Role of PMN-Leukocyte Lysosomes in Tissue Injury, Inflammation And Hypersensitivity. V. Partial Suppression in Leukopenic Rabbits of Vascular Hyperpermeability Due to Thermal Injury.* (31723)

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Two phases of increased vascular permeability in response to a thermal burn were first described by Sevitt(1). These findings were confirmed and extended by others(2,3). The mechanism underlying the early edema is generally accepted to be mediated primarily by histamine(4). However, the mediator or mediators of the late phase of vascular hyperpermeability are unknown, although several possible mechanisms have been suggested(5, 6).

Recent studies in our laboratory have implicated the role of polymorphonuclear (PMN) leukocyte lysosomes in several types of vascular injury(7,8,9). It seemed therefore challenging to examine whether PMN-leukocytes played any role in the vascular changes which follow thermal injury. This was done by comparing the lesions produced by heat in normal rabbits and in rabbits rendered leukopenic by nitrogen mustard. Preliminary findings have been reported recently(10).

Materials and methods. Albino rabbits weighing 2.5-3 kg were used throughout the experiment. The abdomen was shaved with an electric hair clipper and a commercial depilator ("Nair") was applied for 2-3 minutes to the shaved abdomen. The depilator was scraped off with a wooden applicator and the abdomen gently washed with water. Within 30 minutes the abdominal skin of most animals looked normal. Those which developed erythema were discarded.

Heat was applied to the abdominal skin by the method of Sevitt(1) using a hollow brass cylinder through which a current of hot water (56°C) was circulated. The cylinder was closed at one end. This highly polished surface (2 cm in diameter) was used as the burning surface, by applying it firmly to the rabbit's skin.

It was shown by Wilhelm and Mason(3) that in the rabbit 56°C applied for 20 seconds will produce a good late permeability response. Furthermore, Sevitt(11) has shown that the intensity of the lesion will vary with the degree of temperature and the length for which heat is applied. In our experiments the temperature was kept constant at 56°C and 4 types of lesions were produced by applying the heat for 20, 30, 40 and 60 seconds. Two lesions of the same duration were produced with each time of application, thus resulting in 8 burns in each rabbit. A total of 17 rabbits were used in which 34 burns were made (Table I).

The late permeability response reaches a peak in about 4 hours(3). Therefore, Evans blue (20 mg/kg) was administered intravenously 4 hours after induction of the lesions and the animals killed 15 minutes later. The skin lesions were punched out with a cork borer (2.2 cm in diameter) and placed into 4 ml of Formamide for 3 days at 45°C. In this manner the Evans blue can be extracted and quantitated by reading in a calorimeter or spectrophotometer at 620 mμ(12). Normal abdominal skin was punched out from each rabbit and extracted with Formamide. This extract served as the blank. Using a calibration curve, the amount of Evans blue exuded in each lesion could be expressed in micrograms. Since each lesion was produced at 2 sites in one rabbit the average of the 2 sites was recorded.

Leukopenia was produced with nitrogen mustard (Mustargen, Merck, Sharpe and Dohme). It was given in 2 doses, first 1.75 mg/kg, followed 48 hours later by 1 mg/kg. Total white counts and differentials were done on all rabbits before and during treatment with nitrogen mustard. The skin lesions were produced 72 hours after the first dose of

* Supported by Medical Research Council of Canada (MT-1251) and the Banting Res. Foundation.

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TABLE I. Vascular Permeability Induced with Heat in Normal and Leukopenic Rabbits.

Time*	μg Evans blue per lesion							
	20 sec		30 sec		40 sec		60 sec	
	Mean	S.E.†	Mean	S.E.	Mean	S.E.	Mean	S.E.
Normal, n = 9	2.27	1.17	7.38	2.58	11.56	2.33	22.40	7.36
Leukopenic, n = 8	1.30	.82	1.80	1.13	5.70	1.36	9.67	2.16

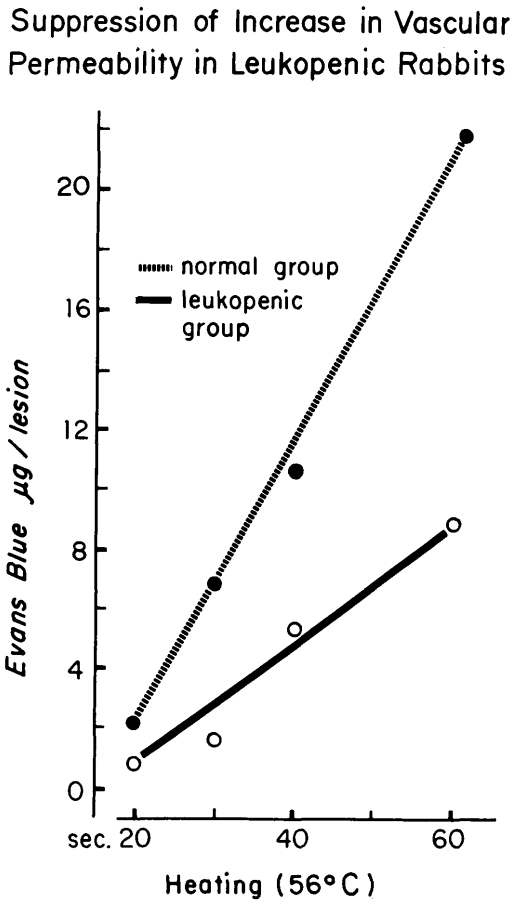
* Length of application of heat (56°C) to skin of rabbits. † Standard error.

nitrogen mustard, when the total WBC count was less than 1000 in most rabbits and the differential count showed less than 10% neutrophils. All rabbits had close to absolute granulocytopenia.

In a few rabbits the Evans blue was mixed with colloidal carbon (C 11-1431a, Pelikan, Guenther Wakner, Hannover, Germany). In these rabbits the skin lesions were biopsied and fixed in Zenker solution and in osmium tetroxide buffered with Tyrode solution. For light microscopy the material fixed in Zenker's was embedded in paraffin and butyl methacrylate. For electron microscopy it was embedded in Epon 812. Some tissue was processed also from animals not receiving colloidal carbon. The sections prepared for ultrastructural studies were stained with lead hydroxide and examined in a Phillips EM 200 electron microscope. For light microscopy paraffin embedded sections were stained with hematoxyline eosin and those embedded in methacrylate were stained with alkalinized 1% Azure II.

Results. There was erythema at all treated sites almost immediately after the application of heat which lasted for several minutes. At site heated for 20 or 30 seconds the skin looked normal within 10-20 minutes after the injury. However, at sites where heat had been applied for 60 seconds and occasionally at the site where it had been applied for 40 seconds, slight to moderate blistering developed within ½ hour. This persisted or even increased over the period of the experiment.

When Evans blue was injected most lesions became blue. Some showed first a ring-shaped or patchy bluing, but eventually the whole lesion became colored. There was a definite gradation, the lesions heated for the longest period being the most blue. A distinct difference could be discerned with the naked eye between the normal and the leukopenic rabbits. However, the difference was more obvious when the exuded dye was extracted with Formamide and estimated spectrophotometrically (Text Fig. 1 and Table I).



Biopsies taken from normal animals showed a moderate degree of infiltration of the dermis by PMN-leukocytes (Fig. 1). Degenerative changes involving the epidermis and skin appendages were present in the lesions produced with 40 or 60 seconds heating (Fig. 2). In-

filtration with PMN-leukocytes was more marked in the latter lesions. Leukopenic rabbits showed the degenerative changes but no infiltration with polymorphs.

Sections of tissue taken from animals injected with colloidal carbon showed accumulation of the tracer in the wall of subepidermal and occasional deeper vessels. Some

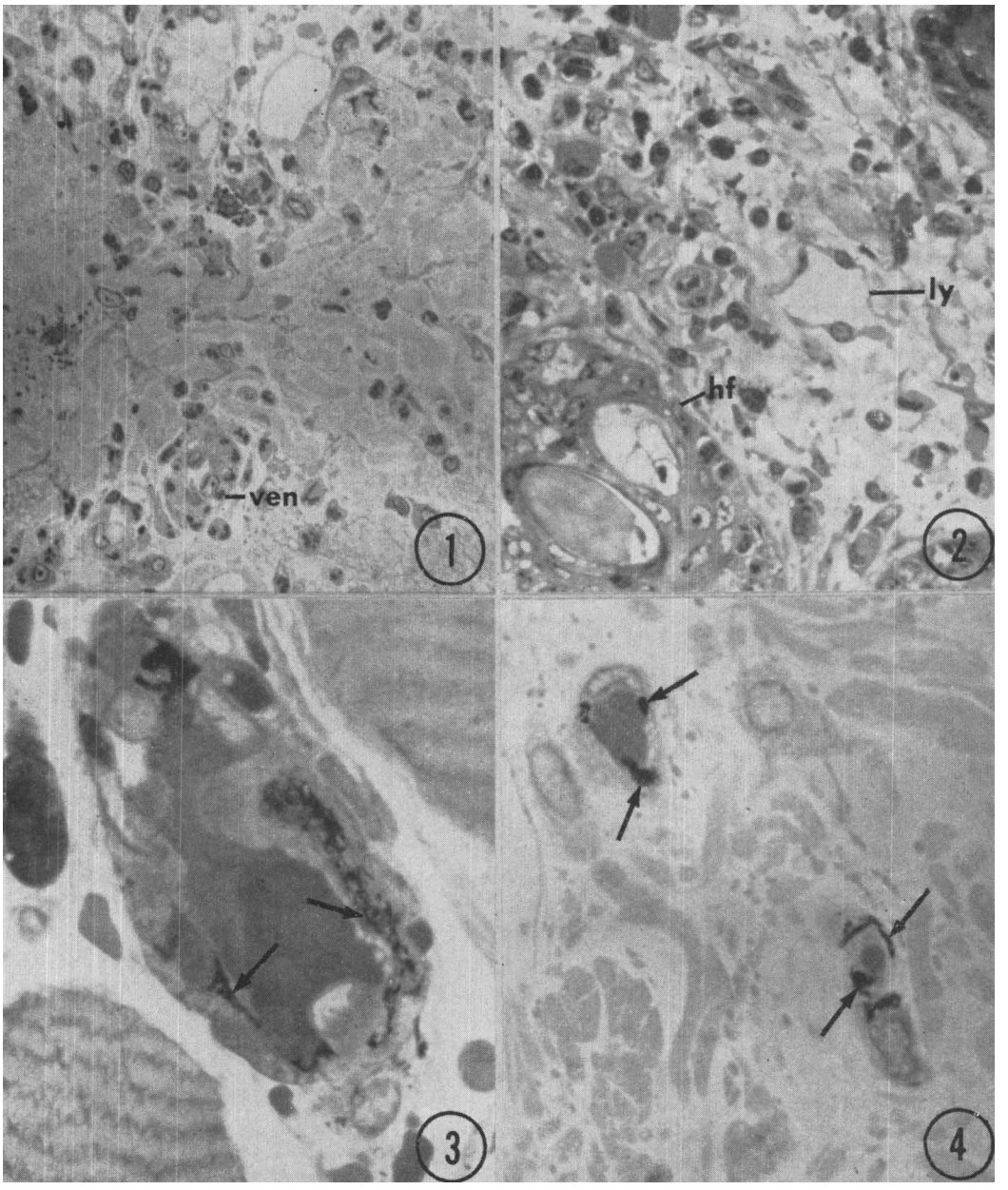


FIG. 1. Skin lesion produced with 56° C heat for 30 sec. Leukocytes are scattered throughout the dermis and are present in the wall of a venue, (ven). × 676.

FIG. 2. Lesion similar to the one in Fig. 1 produced with 56° C heat applied for 60 sec. In addition to accumulation of leukocytes with picnotic nuclei, there are degenerative changes in a hair follicle (hf) and dilation of a lymphatic (ly). × 800.

FIGS. 3-4. Accumulation of colloidal carbon (arrow) in the wall of a venule (Fig. 3) and 2 capillaries (Fig. 4) in skin lesions produced with 56° C heat applied for 30 sec. ×1080.

of these vessels were venules which also contained emigrating PMN-leukocytes in their walls in addition to the tracer (Fig. 3). Most of the superficial vessels were very small, representing presumably capillaries (Fig. 4). These did not contain leukocytes in their walls.

Electron microscopy merely confirmed the presence of tracer in gaps between adjacent endothelial cells below the endothelium and

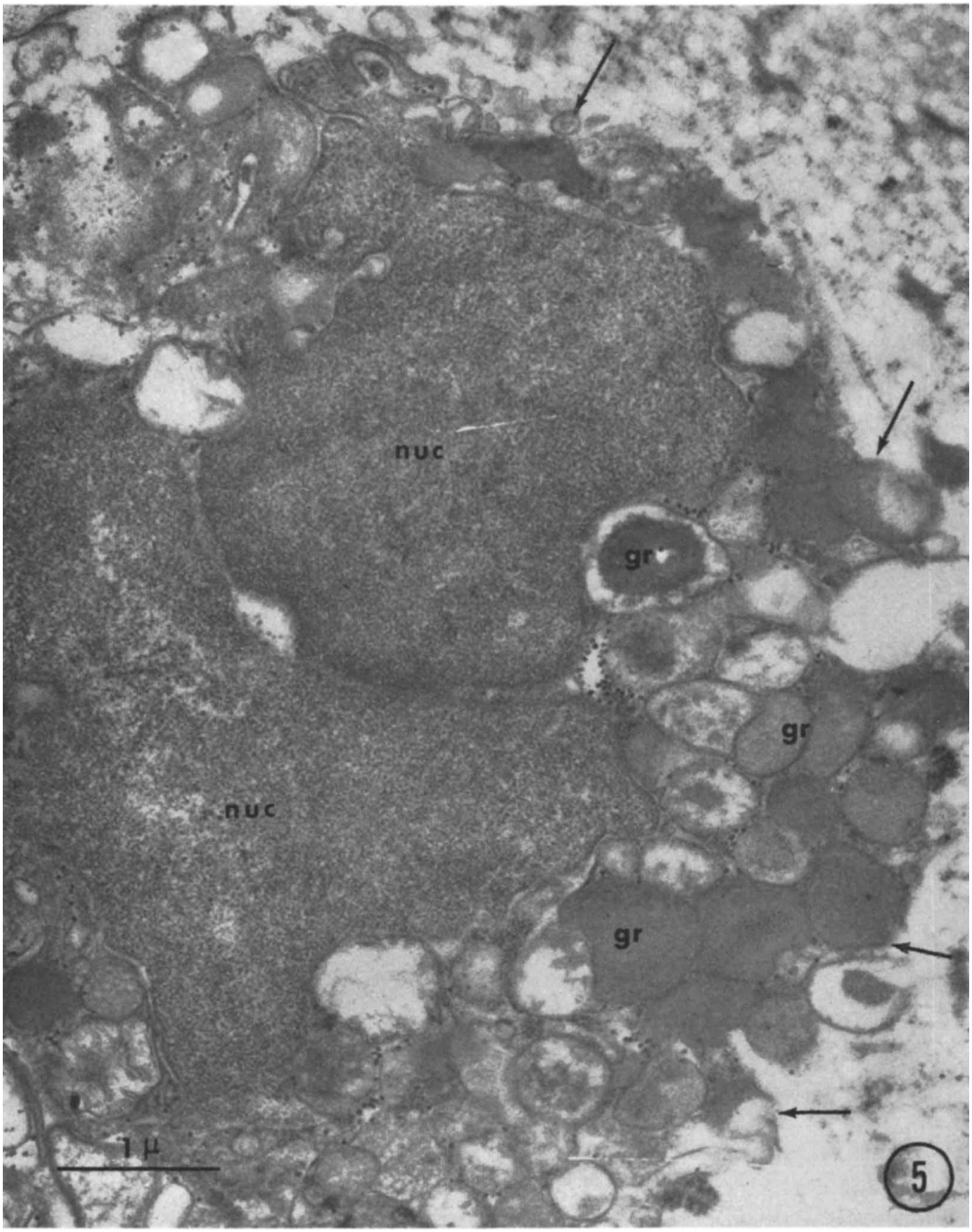


FIG. 5. PMN-leukocyte showing degenerative changes. The nucleus (nuc) is picnotic, granules (gr) are clumped and exposed to the extracellular space (arrows). × 26,400.

occasionally beyond the basement membrane, as described repeatedly in many forms of acute inflammation(13,14,15). Changes in capillaries were similar to those reported by Cotran(16). In addition to this, the ultrastructural studies showed that many of the emigrated PMN-leukocytes had undergone degenerative changes, consisting of nuclear pycnosis, "collapse" of the cytoplasm and extrusion of granules (Fig. 5), not unlike some of the changes observed in the local Shwartzman reaction(17).

Discussion. Stetson in 1951(18) demonstrated the significance of PMN-leukocytes in the pathogenesis of the Shwartzman and Arthus phenomena, by showing that these reactions cannot be elicited in neutropenic rabbits. Recently evidence has accumulated that in these reactions the damaging agent probably derives from the granules or lysosomes of these cells(8,17,19,20,21). Recent studies have also implicated PMN-leukocyte lysosomes in other vascular immune reactions, *i.e.,* passive cutaneous anaphylaxis(7,9). Common to most of these, except the Shwartzman reaction, is the phagocytosis of antigen-antibody complexes and degranulation of polymorphs. However, phagocytosis of bacteria and even of inert (latex) particles likewise results in degranulation of leukocytes, with the presumable release of lysosomal material and subsequent vascular injury(10,22). *In vitro* studies support this concept(23).

The authors believe that the pathogenesis underlying the vascular changes which follow thermal injury are complex and that direct (non-mediated) injury plays an important role. While it has not been excluded that administration of nitrogen mustard may not exert some effect directly on endothelial cells or modify the ability of vessels to respond to subsequent stimuli, there is some suggestion that PMN-leukocytes contribute in some way to the mediation of increase in vessel permeability in heat injury. In their absence the increase in vascular permeability is less pronounced and there is some ultrastructural evidence that they degranulate at the site of injury. It is known that these cells have a short life span(24) and once outside the vascular space they probably rapidly die, which may account for the observed degenerative changes.

The demonstration of PMN-leukocytes in dermal lesions produced with heat is not new. Spector and Willoughby(2) reported an "extensive infiltration with polymorphonuclear leukocytes" after applying 55°C for 27 seconds to the skin of rats.

There is additional indirect evidence that PMN-leukocytes contribute to increased vascular permeability in a lesion akin to the one described here. Although giving it a different interpretation, Logan and Wilhelm (25) showed that tissue leukocytosis preceded and more or less paralleled the increase in vascular permeability induced with ultraviolet irradiation.

The mode of action of PMN-leukocytes in mediating increase in vessel permeability in thermal injury still remains to be elucidated. The most plausible explanation would be that released lysosomal material alters vascular permeability. The mode of action of liberated lysosomal material is now being investigated in several laboratories. There is some evidence that a basic protein or polypeptide(26) in the lysosomes causes increase in vascular permeability in the rabbit(27) and the rat(28). In the latter at least, it acts primarily by liberating histamine from mast cells(28). The proteases or cathepsins of the leukocyte lysosomes(29) may act directly and seem to be responsible for hemorrhagic lesions(22). However, these proteolytic enzymes are capable of forming kinins from appropriate substrates(30) and may release non-specific peptides(29), which in turn could induce increase in vascular permeability.

Summary. Increase in vascular permeability was induced in rabbits with the aid of heat (56°C for 20-60 seconds) applied to the abdominal skin. It was shown that in animals rendered leukopenic the increase in vascular permeability is milder following heat injury. The skin lesions showed infiltration with PMN-leukocytes, which when examined ultrastructurally showed degenerative changes and signs of degranulation. There were no leukocytes in the skin lesions of leukopenic rabbits. It was concluded that the PMN-leukocyte, presumably its lysosomes, contribute

to the pathogenesis of the late phase of increased vascular permeability due to heat injury. The role of these lysosomes in other forms of vascular injury and their possible mode of action was discussed.

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Received August 24, 1966. P.S.E.B.M., 1967, v124.

Biological Properties of Freeze-Dried Attenuated Shigella Vaccines. (31724)

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We have described 2 attenuated strains of *S. flexneri* 2a which when employed as living oral vaccines rendered monkeys resistant against experimental oral challenge(1,2,3). One of these strains was a mutant of a virulent strain and was incapable of causing disease because it had lost the ability to penetrate the intestinal epithelium and reach the lamina propria(3). When fed this organism, experimental animals acted no differently from other animals fed *Escherichia coli*. Five oral doses of the mutant conferred resistance(2). The other vaccine strain was obtained by bacterial recombination of the virulent *S. flexneri* 2a with an Hfr. *E. coli* strain. The hybrid retained the capacity to reach the lamina propria but was unable to multiply to any great extent in this region(1).