockefeller NCS strain) raised and maintained free of ordinary bacterial pathogens, including *Escherichia coli,* were highly resistant to the lethal effect of bacterial endotoxin, but highly susceptible to its infection-enhancing effect. With the acquisition of intestinal flora from other mice the animals became susceptible to the lethal effect of endotoxin. In view of these observations the mechanism of the tumor necrotizing effect of endotoxins (lipopolysaccharides) and of their subfractions, such as lipoid A, deserves further investigation.

Most of the previous investigations on endotoxins were carried out with products obtained from gram-negative bacteria. Recently, a trichloracetic acid extract of *S. aureus* was found to alter dermal reactivity of the rabbit to epinephrine(8), in a fashion comparable to that of *E. coli* lipopolysaccharide and its lipoid A component(7). The same staphylococcal extract also causes hemorrhagic necrosis of mouse sarcoma 180(9, 13). In addition, Higginbotham and Bass found that the Fritchie strain of *S. aureus* has the same activity on this tumor(13). In the present study, the tumor necrotizing efficacy of the staphylococcal extract of strain D was of the same order of magnitude as that of the *E. coli* lipopolysaccharide. Further investigations are necessary to determine the composition of the toxic factor(s) of staphylococci. Definition of this factor as endotoxin must await clarification of the chemical relationship, if any, between the staphylococcal toxin and the endotoxins of gram-negative bacteria.

Summary. The polysaccharide-free lipoid A component of *E. coli* 0111:B4 endotoxin produces hemorrhagic necrosis in sarcoma 180 of mice, but is somewhat less effective than the lipopolysaccharide from which it was derived. A trichloracetic acid extract obtained from *S. aureus* strain D was slightly more effective than the *E. coli* lipopolysaccharide on this tumor.

1. Burrows, W., *Ann. Rev. Microbiol.,* 1951, v5, 181.

2. Thomas, L., *Ann. Rev. Physiol.,* 1954, v16, 467.

3. Springer, G. F., Editor, *Polysaccharides in Biology,* Josiah Macy, Jr. Foundation, New York, 1957.

4. Bennett, I. L., Jr., Cluff, L. E., *Pharmacol. Revs.,* 1957, v9, 427.

5. Atkins, E., *Physiol. Revs.,* 1960, v40, 580.

6. Westphal, O., Nowotny, A., Luderitz, O., Hurni, H., Eichenberger, E., Schonholzer, G., *Pharm. Acta Helv.,* 1958, v33, 401.

7. Neter, E., Anzai, H., Gorzynski, E. A., Nowotny, A., Westphal, O., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 783.

8. Neter, E., Anzai, H., Gorzynski, E. A., *J. Inf. Dis.,* 1960, v107, 332.

9. Mihich, E., Neter, E., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 97.

10. Mihich, E., Nichol, C. A., *Cancer Research*, 1959, v19, 279.

11. Ribi, E., Hoyer, B. H., Milner, K. C., Perrine, T. D., Larson, C. L., Goode, G., *J. Immunol.*, 1960, v84, 32.

12. Schaedler, R. W., Dubos, R. J., *J. Exp. Med.*, 1961, v113, 559.

13. Higginbotham, R. D., Bass, J. A., *Bact. Proc.*, 1961, 109.

Received May 24, 1961. P.S.E.B.M., 1961, v107.

Detoxification of Bacterial Endotoxin with Retention of Ability to Stimulate Non-Specific Resistance to Infection. (26766)

HENRY H. FREEDMAN, BARNET M. SULTZER AND WILLIAM KLEINBERG
(Introduced by A. S. Gordon)
Princeton Labs., Inc., Princeton, N. J.

The highly pyrogenic and toxic endotoxins elicit, at microgram doses, a wide spectrum of biological effects including stimulation of non-specific host resistance to infection(1-3). Attempts at dissociation of *in vivo* properties of endotoxin by chemical modification, largely by hydrolytic measures, have been unsuccessful. This preliminary report of our studies on chemical modification of endotoxin, utilizing reactions resulting in substitution at functional groups known to be present in the molecule, describes a fraction isolated after acylation which is grossly detoxified but retains quantitatively the ability to stimulate resistance to infection with heterologous microorganisms. Recovery of original pyrogenicity and acute toxicity by mild saponification, failure to confer tolerance to the original endotoxin, degree of immunogenicity, retention of tumor-necrotizing activity, as well as the properties of other fractions isolated, will be reported separately. While our manuscripts were in preparation Noll and Braude(4) reported detoxification of endotoxin by reductive cleavage of ester bonds, yielding a product of high immunogenic potency.

Materials and methods. In all procedures, preparative and testing, usual precautions to avoid pyrogen contamination were observed. Preparation was accomplished by adding 25 ml acetic anhydride to 100 mg *S. typhosa* 0-901 endotoxin (Difco) and 10 mg anhydrous sodium acetate, and heating in a boiling water bath for 2 hours. After cooling, the anhydride was removed as a clear solution containing approximately 30% of the starting material, by centrifugation. The residue was dried under N_2 to remove all anhydride, washed 3 times with pyrogen-free saline and finally with water, the washes removing a fraction (20%) yielding a fine suspension like the original endotoxin. The washed residue (50% yield), which is the subject of this report, was dried under N_2 and ground to a fine powder in a mortar. This lyophobic fraction was triturated with small amounts of pyrogen-free saline, then diluted to desired concentrations. Acetyl content, by alkaline hydrolysis, was 12% compared to 1.6% for the original endotoxin.

Tests for acute toxicity and protection against infection were done with ICR mice (Bellewood) weighing 16-18 g. For pyrogen tests 2-2.5 kg male rabbits of mixed breed were used. Dosage, routes of administration, and time schedules are given below. Strains of *Pseudomonas aeruginosa* and *Escherichia coli* from our culture collection were employed in the resistance studies described below. Both organisms were grown in brain heart infusion broth (Difco) for 18 hours at 35°C and appropriately diluted in sterile broth prior to injection of 0.25 ml *i.p.* The *E. coli* inoculum consisted of approximately 2×10^8 viable cells per ml while the *Ps. aeruginosa* viable count amounted to about 6×10^8 cells per ml.

Results. The data in Table I demonstrate the reduction in acute toxicity: the acetyl-