DISSEMINATED INTRAVASCULAR COAGULATION IN RABBITS: SYNERGISTIC ACTIVITY OF MENINGOCOCCAL ENDOTOXIN AND MATERIALS EGESTED FROM LEUCOCYTES CONTAINING MENINGOCOCCI

I. W. DeVoe* AND F. GILKA†

* *Department of Microbiology, Macdonald Campus, McGill University, Montreal, Quebec,* and † *Animal Pathology Division, Health of Animals Branch, Agriculture Canada, Animal Disease Research Institute (E), Box 11300, Station H, Ottawa, Ontario K2H 8P9, Canada*

PLATES XXVII AND XXVIII

FULMINATING meningococcal septicaemia is characterised by marked vascular damage and thrombosis in many organs of the body, and the Waterhouse-Friderichsen syndrome, i.e., haemorrhagic destruction of adrenal glands, is an associated phenomenon (Ferguson and Chapman, 1948). The mechanisms responsible for these pathological effects are not well understood, but their similarity to the generalised Schwartzman reaction in rabbits suggests that meningococcal endotoxin may play a prominent role (Scherp, 1955). Work recently discussed by Horn (1973) implicated polymorphonuclear leucocytes (PMN), and more especially their cationic proteins, in the production of the Schwartzman reaction. Horn (1973) presented a model in which such proteins of PMN sensitise animals to small quantities of endotoxin. Although the presence of PMN in cerebrospinal fluid (CSF) and in thrombi in meningococcal disease is well documented (Ferguson and Chapman, 1948), the involvement of the leucocyte in the pathology of the Schwartzman-like reaction of fulminating meningococcal disease has not been investigated.

Early investigators of meningococcal disease showed interest in the intracellular location of meningococci in PMN from CSF or blood (Flexner, 1907; Murray, 1929), and it is now regarded as a diagnostic feature. Because these intracellular diplococci appeared by light microscopy to be intact, it was generally assumed that they remained viable in leucocytes (Scherp, 1955; Smith, 1968). However, recent experiments indicate that, in in-vitro systems, meningococci are engulfed, killed, and degraded by PMN from human beings, rabbits, or mice (Roberts, 1967; DeVoe, Gilchrist and Storm, 1973; DeVoe, 1976).

Because endotoxin appears to be implicated in the pathogenesis of meningococcal disease, we have been exploring possible mechanisms for the liberation of endotoxin from the meningococcus. DeVoe and Gilchrist (1973, 1975) described a number of strains of meningococci from various serogroups that,

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during active growth, released their endotoxin-containing outer cell-wall layer in the form of "blebs" into the surrounding medium. More recently, we have explored a second possible mechanism. According to Filkins (1971), PMN do not detoxify endotoxin from *Salmonella enteritidis*. Therefore, it seemed reasonable to hypothesise that PMN might themselves play a part in the liberation of endotoxin from meningococci. Support was provided when DeVoe (1976) found that meningococci were degraded inside PMN and then egested into the surrounding medium.

We now present evidence that small quantities of the leucocyte-egested material acting synergistically with endotoxin or with the endotoxin-containing meningococcal cell-wall blebs result in disseminated intravascular coagulation in rabbits.

MATERIALS AND METHODS

Organism. The group-B *Neisseria meningitidis* (strain SDIC) used in these experiments was received from the Neisseria Repository, Naval Medical Research Unit no. 1, School of Public Health, University of California, Berkeley, California. Procedures for examination of strain purity, maintenance of stock and working cultures, and growth and harvesting of the bacterium have been described by DeVoe (1976).

Preparation of polymorphonuclear leucocytes. Methods for the induction, harvesting and preparation of mouse peritoneal-exudate cells were described by DeVoe (1976). Rabbit peritoneal-exudate cells were induced by a similar procedure except that a 25-ml volume of irritant fluid was injected and cells were harvested 12 h later.

In-vitro phagocytosis. Methods for the measurement of in-vitro phagocytosis were reported by DeVoe (1976), but in essence they were as follows. Meningococci labelled with ^{14}C were allowed to react *in vitro* with PMN for 10 min. under controlled conditions to produce by means of phagocytosis a ratio between intracellular meningococci and PMN of 1.0–1.5 : 1.0. Meningococci remaining outside the PMN were rapidly destroyed and removed, and the ^{14}C -labelled meningococcal debris egested during the next 2 h was measured.

2-Keto-3-deoxyoctonate (KDO) determinations. KDO was determined by the method of Weisback and Hurwitz (1959) and Osborn (1963). The colour that developed in the aqueous phase was extracted by an equal volume of butanol. Optical densities were determined on a Gilford spectrophotometer (model 240).

Radioactivity determinations. The methods were those of DeVoe and Gilchrist (1973).

Animals. Swiss albino mice aged 10 weeks and New Zealand rabbits weighing 2 kg were used.

Biological assay of endotoxin. Use was made of the localised Schwartzman reaction. Rabbits were given subcutaneous inoculations of extracted and purified meningococcal lipopolysaccharide (LPS; 10 μg per kg), followed after 24 h by a similar dose intravenously. Extraction and purification of LPS and preparation of cell-wall blebs were as described by DeVoe and Gilchrist (1973).

Products of leucocyte egestion were tested for endotoxin activity by a procedure similar to that described above. After the period of 2 h allowed for egestion, leucocytes were removed by centrifugation at 250 *g*, for 5 min. and the supernatant fluids were centrifuged at 38 000 *g* for 20 min. The radioactive pellets were resuspended in phosphate-buffered saline to a volume appropriate for injection.

Pathological examination. Thirty-two rabbits were given inoculations of meningococcal material. Animals that died or were killed *in extremis* were examined immediately. Tissue from lungs, heart, liver, kidney, and in some instances from duodenum, spleen, adrenals and central nervous system (CNS) was fixed in buffered 10% formaldehyde and embedded in paraffin. Sections (6 μm) were stained with haematoxylin and eosin and sometimes also with Masson's trichrome and phosphotungstic haematoxylin.

RESULTS

Degradation of endotoxin by leucocytes

The ^{14}C -containing supernatant fluids obtained after allowing leucocytes containing meningococci to egest for 2 h were analysed for KDO, a sugar present in LPS (endotoxin). On the assumption that the radioactive counts in the supernatant fluids represented the material derived from a given number of bacteria and from the knowledge of the amount of extractable endotoxin from that number of whole meningococci, it was possible to calculate the theoretical level of KDO to be expected if no intracellular degradation of the endotoxin molecule had occurred. This quantity, when compared with the amount of KDO actually measured in supernatant fluids, might be expected to indicate whether the leucocytes modified the endotoxin. Our calculations indicate that had LPS not been modified, we should have measured 8 μg of KDO from the 2×10^9 bacterial equivalents egested. However, in only one of four experiments were we able to detect KDO ($1.4 \pm 0.2 \mu\text{g}$). Therefore, it appeared that the leucocytes were indeed effective in degrading the endotoxin molecule.

Synergistic activity of LPS and material egested from leucocytes that contained meningococci

It seemed possible that the leucocyte-egested materials—though degraded—might possess endotoxic activity.

With regard to the biological assay of endotoxin, we found that the localised Shwartzman reaction was routinely reproducible (table, group 2) when purified meningococcal endotoxin was used for both injections at a dosage of 10 μg per kg. Furthermore, animals given injections of this quantity of endotoxin experienced only the haemorrhagic spot on the skin with no apparent adverse systemic effects. The intravenous administration to rabbits of a single dose of LPS (10 μg per kg) was without apparent effect (table, group 1), and it should be noted that the LD50 of LPS for rabbits similar to those used in the experiments described was 23 μg per kg. It would have been of interest to treat rabbits subcutaneously with leucocyte-egested material in a dose equivalent to the LPS dose of 10 μg per kg, but this would have required much more egested material than was available. However, the remote possibility existed that the egested endotoxin possessed enhanced toxicity as a result of leucocyte modification.

Egested material from 1×10^7 leucocytes {equivalent to $0.5\text{--}0.75 \times 10^7$ degraded bacteria (DeVoe, 1976)} was given subcutaneously to the third group of rabbits (table). The rabbits showed only slight redness at the site of injection, and this disappeared within hours and no general distress was observed over the 7-day period of observation. Rabbits of a fourth group were given leucocyte-egested material subcutaneously; of the 32 animals so treated, 22 received intravenous inoculations of LPS (10 μg per kg) 24 h later and 10 received an equivalent dose in the form of meningococcal cell-wall blebs (see below) at the same time and by the same route. By the 2nd or 3rd day most

TABLE
Effects in rabbits of meningococcal endotoxin and egested material
 from leucocytes containing intracellular meningococci*

Rabbit group no.	Treatment	Number of rabbits tested	Number of rabbits showing localised Schwartzman reaction	Number of rabbits showing disseminated intravascular coagulation
1	Single intravenous injection of meningococcal LPS (10 µg per kg)	13	0	0
2	Subcutaneous injection of LPS (10 µg per kg) followed after 24 h by intravenous LPS (10 µg per kg)	14	14	0
3	Single subcutaneous injection of leucocyte-egested material only	10	0	0
4	Subcutaneous injection of leucocyte-egested material followed after 24 h by intravenous injection of endotoxin (10 µg per kg) or the equivalent LPS dose as cell-wall blebs†	32	2	12‡

* The material was egested from 1×10^7 loaded leucocytes and its concentration adjusted to the equivalent of 0.05 µg of LPS per kg (disregarding intracellular degradation of LPS).

† Meningococcal cell-wall blebs were administered to 10 of the 32 rabbits.

‡ Five of these 12 rabbits received cell-wall blebs instead of LPS.

Renal cortical necrosis was observed in six animals, three of which had received cell-wall blebs.

of the 32 animals of this fourth group developed obvious signs of physical distress, e.g., loss of appetite, nasal discharge, intermittent rapid breathing disorientation, weakness, diarrhoea and anuria. Twelve of the 32 rabbits developed a disseminated intravascular coagulation and, of these 12, six also exhibited renal cortical necrosis. The remainder showed vascular lesions that were less severe. In the initial experiments, observations were continued until the death of the animals, which usually occurred by the 5th or 6th day. However, in later experiments, animals were killed after 3 days and examined. Macroscopic lesions were found in lungs, livers and kidneys in advanced cases. The lungs showed haemorrhages, oedema and areas of atelectasis and emphysema and the kidneys showed evidence of cortical necrosis (fig. 1). The livers were pale and swollen, and often exhibited lobular necrosis. Although the severity of lesions varied somewhat from animal to animal there was general consistency in their character.

Lung. All rabbits showed localised congestion of the lungs, and alveolar oedema or subsequent atelectasis and emphysema. Alveolar capillaries were infiltrated with PMN (fig. 2). Thrombi were seen in many capillaries or post-capillary venules and in the larger venules (figs. 2 and 3). Slight or distinct haemorrhagic infarcts occurred only occasionally, and were usually at the

sharp borders of the lung where relatively slight collateral vascularisation might be expected (fig. 1).

Myocardium. One-half of the rabbits showed a few foci of myocardial necrosis, infiltrated with PMN, but no thrombi were seen.

Liver. Hepatic necrosis occurred in one-half of the rabbits. Most necrotic lesions were focal, but intermediary or centrilobular necrosis was also present. Dead tissue was invaded by PMN. In protracted cases, larger areas of necrosis demarcated by inflammatory cells occurred. Thrombi in sinuses or venules were seen in only five rabbits. Necrotic lesions, both in the myocardium and liver, had a tendency to calcify in rabbits that survived for a few days.

Kidney. Renal cortical necrosis developed in six rabbits, and were due to thrombosis in glomerular capillaries or afferent arterioles (fig. 4). In protracted cases some necrotic areas were surrounded by inflammatory tissue and appeared to be progressing towards the medulla.

Adrenal. Two rabbits showed small necrotic areas in the cortex, and congestion and haemorrhages were also rare. Thrombosis did not occur, but light focal heterophilic leucocytosis was common in the cortical sinusoids.

Duodenum. A few focal areas of thrombosis in the post-capillary venules of the villi were found in three animals. Focal necrosis in the villi was observed in one rabbit.

Pancreas, spleen and CNS. No lesions were seen.

Meningococcal cell-wall blebs

In the experiments described above we were interested to know if the blebs reported by DeVoe and Gilchrist (1973) could be substituted for the purified LPS. Such blebs originate from the endotoxin-containing outer cell-wall layer. The blebs were first tested for their ability to induce a localised Schwartzman reaction when administered in doses equivalent to 10 μ g per kg of purified LPS. In all instances rabbits developed a haemorrhagic skin reaction within 3 h of the intravenous injection. When blebs were injected intravenously 24 h after a subcutaneous injection of leucocyte-egested material, the reactions in the rabbits were indistinguishable from those in which purified endotoxin was used (table).

Use of rabbit PMN instead of mouse PMN

In most of our experiments at the beginning of this work, rabbits were given subcutaneous injections of leucocyte-egested materials obtained from mouse peritoneal-exudate cells. This egested material by itself was toxic only in conjunction with endotoxin. However, the possibility was considered that the rabbits might have become sensitised to endotoxin by some non-specific inter-species interaction that might represent only a laboratory phenomenon unrelated to leucocyte exocytosis. Therefore, similar experiments were performed with rabbit peritoneal leucocytes. The results of these experiments were identical with those in which leucocytes from mice were used.

Similar experiments with Staphylococcus epidermidis

It was tempting to speculate from the above studies that the leucocytes released some toxic material from meningococci, perhaps a modified form of meningococcal endotoxin. Similar in-vitro phagocytosis experiments were carried out with ^{14}C -labelled *Staphylococcus epidermidis* in place of the meningococcus. Quantitative phagocytosis studies of this bacterium had been carried out previously in this laboratory (DeVoe, Storm and Gilchrist, 1973). As in the experiments with the meningococcus, leucocyte-egested materials appeared in the supernatant fluids. Six rabbits were given subcutaneous injections of the egested materials from a number of leucocytes comparable to that used above for meningococcus-loaded leucocytes, and after 24 h the rabbits were given meningococcal endotoxin (10 μg per kg). These animals remained healthy, and necropsy revealed only a few pinpoint-sized red spots in the lungs of two rabbits. It seemed likely that such small lesions were caused by the barbital used to kill these animals, and not by the egested material containing degraded staphylococci.

DISCUSSION

All evidence to date from in-vitro studies, including the results presented here, indicates that meningococci are engulfed, killed, and degraded in PMN whether from man, rabbits or mice (Roberts, 1967; DeVoe, Gilchrist and Storm, 1973; DeVoe, 1976). However, on these in-vitro findings alone it would be premature to rule out the possibility that PMN provide a temporary haven for meningococci *in vivo*. There is evidence (DeVoe, Gilchrist and Storm, 1973) that human PMN heavily laden with meningococci do undergo lysis. Such lysis could conceivably release viable bacteria.

Our experiments have shown that the material egested from 10^7 PMN is sufficient to sensitise rabbits to endotoxin as evidenced by the production of disseminated intravascular coagulation. In many studies on endotoxin, large quantities have been required to produce severe effects (Ducker and Simmons, 1968).

Lysosomal proteins are known to be excreted from phagocytic cells (Collins and Wood, 1959; Hirsch and Cohn, 1960; Cohn, 1963; Leffell and Spitznagel, 1974). In our experiments material egested from leucocytes loaded with *S. epidermidis* failed to sensitise rabbits to a dose of meningococcal endotoxin. However, degraded meningococci, either alone or in combination with substances originating from the leucocytes (e.g., lysosomal proteins) sensitised rabbits to the subsequent administration of endotoxin.

Filkins (1971) concluded that PMN do not detoxify endotoxin. We set out to determine whether PMN release endotoxin after degrading ingested meningococci. Our findings indicate that endotoxin is modified by PMN, but the material egested after intracellular digestion of the meningococcus was effective as a sensitising agent even though the endotoxin content could not have been greater than 0.5% of that required to sensitise rabbits for the classical Schwartzman reaction. Furthermore, the mechanism of action of the egested

materials appeared to differ from that of the sensitising dose of endotoxin in the classical localised Schwartzman reaction. In only two of more than 30 rabbits tested did a haemorrhagic spot appear at the original site of injection. The reaction differed also from the classical generalised Schwartzman reaction in rabbits in that death occurred after several days rather than after several hours.

The amount of endotoxin contained in the egested material and introduced into each rabbit seemed far too small to account, by itself, for the sensitisation phenomenon. It is possible that a modified form of endotoxin released from PMN was particularly "potent" in sensitising the rabbits. However, a more plausible explanation is that a combination of leucocytic and meningococcal substances in the egested material was responsible for sensitising the rabbits to endotoxin, although this hypothesis has yet to be tested.

We are at present investigating the sequence of events in the rabbit after the introduction of PMN-egested meningococcal materials, i.e., the events leading to sensitisation to endotoxin. Although direct proof is lacking, the accumulating evidence suggests a synergism between PMN and endotoxin in the production of the characteristic disseminated intravascular coagulation of fulminating meningococcal disease.

SUMMARY

Leucocyte-egested material was harvested after the quantitative in-vitro phagocytosis of *Neisseria meningitidis* by rabbit or mouse polymorphonuclear leucocytes. The egested material was injected subcutaneously into rabbits and followed 24 h later with an intravenous injection of what would by itself have been a non-lethal quantity of meningococcal endotoxin, or with an equivalent dose of endotoxin in the form of meningococcal cell-wall blebs. Of 32 rabbits treated in this manner, 12 developed disseminated intravascular coagulation and six of these 12 had renal cortical necrosis. The remainder exhibited less severe lesions resembling those of endotoxaemia. Rabbits were not sensitised to meningococcal endotoxin when materials egested from leucocytes containing *Staphylococcus epidermidis* were used. A description of the pathological findings in the rabbits is presented.

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FIG. 1.—Lungs and kidneys of a rabbit that died 36 h after an injection of LPS. The rabbit had been primed with an injection of material egested from leucocytes containing meningococci. There is developing haemorrhagic infarction in the left cardiac lobe (arrows). Light areas are emphysematous areas in oedematous and atelectatic pulmonary parenchyma. Kidneys show disseminated cortical necrosis.

FIG. 2.—Pulmonary oedema, heterophilic leucocytosis, and thrombosis in a venule. The rabbit was killed 18 h after the second injection (LPS). Haematoxylin and eosin. $\times 250$.

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FIG. 3.—Pulmonary venule with platelet thrombus. The rabbit was killed 18 h after the second injection (LPS). Phosphotungstic haematoxylin. $\times 250$.

FIG. 4.—Renal cortical necrosis with an inflammatory reaction and thrombosis of glomeruli and an efferent arteriole (arrow). The rabbit died 38 h after the second injection (LPS). Phosphotungstic haematoxylin. $\times 250$.