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Effect of Filter Paper, *para*-hydroxybenzoic Acid, and Fixed Tissue on Phagocytosis. (19539)

EDWIN M. LERNER II AND JOSEPH VICTOR.

From the Biological Laboratory, Chemical Corps, Camp Detrick, Frederick, Md.

Wood *et al.* have described "surface phagocytosis" at length under the following conditions(1-4): "The material with surface to be tested was spread flat in the bottom of a Petri dish lined with filter paper previously soaked in Locke's solution. The leukocyte-pneumococcus mixture was distributed over the test surface, and the dish was sealed and placed in the incubator (37°C) for 1 to 3 hours. In this way the preparations were kept moist. When the Petri dishes were opened, impression smears were made from the test surfaces and were stained with methylene blue." Substances such as glass, paraffin, and cellophane were reported as inactive, while a variety of materials including fixed tissue sections, fiber glass, and filter paper alone promoted phagocytosis. The common denominator in the cited experiments was wet filter paper, which might have been responsible for phagocytosis. The inactive materials were those impermeable to liquid.

This report deals with the influence of filter paper on phagocytosis of *E. coli*. During this work, an active principle in filter paper, *para*-hydroxybenzoic acid, was reported by Davis(5,6) as a new bacterial vitamin and antagonist for *para*-aminobenzoic acid. The effects of *para*-hydroxybenzoic acid and of fixed tissues were also examined.

Methods. E. coli suspensions. Cultures were grown for 18-20 hours in tryptose broth containing 1% dextrose, 3.7 μg Fe/ml, and 0.1 μg thiamin HCl/ml. The bacteria were harvested and washed in saline, standardized turbidimetrically to 4 x 10^{10} cells/ml, and checked by dilution plate counts. The bacteria were killed by treatment with 1% CH_2O for 24 hours at room temperature, washed 3 times in saline, and sterility was checked by plating. The number of bacteria employed in each set of comparative experiments was the same but varied among the different sets.

Filter paper. Strips measuring 1 x 4 or 1 x 5 cm of Whatman No. 1 or No. 42 paper were rolled and placed in the bottom of 12 ml tapered centrifuge tubes. Bacterial suspensions containing from 4 x 10^9 to 2.4 x 10^{10} organisms in 0.4 to 0.6 ml were pipetted into these tubes and allowed to remain in contact for 30 to 60 minutes, after which the filter paper was squeezed out and removed. Chamber counts in several instances showed no significant loss of bacteria after this treatment. *Para-hydroxybenzoic acid.* Eastman C.P. *para*-hydroxybenzoic acid, either free or as the Na-salt, was used in H_2O solutions containing from 0.00001 to 1 mg/ml. One to 3 ml of these concentrations of *para*-hydroxybenzoic acid were added to 2.5 x 10^9 to 6 x 10^9 cells for 30 to 60 minutes, centrifuged, and the cells resuspended in saline with or without extra washing in saline or Krebs-gelatin. *Tissue.* Washed frozen sections 5-25 μ thick of for-

TABLE I. Effect of Different Materials on Phagocytosis of *E. coli.*

Test substance	Observations	Percentage neutrophils containing bacteria—Mean ± S.D.	Diff. ± P.E. diff.	Diff. / P.E. diff.
A. Saline	36	36.6 ± 17.7		
B. Filter paper	32	55.4 ± 22.4	B–A 18.8 ± 3.3	B–A 5.7
C. *para*-hydroxybenzoic acid	40	57 ± 18.7	C–A 20.4 ± 2.8	C–A 7.3
D. Tissue	34	21.9 ± 18.9	D–A 14.7 ± 3	D–A 4.9

malin-fixed liver, kidney, lung, spleen, brain, heart, or lymph node were used individually or in combinations, in the same fashion as filter paper.

Controls. Bacterial suspensions were treated with saline, and run concurrently with filter paper, *para*-hydroxybenzoic acid, and tissue.

Phagocytosis. This was measured quantitatively by a previously described method(7), using dog blood cells washed 6 times in Krebs-gelatin solution. The washed dog blood cells were mixed with an equal volume of Krebs-gelatin solution; 0.1 ml of this suspension was added to 0.1 ml of the bacterial suspensions cited above in an 8 mm test tube and mixed in a rotator(8) revolving 24 or 36 RPM for 30 minutes at 37°C. The number of bacteria in each set of comparative experiments ranged from 1 x 10^9 to 4 x 10^9. Smears were prepared and stained with Giemsa-Jenner. The percentage of 50 neutrophils containing bacteria employed in individual tests is indicated in the text.

Results. Under standardized conditions for measuring phagocytosis(7) with washed dog blood cells, 36.6 ± 2% of neutrophils contained *E. coli;* with previous contact with filter paper the mean percentage was 55.4 ± 2.7; with *para*-hydroxybenzoic acid 57.0 ± 2.0; and with tissue sections 21.9 ± 2.2. Thus filter paper and *para*-hydroxybenzoic acid enhanced, and tissue inhibited, phagocytosis (Table I).

The stimulatory effect of *para*-hydroxybenzoic acid was related to the concentration between 0.001 and 1 mg per ml (Table II), and could be plotted as a straight line semilogarithmic ratio.

Filter paper and *para*-hydroxybenzoic acid affected the bacteria directly; no opportunity was afforded for their action on the leukocytes in these experiments. This effect with extremely small amounts was not removed by washing and therefore resembled opsonin action. Since the bacteria were dead during exposure to these "opsonizing" agents, there was no production of opsonizing materials during growth, observed in other unpublished studies.

TABLE II. Effect of *para*-hydroxybenzoic Acid on Phagocytosis of *E. coli.*

No. of *E. coli*	*para*-hydroxybenzoic acid—mg/ml .0	.001	.01	.1	1
	% neutrophils containing *E. coli*				
6 × 10^9	32	50	56	56	72
3 × 10^9	16	40	48	52	68

The effects ascribed to "surface" phenomenon in phagocytosis may have been due to wet filter paper. Surfaces favorable to phagocytosis were freely permeable to liquid, and those unfavorable were impermeable. In the light of these data and Wood's experiments, the claim that a favorable "surface" enhances phagocytosis is open to question.

Conclusions. 1. Phagocytosis of formalin-killed *E. coli* was promoted by previous contact with filter paper or with *para*-hydroxybenzoic acid. The latter, an active principle in filter paper, was effective in concentrations of 1 to 0.001 mg per ml. Fixed tissue inhibited phagocytosis. 2. The activity of the several surfaces reported by Wood *et al.* to promote phagocytosis may be ascribed to the wet filter paper in their experiments. These observations open to question the phenomenon of "surface" phagocytosis.

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Protection of Mice Against X-Irradiation by Spleen Homogenates Administered After Exposure.*† (19540)

LEONARD J. COLE, MAURICE C. FISHLER, MARIE E. ELLIS, AND VICTOR P. BOND.‡
From the Biological and Medical Sciences Division, U. S. Naval Radiological Defense Laboratory, San Francisco, Calif.

Protection of animals against the lethal or deleterious effects of ionizing radiations is now possible by means of several chemical substances administered prior to, or during X-irradiation: for example, cysteine(1), glutathione(2), or sodium nitrite(3). In addition, anoxic anoxia during irradiation is also effective(4). None of these measures, however, has been shown to be protective when carried out *after* irradiation. Recent studies by Jacobson *et al.*(5) suggest the presence of a spleen factor which appears to be effective *after* irradiation exposure. These workers transplanted 4 spleens from young non-irradiated mice (1-12 days old) into the peritoneal cavity of adult CF-1 mice immediately after exposure to 1025 r whole-body X-irradiation. Under these conditions, survival of the irradiated mice was increased, and regeneration of hematopoietic tissue was hastened.

Several initial experiments in this laboratory in which spleens from young mice were implanted into irradiated mice (650 r) confirmed the protective effect of this procedure in LAf_1 mice. The implants became vascularized, grew, and were still present and intact 6 weeks after implantation. As a first approach to isolating and ascertaining the nature of the splenic factor, studies were performed using spleen homogenates.

The preliminary data, described herein, indicate that mice receiving otherwise lethal doses of whole-body X-irradiation are protected by a single intraperitoneal injection of spleen homogenate administered either one hour or as long as 45 hours after radiation exposure.

Materials and methods. LAf_1 mice of both sexes, approximately 7 to 11 weeks old and weighing 20 to 26 g, were used in the irradiation studies. The animals were allowed free access to food (Purina Laboratory Chow) and to tap water. In all experiments the control and experimental animals were matched with respect to sex, age, and body weight; they were irradiated simultaneously and caged together (8 or 10 per cage). A Westinghouse Therapy Unit was used as the radiation source. The radiation factors were: 250 KVP; 15 ma; filter, 0.5 mm Cu plus 1 mm Al; HVL, 1.5 mm Cu; skin to target distance, 100 cm; dosage rate, 25 r per minute, as measured with a Victoreen r-meter placed in air at the position of the mice. Each radiation dose was delivered in a single exposure. During irradiation the mice were contained in individual, perforated lusteroid centrifuge tubes placed radially on a circular wooden turntable platform which rotated at 3.5 r.p.m. to obtain uniformity of radiation dosage.

In a typical experiment, 20 to 30 young LAf_1 mice (1 to 3 weeks old) were sacrificed by severing the cervical spine. The spleens were removed immediately and suspended in

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‡ Lieutenant (Medical Corps), USN.