Effects of Soil Infection Potentiating Factors on Neutrophils in Vitro

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Although the infection-potentiating capacity of soil has long been recognized, it was not until the studies of Rodeheaver et al [*1*] in 1974 that soil silicate clay fractions were identified as the predominant infection-potentiating components. Of these clay fractions, montmorillonite clay, which has the largest surface area and highest cation exchange capacity, proved to be the most potent infection potentiator. Subsequent studies by Haury et al [*2*] demonstrated that leukocytes treated with montmorillonite clay were not able to phagocytose and kill bacteria. In addition, pretreatment of serum with the clay eliminated the serum bacteriocidal effect against gram-negative organisms. These investigators speculated that the clay exerted its adjuvant infectious effect by blocking leukocyte receptor sites that bind bacteria, thereby inhibiting bacterial ingestion. When Cohen et al [*3*] implanted sterile montmorillonite clay into experimental wounds, however, the wounds became inflamed and dehisced within 8 days. Clay-treated wounds showed histologic evidence of neutrophil infiltration and tissue necrosis even in the absence of bacteria. In this report, we describe experiments to further clarify the effects of montmorillonite clay on neutrophil function. We anticipated that the findings might be relevant to the larger question of how foreign bodies in general adversely affect host defenses and potentiate infection.

Material and Methods

Blood specimens were obtained from healthy volunteers by venipuncture, using heparin (10 units/ml) as an anticoagulant. Neutrophils were isolated by the method of Boyum [*4*], with contaminating erythrocytes being eliminated by brief hypotonic lysis. Erythrocytes were obtained by centrifugation to remove leukocytes as buffy coat cells. The medium used for washing and testing of the cells was Dulbecco's phosphate-buffered saline solution (Grand Island Biological Co., Grand Island, NY).

Montmorillonite clay homoionically saturated with magnesium chloride was supplied by one of us (GTR). Previous studies have shown that the type of surface-adsorbed cation is irrelevant to the inflammation-potentiating capacity of the clay [*1*]. To determine the effect of the clay on neutrophil cellular integrity, 10 mg were incubated with 5×10^6 human neutrophils in a total volume of 1 ml. Effects were determined both directly by phase contrast microscopic observation and by measurement of the cytoplasmic marker enzyme, lactate dehydrogenase, in the cell-free medium. Lactate dehydrogenase activity was measured using reagents in a kit for that purpose (Sigma Chemical Co., St. Louis, MO). To determine whether previous treatment of the clay with human serum might influence clay and neutrophil interaction, addtional experiments were conducted using aliquots of clay previously treated with human albumin or heat-decomplemented human serum (Sigma). Pretreatment of the clay with albumin or serum involved incubation of clay with these reagents at a concentration of 1 mg/ml on a rotator for a period of 30 minutes followed by washing the clay three times with saline solution.

Similar experiments were carried out with human erythrocytes using an optical density of 430 nM to measure free hemoglobin as an index of erythrocyte lysis. A million fresh saline-washed erythrocytes were first incubated with varying concentrations of clay suspended in 1 ml of suspension medium. The amount of hemoglobin released was then studied as a function of increasing amounts of clay (0.6 to 20 mg). As with the neutrophil studies, determinations were also carried out using aliquots of clay pretreated with human albumin or human serum.

To investigate the possibility that clay might influence serum complement activity, we examined the effect of clay on zymosan-activated serum. Complement activity in the

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Figure 1. Left, phase contrast photomicrograph of a neutrophil monolayer in suspension medium. Right, same preparation 10 minutes after the addition of clay. The cells have lysed and only their membranes remain.

latter was assessed as a function of its influence on neutrophil chemotaxis using the under agarose technique [5].

Both untreated clay and clay pretreated with human albumin were used to stimulate neutrophil chemiluminescence. The latter was measured using a Beckman scintillation spectrometer in the out-of-coincidence mode as previously described [6]. Each vial that contained neutrophils in 1 ml of fluid volume was counted for a 0.1 minute interval during each 5 minute counting cycle over a 45 minute period. Results were expressed as net counts per minute at the peak response.

Results

In the initial experiments to assess the influence of clay on neutrophil function, we observed macroscopically that the addition of clay to neutrophils suspended in tissue culture medium consistently produced rapid and complete cell lysis. Phase contrast photomicrographs (Figure 1) confirmed this observation. This finding led us to further investigate the cytotoxic influence of clay. To quantify clay-mediated lysis of neutrophils, we attempted to monitor the release of cytoplasmic lactate dehydrogenase into the cell suspension medium. Results of one such experiment are summarized in Figure 2. We observed that, under conditions that provided for virtually complete loss of neutrophil integrity, less than 8 percent of the total lactate dehydrogenase activity available was present in the supernatant fluid. The enzyme was found instead to have been adsorbed in active form onto the sedimented clay. That clay has an ability to adsorb lactate dehydrogenase was confirmed by its ability to effectively remove lactate dehydrogenase from the supernatant fluid of detergent-lysed cells. In an attempt to block adsorption of lactate dehydrogenase to the clay, we exposed the clay to albumin before adding it to neutrophils. Results of an experiment to determine the influence of albuminized clay on neutrophil integrity and lactate dehydrogenase recovery are summarized in Figure 2. We observed that pretreatment of the clay with 1 percent albumin provided for full recovery of lactate dehydrogenase in the supernatant fluid in association with complete physical disruption of the cells. In contrast, clay pretreated with 10 percent human albumin caused neither release of lactate dehydrogenase nor lysis of the cells.

To determine whether neutrophils might be uniquely susceptible to clay-mediated lysis, we also tested the effect of clay on erythrocyte integrity (Figure 3). Incubation of erythrocytes with clay released approximately 30 percent of the total hemoglobin available. Incubation of erythrocytes with clay pretreated with 1 percent albumin released approximately 20 percent of the total hemoglobin. We also observed macroscopically that neither the clay nor the albuminized clay effected complete lysis of the erythrocytes. To consider the possibility that clay might adsorb hemoglobin, reducing the amount of hemoglobin recovered in the supernatant fluid, we compared the amount of hemoglobin in supernatant

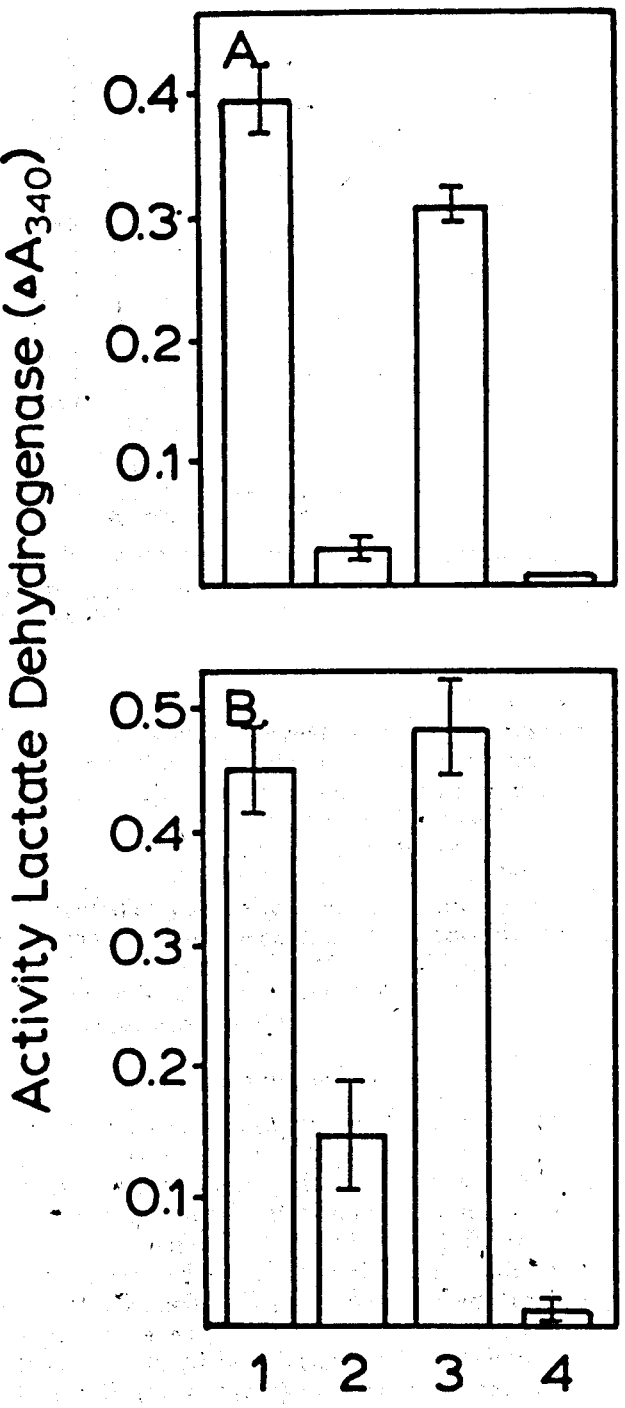

Figure 2. Release of neutrophil lactate dehydrogenase by clay lysis. A: Bar 1, total lactate dehydrogenase released by triton lysis; Bar 2, lactate dehydrogenase activity in supernatant fluid from cells incubated with untreated clay; Bar 3, lactate dehydrogenase activity associated with the sedimented clay; Bar 4, lactate dehydrogenase activity in supernatant after addition of clay to supernatant fluid from triton-lysed cells. B: Bar 1, total lactate dehydrogenase released by triton lysis; Bar 2, lactate dehydrogenase activity in supernatant fluid from cells incubated with untreated clay; Bar 3, lactate dehydrogenase activity in supernatant from cells incubated with 1 percent albumin-pretreated clay; Bar 4, lactate dehydrogenase activity in supernatant from cells incubated with 10 percent albumin-pretreated clay. A_{340} = absorption 340 nm.

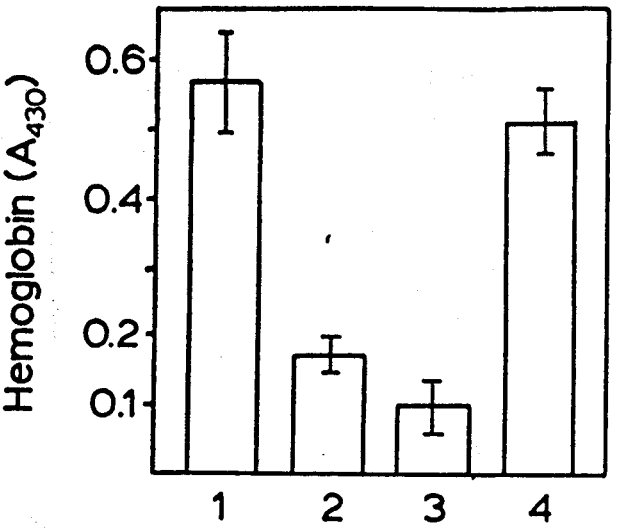

Figure 3. Effect of clay on release of hemoglobin from erythrocytes. Bar 1, total hemoglobin released by triton lysis; Bar 2, hemoglobin in supernatant fluid from cells incubated with untreated clay; Bar 3, hemoglobin in supernatant fluid from cells incubated with 1 percent albumin-pretreated clay; Bar 4, hemoglobin in supernatant after addition of clay to supernatant fluid from triton-lysed cells. A_{430} = absorption 430 nm.

fluid from detergent-lysed cells with the amount remaining after the addition of clay. No difference was observed in the hemoglobin concentrations of these supernatant fluids. Therefore, either erythrocytes are less susceptible to the disruptive influence of clay, or cytoplasmic proteins released from lysed cells effectively reduce the cytolytic property of the clay. That the latter may be true is evidenced by the virtual failure of clay pretreated with 10 percent serum to disrupt erythrocytes under comparable conditions [unpublished data].

The ability of clay to adsorb lactic dehydrogenase and albumin prompted us to consider the possibility that it might also adsorb activated complement or in some way influence complement activity. To investigate this hypothesis, zymosan-activated serum samples were pretreated with increasing concentrations of clay and then tested for neutrophil chemotactic activity (Figure 4). Compared with control values, there was an almost complete disappearance of chemotactic activity when the samples were treated with clay concentrations exceeding 0.6 mg/ml. We have no data on the ability of albuminized clay to influence chemotactic activity.

Chemiluminescence in response to stimulation with clay seemed to increase as the concentration of serum protein used to pretreat the clay decreased. Thus, although clay pretreatment with 5 percent human albumin abolished chemiluminescence, pretreatment with 0.1 percent albumin permitted a dramatic response (Figure 5). It is noteworthy that the degree of chemiluminescence stimulated by untreated clay was intermediate between emission

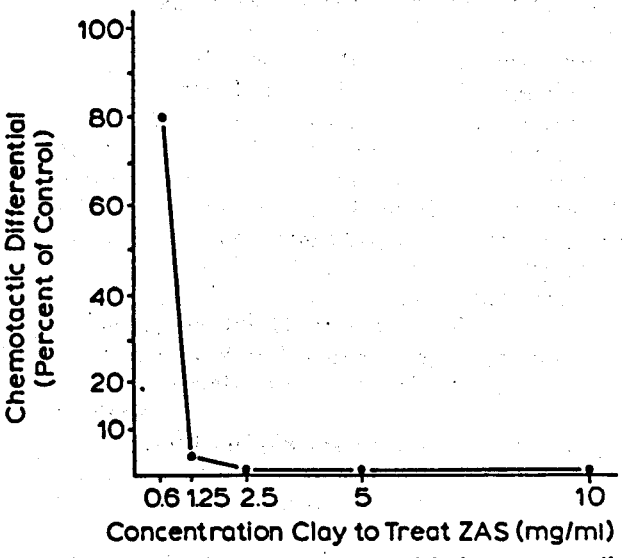

Figure 4. Effect of clay on complement activity in zymosan-activated serum. ZAS = zymosan-activated serum.

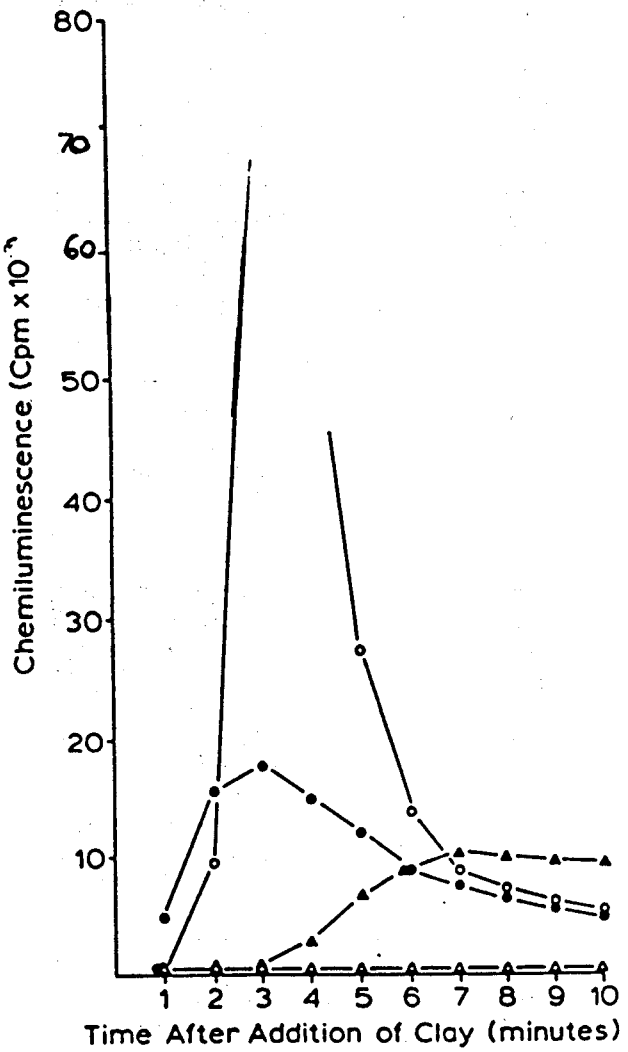

Figure 5. Effect of clay on neutrophil chemiluminescence. Closed circles indicate untreated clay, open circles indicate 0.1 percent albumin-pretreated clay; closed triangles indicate 1 percent albumin-pretreated clay; open triangles indicate clay pretreated with 5 percent albumin or 10 percent pooled human serum.

levels induced by clay pretreated with 0.1 percent albumin and that pretreated with 1 percent albumin. Presumably, as the degree of cellular protection afforded by serum protein treatment decreased, leukocytes were stimulated to increased levels of oxidative metabolic activity. The fact that stimulation with untreated clay actually produced a submaximal response may perhaps be explained by concomitant cell lysis. In other words, the stimulated leukocytes were destroyed by the clay before they could achieve maximum respiratory burst.

Comments

This study has shown that montmorillonite clay, the major infection potentiating fraction of soil, rapidly lyses neutrophils and erythrocytes in vitro. Furthermore, it can stimulate chemiluminescence, the neutrophil oxidative metabolic burst. Pretreatment of the clay with serum or albumin tended to prevent or delay cell lysis, presumably by adsorbing to the clay surface and serving as a buffer between the clay and cells. Although the precise mechanism by which cell lysis occurs is uncertain, the rapidity with which it proceeds suggests a direct toxic effect on the cell membrane before phagocytosis. Since montmorillonite clay is known to have a huge surface area (770 M^2/g) and high cation exchange capacity (90 mEq/100 g) [2], toxicity may be mediated by electrostatic mechanisms. Nash et al [7] have suggested that the cytotoxic effects of silica are due to the capacity of polymeric silicic acid, a product of silica dissolution, to form hydrogen bonds with phospholipids in the cell membrane, thereby modifying membrane surface tension and permeability. The adsorption of serum proteins onto clay may effectively neutralize it by forming so many uniformly strong hydrogen bonds that the complex becomes stable. Indeed, the polymer compound poly-2-vinyl-pyridine-N-oxide adsorbs to the surface of colloidal silica and exerts a cytoprotective effect by just such a mechanism.

These findings may help to explain the infection-potentiating properties of the clay. In a recent monograph [8], two of us proposed that by activating host defenses, reactive foreign bodies such as montmorillonite clay may actually subvert these defenses into injury of the host itself. The extracellular release of lysosomal enzymes attending leukocyte lysis by clay, for example, is presumably toxic to tissue, a phenomenon parallel to tissue injury occurring in autoimmune vasculitis, arthritis, and pneumonitis. In addition to injuring tissue, extruded lysosomal enzymes are also capable of activating the chemo-

tactic complement fraction C5a, thereby attracting more phagocytes. Since high gradients of chemoattractants can trigger degranulation, it may be possible for a self-amplifying cycle of neutrophil chemotaxis, degranulation or lysis, and further chemotaxis to be established, especially if the triggering mechanism (the clay) is not eliminated in the process. The end result would be tissue necrosis, that is, the creation of an environment favorable for bacterial survival. Such a concatenation of events would partly explain the infection-potentiating ability of montmorillonite clay and perhaps of other reactive materials as well [8]. Obviously, the direct cytotoxic effects of such materials on phagocytes may be important as well, since dead phagocytes cannot kill bacteria.

The ability of plasma proteins to suppress clay-mediated cytotoxicity was expected. Surface adsorption of plasma proteins, especially albumin and gamma globulin, appears to minimize phagocyte adhesion to foreign bodies, and many biocompatible foreign bodies bind albumin irreversibly [8]. This buffering effect may have been particularly well demonstrated by our experiments with clay-induced chemiluminescence. The latter was virtually abolished by pretreatment of the clay with 10 percent serum, but compared with untreated clay was enhanced by 0.1 percent albumin pretreatment. If precoating with 0.1 percent albumin approximates conditions in soil-contaminated wounds, plasma protein adsorption onto clay introduced through trauma may paradoxically augment host injury by allowing the respiratory burst and free radical generation to occur before cell lysis. Highly reactive free radicals such as superoxide anions or singlet oxygen acting outside the cell are known to be toxic to tissue [9]. The in vivo toxicity of montmorillonite clay, therefore, may derive from its ability to cause the release from phagocytes of both free radicals and lysosomal enzymes. Interestingly, Cohen et al [3] have shown that montmorillonite clay can be found within foreign body giant cells. Perhaps these cells destroy themselves by ingesting albumin-buffered clay particles and then digesting their protective protein coats in the phagosome. Such a process might occur repeatedly.

Although erythrocytes do not play a direct role as part of the host cellular defense mechanisms, hemolysis by reactive foreign bodies such as montmorillonite clay may potentiate infection through the release of free hemoglobin. The latter has been shown to promote bacterial proliferation [10]. Certain degradation products of hemoglobin may also inhibit neutrophil chemotaxis as well as bacterial phagocytosis and killing [11,12].

Our finding that clay can abolish the chemotactic activity of zymosan-activated serum is of uncertain significance. Clearly, such an event occurring in vivo might compromise the formation of a C5a concentration gradient and thus actually suppress the overall inflammatory response. Alternatively, complement adsorbed to clay might provide a long-term repository of active C5a and thus promote chronic inflammation. The latter notion would certainly be more in accord with clinical findings. It is also possible that clay selectively adsorbs albumin or other proteins in vivo leaving activated complement relatively unaffected. Activated complement adsorbed to clay, of course, would probably be unavailable for bacterial opsonization.

Summary

Although the ability of soil silicate fractions to potentiate infection is well recognized, the precise mechanisms by which they do so remain unexplained. This study was carried out to investigate the effects of montmorillonite clay, the most potent of these soil infection potentiators, on human neutrophils, erythrocytes, and serum complement in vitro. Using phase microscopy, rapid neutrophil lysis was observed when cells were exposed to untreated clay. After lysis, the cytoplasmic marker enzyme lactate dehydrogenase rapidly adsorbed to the surface of the clay. Both enzyme surface adsorption and cell lysis could be blocked, however, by pretreatment of the clay with human albumin. Likewise, neutrophil chemiluminescence could be stimulated by untreated clay, but not by clay pretreated with 5 percent albumin or 10 percent pooled human serum. Maximal chemiluminescence was stimulated by clay pretreated with 0.1 percent albumin, probably because the partially protective albumin coating delayed cell lysis. Compared with the effect on neutrophils, clay lysis of erythrocytes was incomplete. When zymosan-activated serum samples were exposed to clay, complement activity as measured by neutrophil chemotaxis was suppressed in a dose-dependent fashion. We conclude that montmorillonite clay may potentiate infection by a direct cytotoxic effect on the neutrophil, making it unavailable for bacterial phagocytosis, by local reduction in bacterial opsonization due to depletion of activated complement, and by the release of toxic tissue substances, such as lysosomal enzymes and oxygen free radicals, from leukocytes which may damage host tissue and thus create an environment favorable for bacterial survival.

References

1. Rodeheaver GT, Pettry D, Turnbull V, et al. Identification of the wound infection-potentiating factors in soil. Am J Surg 1974;128:8–14.
2. Haury BB, Rodeheaver GT, Pettry D, Edgerton MT, Edlich RF. Inhibition of nonspecific defenses by soil infection potentiating factors. Surg Gynecol Obstet 1977;144:19–24.
3. Cohen IK, Diegelmann RF, Wise WS. Biomaterials and collagen synthesis. J Biomed Mater Res 1976;10:965–70.

4. Boyum A. Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 1968;21(suppl 97):77–89.
5. Nelson RD, Fiegel VD, Herron MJ, Simmons RL. Chemotactic deactivation of human neutrophils: relationship to loss of cytotoxin receptor function and temporal nature of the phenomenon. J Reticuloendothel Soc 1980;28:285–94.
6. Nelson RD, Herron MJ, Schmidtke JR, Simmons RL. Chemiluminescence response of human leukocytes: influence of medium components on light production. Infect Immun 1977;17:513–20.
7. Nash T, Allison AC, Harington JS. Physico-chemical properties of silica in relation to its toxicity. Nature 1966;210:259–61.
8. Dougherty SH, Simmons RL. Infections in bionic man: the pathobiology of infections in prosthetic devices. Part I. Curr Probl Surg 1982;19:218–64.
9. Babior BM. Oxygen-dependent microbial killing by phagocytes. N Engl J Med 1978;298:659–68.
10. Eaton JW, Brandt P, Mahoney JR, Lee JT Jr. Haptoglobin: a natural bacteriostat. Science 1982;215:691–3.
11. Hau T, Nelson RD, Fiegel VD, Levenson R, Simmons RL. Mechanisms of the adjuvant action of hemoglobin in experimental peritonitis. 2. Influence of hemoglobin on human leukocyte chemotaxis in vitro. J Surg Res 1977;22:174–80.
12. Hau T, Simmons RL. Mechanisms of the adjuvant effect of hemoglobin in experimental peritonitis. 3. The influence of hemoglobin on phagocytosis and intracellular killing by human granulocytes. Surgery 1980;87:588–92.