Production of the Shwartzman Reaction with Microbial L Forms

GEORGE M. KALMANSON, MIKE Y. KUBOTA, AND LUCIEN B. GUZE

*Veterans Administration Center, Los Angeles, California 90073, and
UCLA School of Medicine, Los Angeles, California 90024*

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Study of potential pathogenicity of microbial L forms was done by the localized Shwartzman reaction. Stable L forms of *Proteus mirabilis* served as skin preparation in rabbits for induction of Shwartzman reaction by subsequent intravenous injection of either *P. mirabilis* L forms or *Escherichia coli* endotoxin. The intensity of the reaction was positively correlated to numbers of L forms in the skin. L forms also served as the intravenous challenge. In vivo multiplication of L forms was not a prerequisite for the reaction, as it could be produced with nonviable, osmotically lysed L forms. The reaction produced with L forms in the skin was more intense than that produced with the parent bacterial form. These latter observations, coupled with the demonstration that L forms disappeared from the skin (lysed?) after 4 hr, in contrast to bacteria which were recoverable for 72 hr (duration of study), suggest release of endotoxin by L forms as a pathogenic mechanism.

Although the role of microbial L forms in the pathogenesis of disease is uncertain, evidence has accumulated which suggests that L forms may play such a role. (Various terms are used to describe bacteria with all or part of the cell wall missing. These terms include L forms, L colonies, L-phase growth, bacterial variants, protoplasts, and spheroplasts. For purposes of this report, the terms are considered interchangeable. In literature citations the authors' terminology is used.) These forms have been isolated from human clinical material [blood (10, 17, 24, 28, 30, 31), spinal fluid (16, 24, 29), bronchial secretion (5, 21), and genitourinary tract (2, 11, 12, 19, 26)] during various disease states. It was demonstrated that they could be formed in vivo in the peritoneal cavity of experimental animals (4) and in vitro by antibody-complement lysozyme (7, 27). Furthermore, the ability of penicillin to produce these forms in vitro by interfering with cell wall metabolism has been paralleled by their induction in rats with acute enterococcal pyelonephritis after treatment with penicillin (13).

Because they have been isolated clinically, it is important to consider means by which L forms could cause disease. These include direct host-parasite interaction, in vivo reversion to the parent bacterial form, and the production of substances which might be toxic directly or through some immunological mechanism. The endotoxin of gram-negative bacteria is an example of the latter suggestion. One method of demonstrating endotoxin activity is by the Shwartzman reaction. Although the role of this reaction in

human disease has not been absolutely defined, there is an impressive body of evidence, which has been summarized in detail by Hjort and Rapaport (14). We believe there is sufficient evidence that the Shwartzman reaction plays a pathogenic role in gram-negative septicemia of pregnancy, renal cortical necrosis after premature separation of placenta, other gram-negative infections including fulminant diarrhea in infants secondary to *Escherichia coli*, and fulminant meningococcemia.

The present experiments were designed to determine whether the Shwartzman reaction could be elicited with L forms of *Proteus mirabilis*.

MATERIALS AND METHODS

Bacteria and endotoxin. *P. mirabilis* bacteria, strain 9 (ATCC 14168) and stable (considered "stable" because they do not revert to the bacterial form) L forms, strain L9, were obtained through the courtesy of Ruth G. Wittler, Walter Reed Army Medical Center, Washington, D.C. *P. mirabilis* L forms were grown in Heart Infusion Broth or Heart Infusion Agar (Difco) containing 20% human serum (Hyland Laboratories, Los Angeles, Calif.) and 0.5% Oxoid yeast extract (Consolidated Laboratories, Chicago Heights, Ill.). *P. mirabilis* bacteria were grown in Brain Heart Infusion Broth.

P. mirabilis L forms were enumerated by streaking the culture on Heart Infusion Agar containing 5% human serum and 0.5% Oxoid yeast extract. The reduced amount of human serum (5%) gave a less opaque plate than 20% serum, thus facilitating enumeration and morphological study. *P. mirabilis* bacteria were counted on Nutrient Agar (Difco) to prevent swarming.

E. coli endotoxin, 0127:B8 (Difco) was used.

Experimental animals. Male albino New Zealand rabbits were obtained from a single local distributor. Rabbits weighed 2.0 kg (average) at time of delivery and were used within 2 weeks. They were fed Rabbit Chow Checkers (Purina-Ralston, St. Louis, Mo.) and offered water ad libitum.

Production of Shwartzman reaction. Skin preparations were made with *E. coli* endotoxin, *P. mirabilis* bacteria, or *P. mirabilis* L forms. Endotoxin was prepared for intradermal injection by dissolving 10 mg of the endotoxin in 1.25 ml of pyrogen-free 0.85% sodium chloride solution. Three portions of a 48-hr broth culture of *P. mirabilis* L forms were centrifuged, washed three times in fresh medium, and resuspended in (i) original volume, (ii) $\frac{1}{10}$ of original volume, and (iii) $\frac{1}{100}$ of original volume, respectively. A portion of the original culture-centrifuged supernatant fluid was saved for control inoculation. An 18-hr broth culture of *P. mirabilis* bacteria was centrifuged, washed three times in fresh broth, and resuspended to its original volume. Bacterial counts were made in the Petroff-Hauser bacterial counting chamber and the suspension was diluted in fresh broth to approximate the number of L forms. All intradermal inoculations were 0.1 ml in volume.

Challenge endotoxin doses were prepared by dissolving 10 mg of endotoxin in 25 ml of sterile pyrogen-free 0.85% sodium chloride solution. Challenge L form doses were prepared on the day of challenge in a manner similar to the preparatory doses. Challenge was with 1 ml of the endotoxin (400 μ g) or L form suspensions injected through a marginal ear vein. This dosage is somewhat larger than that used by others (1, 3, 32) and probably reflects the quality of the endotoxin preparation, as is also indicated by the fact that no deaths followed challenge. Fukushima et al. (9) noted that biological activity among endotoxin preparations varied according to the method of preparation. Our dosage was determined by preliminary checkerboard titration in skin of rabbits.

Ventral surfaces of rabbits were shaved and various concentrations of L forms, 800 μ g of endotoxin, bacterial suspension, and filter-sterilized supernatant broth from the 48-hr L-form culture were inoculated intradermally on either side of the midline. After 20 to 24 hr, each rabbit was challenged intravenously with L forms or 400 μ g of endotoxin. A control group received no intravenous challenge. As reported by Larson et al. (22), an inflammatory reaction was seen in the skin of rabbits secondary to intradermal inoculation of endotoxin without subsequent intravenous challenge; this averaged 4 to 10 cm² and persisted for several days. No necrosis developed in unchallenged animals. The skin was examined 4 to 6 hr after challenge for early local reaction; none was noted. At 24, 48, and 72 hr after challenge, all Shwartzman reactions were recorded by tracing the necrotic and indurated areas on clear sheets of X-ray film. The area of lesion was determined by retracing the lesions onto graph paper and counting squares. A selected number of animals from each group was then sacrificed and skin reactions were excised. Swab cultures were made of intradermal inoculation sites for recovery of L forms and bacteria as follows. Ventral surfaces of

rabbits were cleaned with 70% ethyl alcohol, rinsed with sterile distilled water, the skin reactions aseptically excised, and tissues placed skin down in sterile petri dishes. With sterile scalpel blades, tissues were sliced with a single pressing and slicing motion through the inoculated site from subcutaneous to dermal layer. Holding the slit open with forceps, a moistened swab was inserted and twirled, and streaked on Heart Infusion Agar containing 5% human serum and 0.5% Oxoid yeast extract. Thickness of the reaction was then measured.

For determination of the presence of preformed endotoxin in *P. mirabilis* L forms, two samples of a 48-hr culture were centrifuged and the sedimented L forms washed three times in fresh broth. One L form sediment was thoroughly drained and resuspended in sterile distilled water at $\frac{1}{25}$ of the original sample volume; as a control, the second sediment was resuspended in fresh medium to a similar volume. Both suspensions were incubated for 24 hr at 37 C. A 0.1-ml amount of the lysed (distilled water) L-form suspension and 0.1 ml of the unlysed (medium) L-form suspension were inoculated intradermally into rabbits as described above. In addition, the animals were prepared with a freshly centrifuged, washed, and 100-fold concentrated L-form suspension, a washed *P. mirabilis* bacterial culture, and an *E. coli* endotoxin solution. A 1.0-ml intravenous challenge dose of endotoxin (400 μ g/ml) was given each rabbit 20 to 24 hr after skin preparation.

RESULTS

When animals were challenged with *E. coli* endotoxin, 1 of 8 rabbits which received 3.5×10^6 L forms in the skin; 8 of 18 with 2.3 to 6.1×10^7 L forms, and 6 of 8 with 4.3×10^8 L forms had positive reactions. No reaction was elicited in 30 animals when skin was prepared with L-form culture supernatant fluid. Three of 10 animals which received 9.3×10^7 bacteria and 14 of 18 animals prepared with *E. coli* endotoxin had positive reactions after challenge with *E. coli* endotoxin. When similarly prepared animals were challenged with 1.5 to 2.3×10^8 L forms, 4 of 34 L-form-prepared animals, 1 of 10 bacteria-prepared animals, and 2 of 10 endotoxin-prepared animals had positive but less intense reactions. Six of eight animals prepared with lysed L forms had positive reactions after challenge with *E. coli* endotoxin. The results of all experiments, as seen at 24 hr, are summarized in Table 1. The appearance of lesions is shown in Fig. 1.

Preliminary studies indicated that L forms were not recoverable from the skin at 24 to 48 hr after inoculation, whether or not the animals had received an intravenous challenge. To study further the rate of microbial disappearance, 10 rabbits were injected with 4.5×10^7 L forms in each of four skin sites. Lesions were excised and examined by swab culture technique at 1, 2, 3, 4,

TABLE 1. *Shwartzman reaction with L forms of P. mirabilis*

Skin prepn	Shwartzman reaction					
	Challenged ^a with 400 μ g of <i>E. coli</i> endotoxin			Challenged with 1.5 to 2.3 $\times 10^8$ L forms		
	No. ^b	Avg area	Avg volume	No. ^b	Avg area	Avg volume
		mm ²	mm ³		mm ²	mm ³
L forms						
3.5 $\times 10^{10c}$	1/8	137	137	0/8		
2.3 to 6.1 $\times 10^7$	8/18	99	264	3/18	22	43
4.3 $\times 10^8$	6/8	171	495	1/8	25	49
L forms (lysed) ^d						
1.8 $\times 10^7$	6/8	61	155			
Bacteria						
9.3 $\times 10^7$	3/10	85	283	1/10	13	21
<i>E. coli</i> endotoxin (800 μ g)	14/18	217	707	2/10	32	74

^a Intravenous challenge.^b Number of animals with positive reaction expressed as a proportion of animals tested.^c Number of organisms injected.^d After 24 hr in distilled water. None remained viable.

and 24 hr. It was found that L forms could be recovered up to 4 but not at 24 hr after injection. In an analogous experiment, the bacterial form could be recovered for 72 hr (duration of study). An explanation for the rapid disappearance of L forms in the skin was sought by determining the cidal power of normal rabbit serum against the organism by previously described methods (20). It was found that normal rabbit serum killed *P. mirabilis* L forms but not bacteria within 1 hr (unpublished results).

DISCUSSION

This study has demonstrated that a local Shwartzman reaction could be produced in the skin of rabbits prepared by intradermal inoculation of a stable L form of *P. mirabilis* when the animals were given *E. coli* endotoxin intravenously 20 to 24 hr later. The intensity of the Shwartzman reaction was positively related to the number of organisms in the skin. Osmotically lysed organisms could serve for skin preparation, indicating that in vivo multiplication was not necessary. The Shwartzman reaction could be produced in similarly prepared animals by intravenous challenge with the L forms, but it was of lesser frequency and intensity.

Shwartzman reaction could also be produced by skin preparation with the parent bacterial form, but this occurred less frequently than was noted in the L-form-prepared animals, perhaps indicating less release of endotoxin. This explanation

appears more likely in view of the fact that viable L forms could be recovered from rabbit skin for 4 but not 24 hr, whereas bacteria persisted for 72 hr. The disappearance (lysis?) from the skin may be related to the susceptibility of the L form to rabbit serum as demonstrated in vitro, and thus account for the local release of endotoxin.

Endotoxin-like activity of L forms has been previously suggested. Minck and Fruhling (25) were able to produce skin lesions in rabbits with L forms of *Vibrio cholera* and a strain of *Proteus*. When the parent bacterial form of *V. cholera* was injected, only a transitory edema and congestion of 3 to 4 days' duration ensued. When the parent bacterial form of *Proteus* was injected, there was usually a severe spreading necrotic reaction, most frequently followed by death of the animals. When L forms of both organisms were injected, the results were in all respects comparable with each other but different from results obtained with bacteria. Some hours after inoculation, edema and congestion appeared, sometimes with hemorrhagic plaques. Edema and congestion began to regress in 24 to 48 hr; at the same time, there appeared at the site of injection a small nodule which gradually increased in size over 4 to

FIG. 1. *Shwartzman reaction in skin of rabbits.* Skin preparation: (A) 4.3 $\times 10^8$ *P. mirabilis* L-forms; (B) 3.5 $\times 10^8$ *P. mirabilis* L-forms; (C) 6.1 $\times 10^7$ *P. mirabilis* L-forms; (D) 800 μ g of *E. coli* endotoxin. Intravenous challenge 20 hr later: 400 μ g of *E. coli* endotoxin.

5 days, then gradually regressed over a period of several weeks. The nodules were filled with a yellowish-white fatty viscous material. Occasionally fistulae occurred before regression. The early lesions contained polymorphonuclear leukocytes; later they were surrounded by macrophages with clear cytoplasm containing inclusion bodies. Neither L forms nor bacterial revertants could be isolated from the lesions. This finding, together with the ability to reproduce the pathological sequence with ether and chloroform extracts of the L form of *Proteus*, suggested to us that in vivo multiplication was unnecessary.

Tulasne and Lavillaureix (34) studied the pathological sequelae of injection of a stable L form (nonreverting in vitro for more than 1 year of serial transfers) of a "water vibrio" into mice by a variety of routes. Inoculation subcutaneously, intraperitoneally, intravenously, intracerebrally, and into the lungs led to rapid death of animals, usually within 24 hr. Regardless of the route of injection, the pathological pictures were similar: there was intense congestion of the visceral organs, particularly the intestines, which were filled with liquid resembling "melon juice." Recovery of L forms depended on route of inoculation. After subcutaneous inoculation, they were isolated from the local lesion and from "pus." After intraperitoneal inoculation, L forms were present in the peritoneal fluid. They could not be recovered after intravenous or intrapulmonary inoculation but, after intracerebral inoculation, were isolated from the local lesion and "the usual organ lesions and the intestine." In a subsequent study, the authors (35) could reproduce the pathological effects with either heat-killed L forms or with endotoxin prepared therefrom.

Timakov and Kagan (33) found that repetitive inoculations of living cultures of avirulent strains of *Salmonella typhimurium* L forms led to sterile abscesses at the site of inoculation and, after the second and third injections, almost half of the animals died.

Dasinger and Suter (6) compared toxicity of bacteria and L forms of *S. paratyphi* B. Endotoxic activity was measured in mice made hyperreactive to endotoxin by intravenous injection of BCG 10 days prior to challenge. The L forms were noted to be less toxic than their parent bacterial form.

Kagan (15) studied the Shwartzman reaction in the knee joints of rabbits. Antigen was prepared by alternate freezing and thawing (from -7 to $+37$ C) an L form of Group A β -hemolytic streptococci. The sensitizing substrate was injected into the knee joint and the "resolving" dose was given intravenously 18 hr later. Reaction in the joint consisted of turbidity of the surface of the joint with sero- to seropurulent exudate and

"strongly infiltrated surrounding tissue." The reactions were somewhat stronger when the antigen was prepared from the parent bacterial form. A cross-sensitizing effect was found between the bacterial and L forms.

Further evidence of the toxicity of L forms has been derived from tissue culture study. Lavillaureix (23) studied four stable strains of L forms which he termed pathogenic, i.e., they could produce an endotoxin-like death of mice. He found that these L forms were toxic for two tissue culture strains, HeLa and KB both originally derived from the epidermoid carcinoma of human cervix. The tissue cells became pyknotic and developed vacuoles and inclusion bodies. After 15 serial passages, the organisms could be recovered in culture media and were able to produce death in mice with congestion of the organs and "melon-juice" liquid in the intestines. On the contrary, two "non-pathogenic" L forms derived from *Proteus* were not toxic for tissue culture and could not be recovered from the tissue. Freeman and Rumack (8) found that spheroplasts prepared from *Brucella* were more destructive than the bacterial form to cultures of mononuclear phagocytes prepared from guinea pig peritoneum. They suggested that "removal of surface component, in general, results in cells that are increasingly cytopathogenic."

Kagan and Rakovskaya (18) found that the pathogenicity of L-forms for tissue culture varied, not only with the particular L-form (those made in vitro were, in general, less pathogenic than those isolated from patients), but also with the type of tissue culture used. The effect on chick embryo fibroblasts was greater than that on a culture of *Macacus rhesus* kidney cells.

The data presented in this study, together with the experiences cited from the literature, indicate that L-forms may be toxic. This toxicity can be manifested either directly or through the mechanism of the Shwartzman reaction. Speculation may be made concerning the possible role of L forms in initiation or contribution to progression of disease. During the course of an infection, as for example with gram-negative bacteria in the kidney, L forms would be formed either through the natural defenses of the body, i.e., antibody-complement-lysozyme, or following the use of cell wall active antibiotics. Local lesions could then occur either directly or through the mechanism of the Shwartzman reaction following release of endotoxin-like material. It is conceivable that repetitive insults of this kind could lead to eventual establishment of clinical disease in the target organ.

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