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Inflammation and Tissue Injury II. Local Release of Lysosomal Enzymes During Mixed Bacterial Infection in the Skin of Rabbits.* (32315)

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It has been shown(1) that the degree of local tissue injury (*e.g.*, increased vascular permeability, hemorrhage, etc.) in response to infection was dependent upon the presence of polymorphonuclear leukocytes. Normal and leukopenic (produced with nitrogen mustard) rabbits were inoculated intradermally with "viable" and "heat-killed" human dento-gingival plaque. In normal animals, both viable and heat-killed bacterial suspensions produced marked inflammatory reactions culminating in local abscess formation. On the other hand, in leukopenic animals vascular permeability in response to viable bacterial plaque suspensions was only about half that seen in normal animals. Hemorrhage was minimal or absent. This difference between the response in normal and leukopenic rabbits was even more dramatically illustrated following injection of heat-killed plaque material. No detectable response could be seen in the skin of the leukopenic rabbits, even after 24 hours. At the ultrastructural level, infiltrating PMN-leukocytes in normal animals had phagocytosed the injected materials and subsequently underwent degranulation and lysis. Leukopenic animals showed little or no cellular activity. The process of phagocytosis, degranulation and cell lysis in PMN-leukocytes has been associated with the extracellular release of lysosomal hydrolases(2,3). Thus, it was speculated that lysosomal enzymes, especially proteases(4), played an important role in the type of tissue injury observed in the normal animal. If this reasoning was correct, then one would expect to find changes indicative of extracellular release of lysosomal enzymes in the area of tissue injury. The following experiments were designed to test this hypothesis.

Methods. Bacterial plaque adjacent to the gingival margin was obtained from humans with clinically normal and diseased gingiva. This material was pooled, weighed (wet weight), and suspended in saline to provide a concentration of 35 mg/ml. A portion was placed in a boiling water bath for one hour to provide the "heat-killed" bacterial suspension. The abdomen of 14 normal and 20 leukopenic rabbits (2-3 kg) was shaved prior to the start of each experiment. Each animal received an injection of viable plaque (0.2 ml) and heat-killed plaque (0.2 ml) on either side of the mid-line. Leukopenia was induced by administration of nitrogen mustard as outlined previously(1). Twenty-four hours after local inoculation, animals were killed with sodium pentobarbital and the injection sites were excised. An uninjected portion of abdominal skin was excised to serve as control. Each sample was minced with scissors and immediately placed into 5 ml of a tissue culture medium consisting of tyrode buffer-Eagle's medium, 500 units Penicillin, 750 μg Streptomycin, 25 μg Fungizone, 50 μg Colimycin and 10% Seitz-filtered normal rabbit serum. The tubes were stoppered and placed in a 37°C water bath for 18 hours, following which the

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mixture was filtered through Whatman No. 40 filter paper. It was assumed that free enzyme had been eluted from the tissue and would be found in the filtrate. Enzymatic activity was expressed as a function of the dry weight of the excised tissue after being dried in a 65°C oven for 5 days.

The filtrates were assayed for *protease activity* by a modification(5) of Anson's method (6). Either 2% denatured hemoglobin (Worthington Biochemical Corp., Freehold, N. J.) or 3% bovine plasma albumin (Armour Pharmaceutical Co., Kankakee, Ill.) was used as substrate. The filtrate and substrate were added to 0.1 M citrate buffer (pH 4) and incubated for 18 hours at 37°C. The reaction was stopped by adding 2 ml of 10% trichloracetic acid (TCA) and filtered through Whatman No. 42 filter paper. The TCA-soluble products in the filtrate were measured by their absorption at 280 mμ in a Zeiss PQM II spectrophotometer.

Beta-glucuronidase activity was measured by a modification of Fishman's technique(7). The test mixture consisted of 0.1 ml of 0.01 M phenolphthalein-glucuronic acid (substrate) (Sigma Chem. Co., St. Louis, Mo.), 0.1 ml of the tissue extract and 0.8 ml of 0.1 M acetate buffer (pH 4.5). After incubation for 1 hour at 37°C, 1 ml of 5% TCA was added to stop the reaction and each mixture was filtered through Whatman No. 42 filter paper. The amount of phenolphthalein liberated was measured at 550 mμ within 5 minutes after addition of 2.0 ml glycine buffer (pH 11.2).

Acid phosphatase activity was measured by a modification of the technique of Bessey *et al* (8). The substrate consisted of disodium-p-nitrophenol phosphate (Sigma Chem. Co., St. Louis, Mo.) in a citric acid buffer (pH 4.8). After incubation at 37°C for 1 hr the reaction was stopped by addition of 0.1 N sodium hydroxide and the optical density was measured at 410 mμ.

The results were expressed in enzyme units. One enzyme unit produced an optical density of 1×10^{-5} per hour of incubation at 37°C per mg dried weight of skin.

Results. The results are summarized in Table I. Fig. 1 represents the results of the hemoglobin assays. The injection of viable bacteria in normal animals caused an average increase in proteolytic activity that was almost 2-fold that of the control sites. In the leukopenic rabbits only a slight rise in proteolytic activity was observed, the mean values being relatively similar to that of uninoculated skin in the normal animals.

The effect of heat-killed plaque injections in normal animals also resulted in a substantial increase in proteolytic activity, although not as marked as that observed with viable bacteria. Leukopenia did not appear to suppress proteolytic activity in response to heat-killed plaque when hemoglobin was the substrate.

Albumin assays were performed in order to demonstrate that the proteolytic effect was

TABLE I. Enzymatic Activity in Uninoculated Skin Sites and Those Injected with Viable or Heat-Killed Bacterial Plaque in Normal and Leukopenic Rabbits.

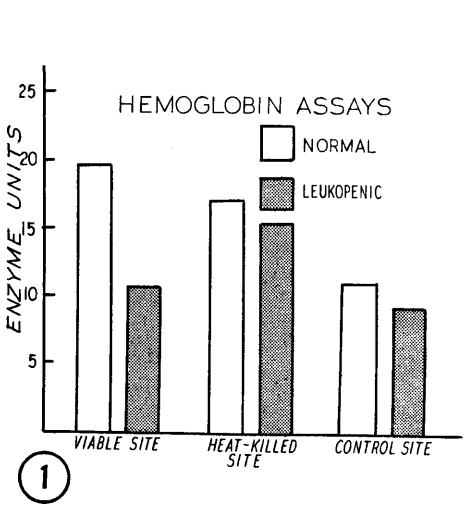
		Normal			Leukopenic			
Assay	Inoculation	No.	Mean	S.E.	No.	Mean	S.E.	Signif.*
Protease (hemoglobin)	Viable	9	19.72	3.37	12	10.71	1.57	Yes
	Heat-killed	9	16.87	2.17	12	15 27	2.23	—
	Control	9	11.00	1.80	12	9.32	1.15	—
Protease (albumin)	Viable	4	2.77	.95	5	1.51	.73	—
	Heat-killed	4	2.88	.90	5	1.61	.24	—
	Control	4	1.64	.57	5	.87	.24	—
Beta glucuronidase	Viable	9	21.01	3.82	11	2.77	1.23	Yes
	Heat-killed	9	12.87	3.29	9	3.03	.42	Yes
	Control	7	3.06	1.37	6	2.83	.38	—
Acid phosphatase	Viable	8	5.33	.78	6	3.70	.41	—
	Heat-killed	7	5.02	.88	6	4.83	.46	—
	Control	8	3.90	.55	6	3.40	1.34	—

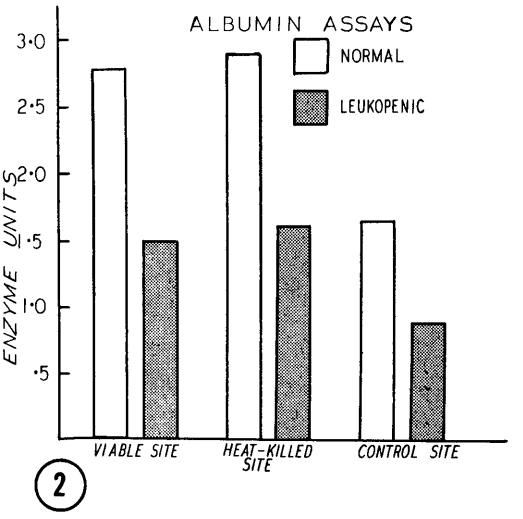
* Statistically significant difference between normal and leukopenic rabbits using a "t" test.

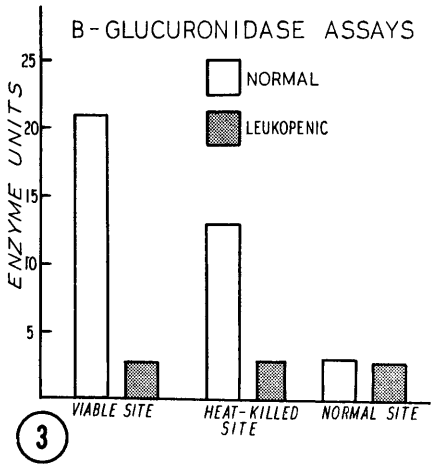
not specific for hemoglobin. As can be seen in Fig. 2 the optical density readings for albumin were considerably lower than those obtained with hemoglobin as the substrate. However, the general trend was similar to that observed with hemoglobin.

β-glucuronidase activity in infected sites is shown in Fig. 3. Where viable bacteria were injected in normal animals β-glucuronidase activity was dramatically elevated to approximately 7-fold that found in the normal skin control. Heat-killed plaque also increased the enzyme activity considerably but not to the same extent as in viable sites. In extracts from leukopenic animals, both viable and heat-killed bacterial infections showed little change in enzyme activity as compared to controls.

Fig. 4 represents the results of the acid phosphatase assays. As was obvious in all the assays, inoculation by viable and heat-killed







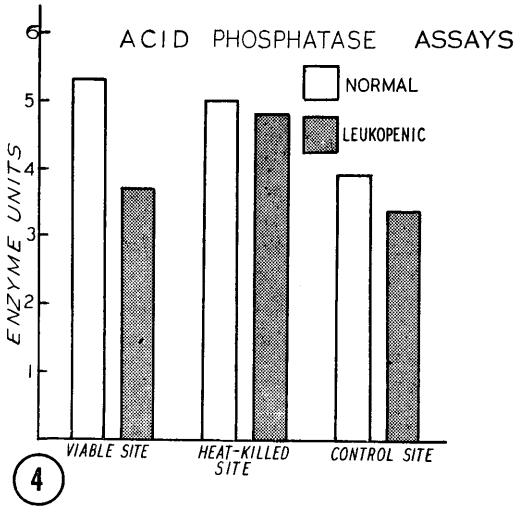


FIG. 1. Protease activity in uninoculated skin sites and those injected with viable or heat-killed bacterial plaque in normal and leukopenic rabbits (2% hemoglobin as substrate).

FIG. 2. Protease activity in uninoculated skin sites and those injected with viable or heat-killed bacterial plaque in normal and leukopenic rabbits (3% albumin as substrate).

FIG. 3. Beta-glucuronidase activity in uninoculated skin sites and those injected with viable or heat-killed bacterial plaque in normal and leukopenic rabbits.

FIG. 4. Acid phosphatase activity in uninoculated skin sites and those injected with viable or heat-killed bacterial plaque in normal and leukopenic rabbits.

bacteria caused the acid phosphatase levels to be elevated in the normal animals. In the leukopenic rabbits the viable infection showed little or no change as compared to the control. However, differences between normal and leukopenic animals were less sharply defined with this enzyme.

Discussion. It was evident from these results that marked differences in enzyme activity exist between bacterial lesions in normal and leukopenic animals. The uninoculated control sites indicated that a slight suppression of enzymatic activity occurred in the leukopenic animals. If one assumes that nitrogen mustard exerts no other relevant effect than inducing a leukopenia, then these gross differences in enzyme activity must primarily be due to the presence or absence of leukocytes in the lesions. We concede that enzymes derived from bacteria may be a contributing factor *in vivo;* however, in the *in vitro* system living micro-organisms were eliminated by the presence of antibiotics. This was substantiated by streaking random samples of the filtrates onto nutrient agar plates. No aerobic growth was evident after incubation at 37°C.

It seems reasonable to assume that the lysosomes of PMN-leukocytes(10) are the major factor in accounting for the elevation of hydrolytic activity. Ultrastructural evidence, as previously described(1), indicated that these were the predominant cell type in lesions taken from normal animals. No obvious inflammatory cell activity was observed in leukopenic rabbits injected with either viable or heat-killed bacterial suspensions. Further, analysis of these lesions by Ouchterlony gel diffusion indicated that PMN-leukocyte lysosomal material was demonstrable only in experimental lesions taken from normal animals(9). Thus, the rise in enzyme activity in response to bacterial infection was intimately related to the presence and subsequent lysis of PMN-leukocytes. Basic proteins or polypeptides(11), which would act to increase vascular permeability, and a variety of hydrolytic enzymes, capable of degrading various tissue components including proteins, would be liberated by degenerating polymorphs. The result is local tissue injury.

PMN-leukocyte hydrolases have been implicated in the mediation of numerous other forms of inflammatory tissue injury: passive cutaneous anaphylaxis in guinea pigs(12) and rats(13), the Arthus phenomenon(14,15) and the local Shwartzman reaction(16,17). Other studies have indicated that acid proteases are the crucial mechanism whereby tissue breakdown occurs in various inflammatory states(4, 18,19,20,21).

In infection there has been a tendency to stress the role of noxious factors emanating from bacteria as being primary mediators of tissue injury. The result of this study indicates that inflammatory cells of the host also play an important role.

An unanticipated finding was the elevation of protease activity (hemoglobin substrate) at sites injected with heat-killed plaque in leukopenic animals. This would indicate that other cells, perhaps tissue histiocytes, fibroblasts, etc. may be elaborating and releasing proteases in response to local infection. On the other hand, viable bacteria might have destroyed such cells in these animals and therefore elevated enzyme levels could not be demonstrated.

Summary. Viable and heat-killed dentogingival bacterial plaque was injected into the skin of normal and leukopenic rabbits. Increased levels of various lysosomal hydrolases (acid protease, β-glucuronidase and acid phosphatase) were demonstrated in lesions taken from normal animals, but, in general, not from leukopenic rabbits. It was suggested that tissue injury may be mediated by the release of lysosomal enzymes from degenerating PMN-leukocytes.

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A Quantitative Assay for Avian Myeloblastosis Virus.* (32316)

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Various methods for the assay of avian myeloblastosis virus (AMV) have been reported in the literature. Direct counts of virus particles with the electron microscope and assays of virus-associated adenosine triphosphatase have been used by Mommaerts *et al* (1) and by Beaudreau and Becker(2). Tissue culture techniques for the assay of infectious AMV include the interference test with Rous sarcoma virus and the fluorescent focus test described by Vogt and Rubin(3). The neoplastic potential of AMV has so far been assayed only with endpoint procedures performed *in vivo* or *in vitro*(4,5,6). Since endpoint techniques lack precision and economy, it was desirable to design an assay based on enumeration of individual lesions caused by the oncogenic action of the virus *in vitro*. Such an assay is described here.

Materials and method. The stock of AMV used to infect chicken yolk sac and bone marrow cultures consisted of plasma obtained from leukemic birds. The details of the preparation of the virus stock have been published (7). The modified Eagle's medium and serum supplements were those recommended by Baluda and Goetz(6). Chicken serum added to the tissue culture medium was always pretested for the presence of antibody against AMV and for cell toxicity. Primary yolk sac cultures were prepared from individual fertile chicken eggs on the 14th day of incubation according to a procedure reported in the literature(6). From the same eggs, embryos were excised and used for fibroblast cultures in order to determine genetic susceptibility to avian tumor viruses by challenging with Rous sarcoma virus pseudotypes of the A and B subgroups(8). By this technique most of the embryos as well as the corresponding yolk sac cultures were susceptible to all serotypes of subgroups A and B but some embryos and their respective yolk sac cultures were resistant to the subgroup B serotypes of avian leukosis viruses. In accordance with published work, fully susceptible embryos were designated as C/O and embryos showing selective genetic resistance to subgroup B were termed C/B(8). The source of chick embryos was

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