Action of Glycine and a Lysozyme-like Agent from Rabbit Monocytes in Destruction of Brucella.* (25874)

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Results of studies carried out with vaccine strain of *Brucella melitensis* Rev. Is suggest that death of these bacteria may be produced in stepwise fashion by action of enzyme on its wall, but only after the substrate has been made available by an agent which causes preliminary damage. This study summarizes experiments which indicate that normal rabbit monocytes contain a lysozyme-like material capable of attacking Brucella cells. Used alone, this agent exhibits no inhibition of living Brucella, presumably because the specific configuration of cell wall prevents its action. When the enzyme is combined with the amino acid, glycine, death and lysis take place. Evidence is presented to indicate that a similar reaction may take place intracellularly, in monocytes parasitized with Brucella, when tissue culture medium is supplemented with sufficient amounts of glycine. These studies place emphasis on the possible role of similar agents, which, under physiological conditions existing in intact animal, may contribute to inhibition of growth and ultimate disposal of organisms responsible for Brucellosis.

Methods. Bacterial cultures. Smooth cultures of *B. melitensis* Rev Is(1) were maintained on Albimi Brucella agar slants. For tests of enzyme action on killed cells, growth was harvested after 3 days at 37°C into saline or Zobell's solution ($K_2HPO_4 \cdot 3H_2O$, 1.31 g; KH_2PO_4, 0.75 g; NaCl 2.5 g; $MgSO_4 \cdot 7H_2O$, 0.10 g, $CaCl_2 \cdot 2H_2O$, 0.0056 g; Cysteine-HCl, 0.10 g, H_2O, distilled, 1 liter; pH 7.0-7.4). Suspensions containing approximately 10^{10} bacteria/ml were heated 2 hr at 60°C, washed in saline, centrifuged, and the pellets treated with N-butanol at room temperature for ½ hr and sometimes overnight at 4°C. Butanol was removed; cells were resuspended in saline and stored in the cold. For tests on living Brucella, cells were harvested either from shaken cultures in Albimi broth at 37°C or from young growth on Albimi agar (14-24 hrs). For infection of monocytes, cells were harvested after 1-3 days on Albimi agar, suspended in Tyrode's solution to approximately 10^9/ml, and diluted in tissue culture medium. *Preparation of normal rabbit monocytes.* Peritoneal exudates were collected 5 days after injection of 40-50 ml Klearol into 3-5 lb rabbits. Previous experiments have shown that such exudates contain 85% or more monocytes(2). The peritoneal cavities were washed with either saline or Tyrode's, and exudate cells were aspirated through sterile cheese cloth filters into separatory funnels. The oil was discarded; cells were centrifuged 10 minutes at approximately 250 g in the cold, washed once in saline or Tyrode's, counted in Petroff Hausser Chamber, and used for extraction or for monocyte infection experiments. In the latter case, cells were harvested in Tyrode's solution. In general, extreme washing was avoided, as was treatment with proteolytic enzymes, to reduce chances of removing active factors. *Monocyte maintenance medium.* Parasitization of monocytes and maintenance was carried out in medium composed of 40% fresh, unfiltered, normal rabbit serum and 60% Tyrode's, made as follows: NaCl, 7.7 g; KCl, 0.2 g; NaH_2PO_4, 0.05 g; $MgSO_4$, 0.1 g; Dextrose, 4 g; phenol red, 1 ml of 1% solution; distilled water, 990 ml. After autoclaving, sterile $NaHCO_3$ was added to final concentration of 0.5 g and $CaCl_2$ to final concentration of 0.14 g. When Tyrode's was used for harvesting monocytes, calcium was omitted. pH was adjusted to 7.0-7.4 with sterile H_3PO_4. Monocyte cultures were set up in 25 ml Erlenmeyers layered with 2 to 3 ml medium containing parasitized monocytes. On some occasions solutions were distributed into 12 ml

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TABLE I. Effect of Monocyte Extracts from Normal and Immune Rabbits on Heat-Killed, Butanol-Extracted Brucella.

Source	Dilution of extract	% lysis, 18 hr 37°	Viscosity	Reaction with Alcian Blue	Residual cells remaining after treatment with extract: % lysis upon addition of crystalline lysozyme, 40 μg/ml	Residual cells remaining after treatment with extract: Viscosity
Immune	1/40	21	+	+	0	—
	1/80	25	+	+	0	—
	1/160	24	+	+	0	—
	Control	6	—	—	24	+
Normal	1/40	25	+	+	not tested	not tested
	1/80	24	+	+	*idem*	*idem*
	1/160	24	+	+	"	"

conical centrifuge tubes and incubated slantwise. Viability of monocytes was ascertained by staining suspensions with eosin, after method of Hanks(3).

Results. Preparation and properties of a lysozyme-like agent from rabbit monocytes. The agent may be extracted from normal and immune rabbit monocytes by procedures whereby the cells are broken open by freeze-thaw or Mickle disintegration technics. Usually active material has been obtained from extracts prepared by freeze-thawing 2 to 5 $\times$ 10^8 monocytes in 5 to 10 ml N/1 or N/10 acetic acid solution, pH 3.6, mixed with equal parts of physiological saline, as used by Amano, *et al.*(4); by freeze-thawing of monocytes that had been stored in Tyrode's solution for 1-2 weeks at 4°C; or by freeze-thawing in "intracellular salts" solution, according to procedure of Hirsch(5). An active material has also been obtained by Mickle disintegration in phosphate buffer at pH 7.0 or in distilled water, but these preparations lose activity on storage. Extracts were adjusted to pH 6.8 to 7.0 with NaOH; precipitates and debris were removed by centrifugation. Yields from 2-3 $\times$ 10^8 monocytes exhibited lysozyme activity for *M. lysodeikticus* comparable to concentrations of crystalline egg white lysozyme (Worthington) in the order of 500 μg.

Action on Brucella cells. Heat-killed and Butanol-, Acetone-, or Chloroform-Extracted. Cells suspended in phosphate buffer at pH 8.0 on treatment with the extract undergo reduction in turbidity (Table I); the extent varies with the particular cell preparation. This is accompanied by release of a viscous material, which is destroyed by crystalline DNAse, indicating it is bacterial DNA. Crystalline lysozyme causes a similar reaction. Trypsin, chymotrypsin, and pancreatic lipase also cause reduction in turbidity, but, in these cases, cause no release of viscous material. When washed out of the monocyte extract or lysozyme reaction mixture and resuspended in distilled water, the cell residue is not osmotically fragile, in contrast to results obtained with living cells. Table I also indicates that extracts prepared from exudates produced in Rev Is immunized rabbits contain comparable amounts of the agent. The viscous material released from cells reacts with Alcian Blue dye to form a stringy precipitate.

Action on living Brucella cells. The material in monocyte extracts has no killing or lytic effect when tested alone in living cells, either in Albimi media, or in phosphate or veronal buffers, pH 7.0 to 8.0. When combined with glycine, an accelerated lysis and death takes place (Fig. 1). This effect takes place more rapidly with fast growing, young cells, than with stationary phase preparations. Rough cells appear to be more susceptible than smooth, and tests with a virulent strain *B. melitensis* indicate that cells of a comparable age are more resistant than either rough or smooth Rev Is. With living cells, the monocyte extract also brings about a release of bacterial DNA; lysozyme produces a similar effect. This response is not observed with the following enzymes: pancreatic lipase, trypsin, chymotrypsin, RNAse, DNAse or

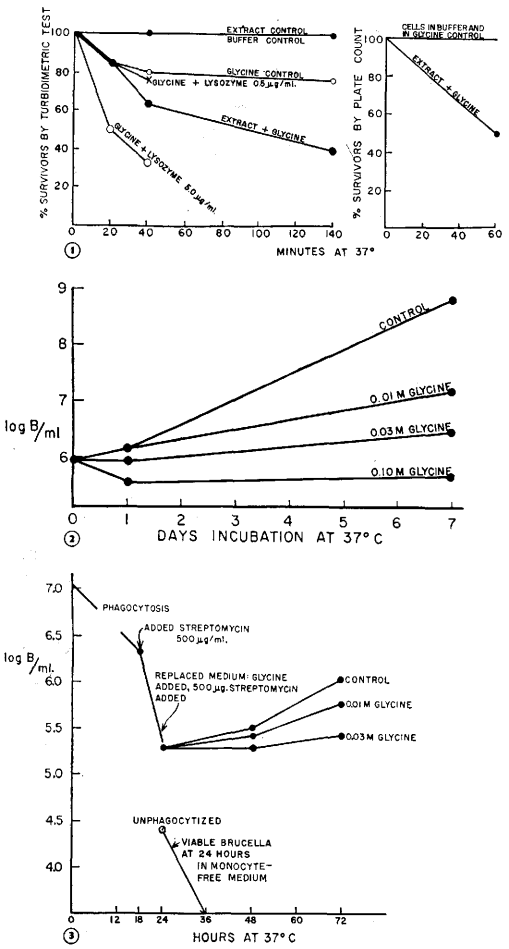

FIG. 1. Effect of glycine, monocyte extract or lysozyme, and their combination on smooth cells of Rev 1s, in buffer-Tyrode's solution, at pH 6.2.

FIG. 2. Intracellular brucella in normal rabbit monocytes treated with varying amounts of glycine, in Tyrode's serum medium.

FIG. 3. Intracellular multiplication of Rev 1s in rabbit monocytes treated with varying concentrations of glycine in presence of 500 µg/ml streptomycin.

D-amino acid oxidase. This suggests that the monocytic agent is a cellular lysozyme. Other properties which suggest its lysozyme-like nature include: (1) its resistance to trypsin, (2) heat stability, (3) action on *M. lysodeikticus*, and (4) its action on cell wall preparations of Brucella, *Micrococcus lysodeikticus*, and *S. aureus*, all containing the mucopolysaccharide substrate for lysozyme. Further details of these studies will be published.

Action of glycine on growth of Rev 1s in monocytes in tissue culture medium. To determine whether glycine might also act together with the lysozyme-like agent in parasitized monocytes, a suspension of 1×10^6/ml of these cells was exposed to viable bacteria, and, after phagocytosis, the infected monocytes were washed and resuspended in fresh medium, to which was added varying concentrations of glycine, ranging from 0.01 to 0.3 M (Fig. 2). In one set of cultures, streptomycin was included to prevent extracellular multiplication of bacteria. Fig. 3 shows effects of streptomycin treatment on yields of intracellular bacteria. The data suggest that glycine exerts some action on the monocyte culture, in that growth of bacteria is reduced in presence of glycine. In other experiments, addition of 0.03 M glycine completely prevented bacterial multiplication in parasitized monocytes. Examination of stained preparations revealed extensive multiplication in untreated control cells. Concentrations of glycine used exerted no deleterious effects on monocytes themselves.

Discussion. These results suggest that extracts of monocytes contain an agent which resembles a lysozyme. The living Brucella is very resistant to the agent, but, when treated with glycine, a damage occurs which then allows the extract to lyse the cells. This indicates that, within the living monocyte, the agent can operate only when a similar damage takes place. It is possible that glycine plays a role similar to versene in that it chelates essential metal ions and renders the Gram-negative wall susceptible to lysozyme, in a manner observed with other bacteria(6). On the other hand, it may act by disturbing wall synthesis, thereby exposing the substrate in a form accessible to the agent. Glycine has been observed to produce protoplast-like structures of Brucella by Gerhardt(7) perhaps by this mechanism. Gary, *et al.*(8), working with *B. abortus* reported that addition of amino acid supplements, especially containing DL-valine and L-lysine, tend to inhibit total polysaccharide synthesis in growth media containing glucose, yeast autolysate, and casein hydrolysate. The work of Shockman, *et al.*(9), on conditions leading to cell lysis of *S. faecalis*, also suggests that the

physiological balance of growth medium may lead to imbalance of wall synthesis and cell lysis. Related effects may be occurring in those intracellular systems which prevent multiplication of Brucella, thereby rendering the bacteria more accessible to intracellular enzymes. The results of these experiments suggest that *in vitro* attempts to reconstitute intracellular conditions must take into account the necessity for establishing the proper physiological test environment; that death of cells may be a stepwise series of reactions; and that omission of any of the necessary components may result in failure to demonstrate the factors concerned. Operation of a mechanism similar to that produced by glycine in monocytes may be involved in natural resistance to infection. It is non-specific and perhaps dependent upon day-to-day variations in physiological levels of at least 2 agents.

Summary. A lysozyme-like material from rabbit monocytes is described. It acts on glycine-treated brucella to cause lysis and death. When parasitized monocytes containing this agent are treated with low concentrations of glycine, intracellular growth and yields of smooth *Brucella melitensis,* Rev Is is suppressed. The theory is proposed that intraphagocytic death involves a stepwise alteration of the wall, followed by enzymatic degradation of the mucopolysaccharide component responsible for structural integrity of the bacteria.

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Effect of Metabolic "Stress" on Development of Tumor Following Inoculation of Walker Carcinosarcoma Cells.* (25875)

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The effect of dietary deficiency in production of spontaneous tumors has been well described by Tannenbaum and Silverstone(1) who demonstrated that prolonged restricted caloric intake and malnutrition in laboratory animals will diminish induction and growth of spontaneous tumors. However, to our knowledge the influence of acute metabolic changes has not been investigated in relationship to increased susceptibility of rats to inoculation of transmissible malignant tumor cells. In this present investigation the effect of "acute" metabolic changes, such as starvation and dehydration, which can be considered a form of metabolic "stress," have been studied in relation to the animals' susceptibility to inoculation of Walker 256 carcinosarcoma.

Method. Female Holtzman white rats weighing between 215 and 230 g were divided into 5 groups and housed in the same room with identical diet (Purina lab-chow) for 3 weeks. They were weighed every day to be certain a similar weight gain was obtained in all animals before experiment was started. At end of this period, the groups were treated as follows: In *Group 1* water bottles were removed for 48 hours but "chow" feed was still available; in *Group 2* food was taken away

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