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Endotoxin as Adjuvant in Autoimmunity to Cardiac Tissue.* (28721)

A. MICHAEL DAVIES, IGAL GERY, E. ROSENMANN AND A. LAUFER
(Introduced by Jack Gross)
Departments of Preventive Medicine and Pathology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Repeated injections of rabbits(1) and rats (2) with preparations of homologous heart tissue mixed with Freund's complete adjuvant, lead to the production of circulating antibodies specific to heart. Patients with chronic heart disease may also show anti-heart antibodies in their sera(3) and here, the antibody stimulating activity of Freund's adjuvant cannot be invoked in explanation of the auto-immune reaction. One possible mechanism is that chemical changes in, for example, an infarct, may convert it into a form antigenic for the patient(2). A supplementary mechanism may be the "adjuvant" effect of circulating endotoxin, released in patients shocked after myocardial infarction or cardiotomy.

Endotoxin has been demonstrated in the blood and myocardium of shocked animals (4) and these compounds are known to stimulate antibody production to a variety of heterologous antigens(5,6). The following experiments demonstrate the ability of endotoxin to act as adjuvant in immunity to homologous tissue antigen.

Materials and methods. Male albino rats of the Hebrew University stock, weighing 80-90 g, were given 6 weekly injections of pooled rat heart homogenate mixed with Freund's complete adjuvant (Difco) or with endotoxin. Controls received heart homogenate alone, endotoxin, or adjuvant mixed with saline.

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The preparation of the heart homogenate was as previously described(1) and an amount equivalent to 5 mg protein was given per injection.

Endotoxin (kindly provided by Prof. Moshe Shilo) was a highly purified preparation from *E. coli* having an LD_{50} of about 7 μg/kg for rabbits when given intravenously. Rats, given 100 μg/kg intraperitoneally, appeared sick for a few hours but always recovered; this dose level was used for immunization.

In further experiments (Table III) heart homogenate and endotoxin were given at different sites and at different times.

Blood samples were drawn from the tail veins at different intervals and one week after the last injection, the animals were bled out from the throat and the hearts removed, washed in saline and fixed in 10% formalin. Sections of heart tissue were stained with hematoxylin and eosin.

Sera were examined for heart-specific antibodies by the hemagglutination and hemagglutination-inhibition tests previously described(1), using tanned human erythrocytes sensitized with extracts of rabbit or rat heart. Extracts of fresh heart were poor sensitizing antigens for the tanned cells but use of extracts of rat heart homogenate that had been frozen to $-80°$ and thawed to room temperature 5 times increased the sensitivity of the test. The results cited are those with the frozen-thawed rat heart extracts unless otherwise stated.

Results. (Table I). None of the rats re-

TABLE I. Hemagglutination Titers of Heart-Specific Antibodies in Sera of Rats After 6 Injections of Rat-Heart Homogenate With and Without Freund's Adjuvant and Endotoxin.

Antigen	No. of rats	Hemagglutination titer*					
		<1:20	20–	40–	80–	160–	320
Rat heart alone	20	20	0	0	0	0	0
Heart + Freund's adjuvant	17	14	3	2	0	0	0
Heart + endotoxin	37	20	7	1	3	1	5†
Freund's adjuvant	15	15	0	0	0	0	0
Endotoxin	12	12	0	0	0	0	0

* Titer with tanned erythrocytes sensitized with rat heart extract.
† Two animals reached titers of 1:2560.

ceiving injections of pooled fresh heart homogenate showed antibody titers at the critical dilution of 1:20. Less than a third of those given heart homogenate with Freund's complete adjuvant showed circulating anti-heart antibodies to low titers (geometric mean titer = 5.4), while endotoxin raised the proportion of positives to nearly a half and the mean titer to 11.4. Two of the rats of this latter group attained titers of over 1:2,500 and represent the highest levels seen in hundreds of rats immunized with different preparations of homologous heart over the past 2 years.

Adjuvant and endotoxin controls showed no circulating antibodies.

All sera that agglutinated erythrocytes sensitized with rat-heart preparations also reacted with cells sensitized with rabbit heart, usually to a slightly higher dilution. The heart specificity of the antibodies was also demonstrated by hemagglutination-inhibition tests using both homologous and heterologous heart preparations. In 2 animals the presence of true auto-antibodies was confirmed by the fact that erythrocytes sensitized with the animal's own heart were agglutinated by its serum to the same titer as other sensitized cells.

TABLE II. Myocarditis in Rats Given 6 Injections of Rat-Heart Homogenate With and Without Adjuvant and Endotoxin.

Antigen	No. of rats	No. of minimal or doubtful changes*	No. with moderate or marked myocarditis*
Rat heart alone	13	2	1
Heart + Freund's adjuvant	9	1	1
Heart + endotoxin	28	4	1
Freund's adjuvant	15	2	0
Endotoxin	10	1	2

* Defined in text.

Pathologic changes were seen in some of the hearts (Table II) although there was no correlation between the appearance of lesions and the production of humoral antibodies. The hearts appeared macroscopically normal but the histologic picture showed foci of infiltrative round cell myocarditis of varying degrees. Rats of the stock used show, rarely, sparse and diffuse round cell infiltrations of the myocardium and in the animals designated as having "minimal/doubtful changes," lesions of this type were seen (Fig. 1 A).

Animals in the group defined as "moderate or marked myocarditis" showed foci of interstitial infiltration of small lymphocytes, varying in intensity and number and occasionally accompanied by histocytes (Fig. 1 B). Subendocardial foci of necrosis with lymphohistiocytic reaction were occasionally present (Fig. 1 C).

Cases of moderate or marked myocarditis occurred in all groups receiving antigen as well as in controls receiving endotoxin only (Table II). The histologic pattern was the same in all the affected animals.

An indication of the mode of action of endotoxin in homo-immunity is illustrated in Table III. When mixed before injection, the endotoxin showed its expected adjuvant effect. When given separately, 8 hours apart, this effect was lost, indicating the need for contact between the two substances.

Discussion. Bacterial endotoxins are known to enhance the antibody response of the in-

tact animal in both specific and non-specific ways(7) and formation of germinal centers in the spleen in the presence of antigen is speeded up(8). There is little information on the effects of endotoxin in the rat and there may be a species difference(cf. 6) especially as our animals were extremely resistant to the toxic effects. In the rabbit, the endotoxin acts when given from 6 hours before the antigen to 24 or 48 hours after(6,9). Such was not the case in the present experiments and a different mechanism may be involved which depends on interaction between endotoxin and the tissue antigen.

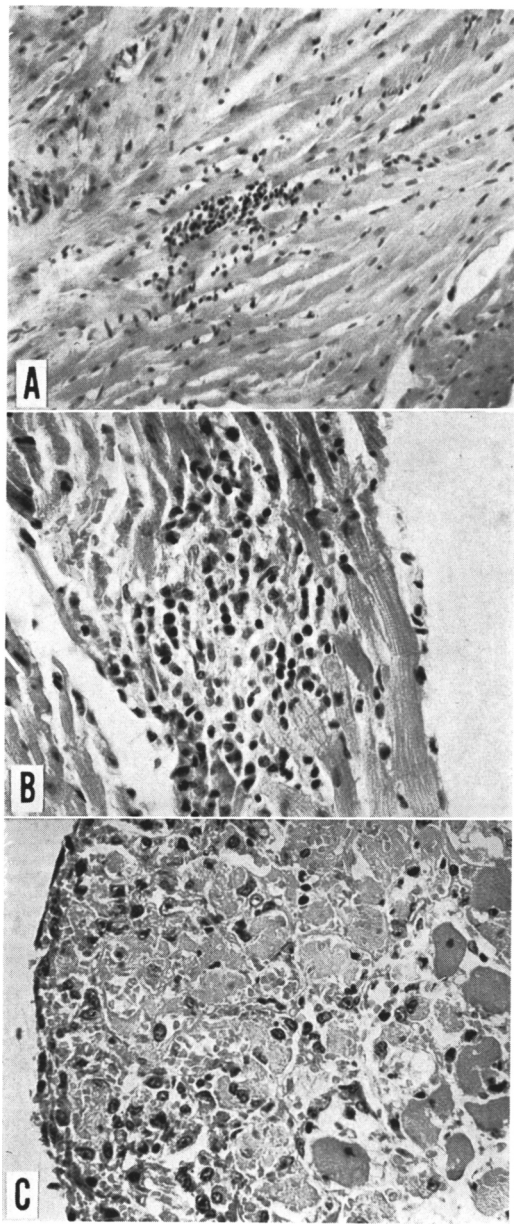

FIG. 1. A. Single myocardial focus of lymphocytic infiltration of the type designated minimal/doubtful changes. 147 ×. B. Part of area of moderate myocarditis showing mainly lymphocytic infiltration with a few histocytes. 318 ×. C. Subendocardial focus of necrosis with mainly histiocytic infiltration. 318 ×.

Rats given repeated injections of endotoxin showed non specific hemagglutinins that reacted also with rat cells. This finding, confirmed by others(10), strengthens the possibility that endotoxin may be involved in production of autoantibody, perhaps by combining with tissue components. It has been suggested(11) that immunity to cancer cells is elicited by changes in the surface antigens and a linkage of tissue antigen to bacterial products, with antigenic modification, may be the explanation of certain of the autoimmune diseases(12).

Kováts(13) has reported the production of myocarditis in rabbits given endotoxin and heart homogenate. The lesions described in his paper differ, however, from those obtained in our experiments, and he failed to produce myocarditis by administration of endotoxin alone, in contrast to the present results, our own results in rabbits and those reported by other investigators(14,15). The production or otherwise of immunopathological lesions does not, of course, conflict with the serological findings of immunity, for in the present experiments, as in other studies (2), the two phenomena show no interdependence.

Summary and conclusion. Endotoxin administered together with homologous heart tissue homogenate in rats had a marked ad-

TABLE III. Effect of Mode of Injection of Heart Homogenate and Endotoxin.

No. of rats	Mode of injection*	No. with titer ≧ 20
28	Homogenate mixed with endotoxin	12
10	Homogenate before endotoxin	1
18	Homogenate after endotoxin	0

* The first group received heart homogenate mixed with endotoxin before injection. In the other cases homogenate and endotoxin were injected into separate peritoneal sites 8 hr apart.

juvant effect in production of circulating anti-heart antibodies. This effect was greater than that of Freund's complete adjuvant and was abolished when the endotoxin was given separately, before or after the homogenate. Focal infiltrative myocarditis was seen in some of the animals but could be attributed equally to the endotoxin as to the administered antigen. It is suggested that the autoimmune process can be partially explained on the basis of modification of tissue antigen by linkage to endotoxin.

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Influence of β-mercaptoethylamine, Mesenteric Vessel Clamping and pH on Intestinal Absorption of Fe^{59} in Rats.* (28722)

KEDAR N. PRASAD† AND JAMES W. OSBORNE
(Introduced by T. C. Evans)
Radiation Research Laboratory, College of Medicine, University of Iowa, Iowa City

Iron absorption from the small intestine is oxygen dependent(1), but the mechanism is not known. Hypoxia has been reported to increase iron absorption in rats(2,3,4,5). It has been postulated that O_2 content of the intestinal mucosal cell may influence absorption of iron by modification of the redox potential within the cell(2,4).

β-mercaptoethylamine (MEA) has been reported to reduce tissue oxygen tension(6, 7). Clamping of the superior mesenteric vessels produces circulatory hypoxia in the gut (8). Therefore, the effects of MEA and mesenteric vessel clamping on intestinal iron absorption were studied. As a byproduct of the investigation to see whether MEA and mesenteric vessel clamping would influence iron absorption, the effect of pH on the process was studied. A perfusion technique was used to determine the effects of MEA, mesenteric vessel clamping, and pH upon iron absorption from the small bowel.

Materials and methods. Male Holtzman rats weighing from 200 to 250 g were used. In one group of animals, MEA (100 mg/kg) was given intraperitoneally fifteen minutes before perfusion of $Fe^{59}SO_4$. In another group, superior mesenteric vessels were

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† Present address: Medical Research Center, Brookhaven National Laboratory, Upton, N. Y. The study was conducted in partial fulfillment of the requirements for a Ph.D. degree in Radiation Biology at Univ. of Iowa.