Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 105 DECEMBER 1960 No. 3

SECTION MEETINGS

MINNESOTA
Mayo Foundation October 7, 1960
NORTHWEST
University of Oregon Medical School November 12, 1960

Absence of Endotoxic Activity in Materials Derived Without Bacterial Contamination from Mammalian Tissue. (26134)

E. MERLER,* A. PERRAULT, R-J. TRAPANI, M. LANDY AND M. J. SHEAR
Laboratory of Chemical Pharmacology, National Cancer Institute,† Bethesda, Md.

Much literature has accumulated on the biologic effects of endotoxins, which are recognized as deriving mainly, if not exclusively, from Gram-negative bacteria. There are, however, a number of reports on endotoxic properties of materials extracted from mammalian tissues. Atkins(1) has summarized these contributions. In addition, Antopol and coworkers(2-4) found that extracts of normal and malignant tissues were capable of eliciting several characteristic endotoxic reactions, *viz.*, the Shwartzman phenomena in rabbits and tumor damage in mice; more recently, Martin and Spielmann obtained(5) materials with pyrogenic potency from human erythrocytes.

Earlier experiments(6) in this laboratory had yielded preparations, from mammalian tissues, which exhibited 3 endotoxic properties. Some of these preparations were later found(7) to induce the entire array of host reactions as do bacterial endotoxins.

To obtain a reproducible methodology, the procedures first employed were subjected to systematic study in which the effects of variations in technic were examined. This communication reports that with some procedures the final preparations were devoid of biologic activity; when the products displayed endotoxic potency, it was found that the variations in technic had permitted bacterial growth.

Materials and methods. Various tissues (whole blood, red cells, red cell stroma, and tumor) from several mammalian species (mouse, rabbit, and man), after immersion in an aqueous medium containing phenol and trichloroacetic acid, were treated as outlined

* Present address: Children's Hospital, Boston, Mass.

† Nat. Inst. of Health, P.H.S., U. S. Dept. of H.E.W.

previously(8). Other precautions, taken to preclude bacterial growth during protracted chemical manipulations, included: buffer solutions were freshly prepared and autoclaved; the trypsin (pancreatin), after purification to remove polysaccharides, was sterile, and negative in the tumor assay even at the high dose of 20 mg per mouse; additional phenol was employed during dialysis after treatment with trypsin; and pyrogen-free water was employed throughout.

Among the variations in technic examined for their influence upon the properties of the final product was omission of layering with toluene during treatment with trypsin.

Small aliquots of all solutions, and of the materials at all stages of the manipulations, were examined for presence of bacteria by inoculation into thioglycollate fluid medium or semi-solid medium and by subsequent plating on nutrient, EMB or blood agar. When bacterial growth was evident, the organisms were isolated and their reaction to the Gram stain determined.

Elicitation of hemorrhagic necrosis(9) in sarcoma 37 was the biological activity measured in the bioassays.

Results. In experiments with red cell stroma, the product obtained when toluene had been employed was found to be inactive; a parallel experiment, in which toluene had been omitted, yielded a product active in the tumor assay. Culture of the tryptic digest was negative in the former instance; bacterial contamination with Gram-negative species was evident in the latter.

The lack of activity of this non-contaminated preparation from stroma raised the question as to whether contamination inadvertently occurred with other preparations despite the precautions taken. Accordingly, systematic bacteriologic examination was instituted at the various stages of the processing of materials. It was found that all steps in the protracted manipulations could be carried out, repeatedly, with exclusion of bacteria in the open chemical laboratory. However, the end products, when obtained under this kind of bacteriologic control, displayed no endotoxic activity as assessed in the tumor assay.

The products we prepared from human red cell stroma by Martin's method(5) also were inactive in the tumor assay even when as large a dose as 5 mg per mouse was employed; a low order of pyrogenicity was exhibited by this product which had a M.P.D. of 150 μg.

The negative products obtained under satisfactory bacteriologic controls were high in nitrogen, yielding values commonly found in mucoproteins.

Discussion. Inasmuch as this kind of bacteriologic data is not available on the earlier preparations(6), of which some may have been contaminated, the significance of their endotoxic properties is now reassessed. It was stated(7): that the micro-organisms which plants and animals ordinarily contain might be the source of activity of the polysaccharide complexes obtained from their tissues; and that pyrogenic bacteria, inadvertently introduced during handling of the source materials, were to be reckoned with in view of their ubiquity, high potency, and relative ease of contamination by them. However, because of the considerations stated, these factors did not appear to account for the results obtained. Results(7) reported for the earlier preparations are now viewed as possibly having been due, in an undeterminable number of instances, to inadvertent introduction of bacteria.

Nevertheless, it would be premature to conclude that tissues do not contain components with endotoxic properties. For one thing, Bennett has, under strictly controlled conditions, obtained pyrogen from turpentine-induced exudates(10) in a critical extension of Menkin's(11) finding, and also from polymorphonuclear leucocytes(12). We have speculated(7) that, if tissues contain endotoxic polysaccharides, they would not exhibit their characteristic properties in their usual state of combination with protein but only after they were separated from the complex. The high nitrogen values obtained under current conditions of our experiments indicate that the enzymes employed apparently did not split off the protein moiety with consequent liberation of polysaccharide. This experience is consistent with those of others

who have also encountered resistance of mammalian mucoproteins to proteolytic enzymes.

Whether the polysaccharides in these inactive complexes would, or would not, exhibit endotoxic behavior when success is achieved in liberating them from combination is not known. This is being investigated.

Summary. In the attempted isolation from mammalian tissues of polysaccharides with endotoxic properties, only materials devoid of such activity were obtained under conditions in which exclusion of bacteria was demonstrated.

1. Atkins, E., *Physiol. Rev.*, 1960, v40, 580.
2. Antopol, W., *J. Inf. Dis.*, 1937, v61, 334.
3. Antopol, W., Glick, D., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 346.
4. Antopol, W., Chryssanthou, C., *ibid.*, 1960, v103, 725.
5. Martin, H., Spielmann, W., *Klin. Wochnschr.*, 1958, v36, 491.
6. Perrault, A., Shear, M. J., *Proc. Am. Assn. Cancer Res.*, 1955, v2, 39.
7. Landy, M., Shear, M. J., *J. Exp. Med.*, 1957, v106, 77.
8. Perrault, A., Shear, M. J., *Cancer Res.*, 1949, v9, 626.
9. Leiter, J., Downing, V., Hartwell, J. L., Shear, M. J., *J. Nat. Cancer Inst.*, 1950, v10, 1273.
10. Bennett, I. L., Jr., *J. Exp. Med.*, 1948, v48, 279.
11. Menkin, V., *Arch. Path.*, 1945, v39, 28.
12. Bennett, I. L., Jr., Beeson, P. B., *J. Exp. Med.*, 1953, v98, 493.

Received Sept. 26, 1960. P.S.E.B.M., 1960, v105.

Effect of Nitrogen Mustard in Rabbits Following Exposure to X-Irradiation. (26135)

O'NEILL BARRETT, JR. (Introduced by W. H. Crosby)
(With technical assistance of Robert Packard)
Department of Hematology, Walter Reed Army Institute of Research, Washington, D. C.

Bone marrow depression represents one of the problems encountered in the use of chemotherapeutic agents in treatment of malignant disease. At this Institution we have treated several patients with large doses of nitrogen mustard (0.95-1.8 mg/kg) for Hodgkin's disease(1). Results of such therapy suggest that patients who have received prior x-irradiation have a more severe and prolonged bone marrow depression than those not previously exposed. Because of the small number of patients and variation in the clinical setting, definite conclusions concerning this point have been difficult to reach. This report deals with the results of a comparable study done in experimental animals in an attempt to determine whether or not there is a difference in response between irradiated and non-irradiated animals following administration of nitrogen mustard.

Methods. Ten adult, female rabbits used in the study were divided into 2 groups of 5 each. One group was given x-irradiation while the other served as control. Two courses of x-ray were given, 250 r whole body irradiation over a 10-minute period. Following each course of exposure, a suitable period of 5-6 weeks was allowed for recovery of bone marrow as manifested by return of normal peripheral blood counts. Five weeks following the last dose of x-ray, both groups of animals were given 1.5 mg/kg nitrogen mustard intravenously. Peripheral blood counts, including red blood cell count, hemoglobin, hematocrit, white blood cell count and platelet counts (direct), were performed prior to start of the study, at weekly intervals during experiment and one week following administration of nitrogen mustard. All blood specimens were obtained by cardiac puncture without difficulty. Bone marrow aspiration was performed prior to x-ray exposure and one week following nitrogen mustard administration in the surviving animals.

Results. In the control group one animal sustained a cutaneous injury which became