Identification of a reticuloendothelial stimulating agent in zymosan

S. J. RIGGI AND N. R. DI LUZIO
Department of Physiology, University of Tennessee
Medical Units, Memphis, Tennessee

RIGGI, S. J. AND N. R. DI LUZIO. Identification of a reticuloendothelial stimulating agent in zymosan. Am. J. Physiol. 200(2): 297-300. 1961.—The functional activity of the reticuloendothelial system (RES), as determined by the intravascular clearance of colloidal carbon, and the degree of induced RE hyperplasia were studied following the intravenous injection of various constituents of zymosan. The readily extractable lipid component from the yeast cell wall was inactive, as was the polysaccharide, mannan. Stimulatory activity was still present in the zymosan residue after removal of free and bound lipids. The administration of glucan derived either from yeast, or its cell wall, resulted in marked RE activation and induced hyperplasia, demonstrating it to be the active RE stimulating agent. The relationship of glucan's chemical structure to its ability to induce RE hyperfunction and hyperplasia is discussed.

ZYMOSAN, a 3-μ cell wall preparation derived from Saccharomyces cerevisiae, has been demonstrated to produce marked hyperplasia and hyperfunction (1-5) of the reticuloendothelial system (RES). Zymosan has previously been employed to inactivate the third component of complement (6, 7), enhance antibody formation (3), promote survival following exposure to ionizing radiation (8), increase resistance to a number of bacterial infections (9), inhibit tumor development (10) and inhibit dietary-induced hypercholesterolemia and cholesterolosis (4, 5). Whether all of these phenomena reflect the stimulating influence of zymosan on the RES must await further study.

Zymosan has been analyzed to contain polysaccharides, proteins, fats and inorganic elements (11). The specific chemical fraction responsible for the above effects, as well as activation and proliferation of the RES, has not been identified. In view of the many important biological activities produced by the parenteral administration of zymosan, a study was undertaken to determine the specific fraction responsible for the activation and proliferation of the RES in anticipation that many of the possible harmful side effects such as pyrexia, anemia, pulmonary hyperplasia and microemboli might be eliminated. These studies also contribute to a further understanding of the relationship of chemical structure to activation and proliferation of the RES. During the progress of these experiments it was reported that the active agent in zymosan was a lipid or a mixture of lipids and that the polysaccharide-protein-ash residue was inactive (12). The results of our studies indicate that the stimulatory effects of zymosan on the RES can be reproduced by a specific lipid-free polysaccharide obtained from the cell wall of Saccharomyces cerevisiae.

METHODS

Male and female rats (Holzmun) were injected intravenously with 1 mg/100 gm body weight of the various fractions derived from zymosan. All yeast cell fractions were suspended in 0.05% Tween 80 (polyoxyethylene sorbitan mono-oleate) in a concentration of 5 mg/ml. The control rats were injected with equivalent volumes of 0.05% Tween 80. All rats were injected daily for 5 or 6 days while under light ether anesthesia. On the following day the phagocytic activity of the RES was evaluated by measuring the intravascular removal rate (1) of colloidal carbon (Gunther Wagner, Hanover, Germany; preparation C 11/1431a). The rats received an injection of 8 mg of colloidal carbon/100 gm body weight. The colloidal carbon was injected into an exposed saphenous vein, and a series of blood samples were obtained from the tail veins. The samples were hemolyzed in 0.1% Na₂CO₃ and the colloidal carbon concentration determined spectrophotometrically. The half-time (τ/2), the global phagocytic index K, and the corrected phagocytic index α were calculated (1). The zymosan employed was Standard Brands, type A, which was suspended in saline and injected intravenously daily for 5 days. The dose of zymosan was 4 mg/100 gm. Control rats received equivalent volumes of saline.

A chloroform-ether extractable lipid fraction (lipid 1), and the residue after the free lipids were removed (resi-
TABLE I. Influence of Various Agents on Reticuloendothelial System

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial Wt, gm</th>
<th>Final Wt, gm</th>
<th>% Body Weight</th>
<th>t/2 min.</th>
<th>K Index</th>
<th>α Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Spleen</td>
<td>Lung</td>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Male rats injected for 5 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (10)</td>
<td>203±4</td>
<td>230±6</td>
<td>4.58±0.13</td>
<td>0.33±0.02</td>
<td>0.54±0.02</td>
<td>0.82±0.02</td>
</tr>
<tr>
<td>Zymosan (10)</td>
<td>187±3</td>
<td>190±7</td>
<td>5.32±0.13</td>
<td>0.32±0.06</td>
<td>0.40±0.04</td>
<td>0.86±0.09</td>
</tr>
<tr>
<td>Tween 80 (6)</td>
<td>255±10.6</td>
<td>240±0.2</td>
<td>3.24±0.01</td>
<td>0.32±0.01</td>
<td>0.35±0.01</td>
<td>0.74±0.04</td>
</tr>
<tr>
<td>B. Female rats injected for 5 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid I (7)</td>
<td>248±11.1</td>
<td>239±9.7</td>
<td>3.31±0.16</td>
<td>0.32±0.02</td>
<td>0.64±0.03</td>
<td>0.74±0.04</td>
</tr>
<tr>
<td>Residue A (7)</td>
<td>240±10.4</td>
<td>236±8.6</td>
<td>4.65±0.17</td>
<td>0.80±0.03</td>
<td>0.74±0.04</td>
<td>0.74±0.03</td>
</tr>
<tr>
<td>Residue B (7)</td>
<td>249±8.7</td>
<td>231±6.6</td>
<td>4.36±0.18</td>
<td>0.78±0.06</td>
<td>1.47±0.07</td>
<td>0.63±0.08</td>
</tr>
<tr>
<td>C. Male rats injected for 6 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tween 80 (9)</td>
<td>256±9.1</td>
<td>246±8.9</td>
<td>4.31±0.12</td>
<td>0.34±0.01</td>
<td>0.49±0.02</td>
<td>0.74±0.04</td>
</tr>
<tr>
<td>Mannan (9)</td>
<td>259±10.7</td>
<td>248±9.3</td>
<td>4.19±0.07</td>
<td>0.33±0.01</td>
<td>0.51±0.01</td>
<td>0.74±0.04</td>
</tr>
<tr>
<td>Glucan yeast (9)</td>
<td>267±9.4</td>
<td>253±8.7</td>
<td>4.19±0.07</td>
<td>0.53±0.02</td>
<td>1.14±0.05</td>
<td>0.63±0.08</td>
</tr>
<tr>
<td>Glucan zymosan (9)</td>
<td>265±11.8</td>
<td>264±11.1</td>
<td>4.19±0.03</td>
<td>0.83±0.03</td>
<td>0.95±0.05</td>
<td>0.8±0.11</td>
</tr>
</tbody>
</table>

Values are means ± standard error. * Figures in parentheses are no. of rats in group.

Due A) were evaluated. Residue A was further subjected to mild acid hydrolysis and subsequent lipid extraction to yield a lipid free fraction (residue B). Based on the initial observations, three other fractions were tested in respect to their ability to activate the RES. These included glucan isolated from yeast cells and yeast cell walls by the method of Hassid et al. (13) and mannan prepared from yeast by the method of Haworth et al. (14).

All data were statistically analyzed at the 95 % level of confidence. The influences of the various zymosan fractions (table IB and IC) were analyzed according to the analysis of variance, while the comparison of zymosan and saline (table IA) was analyzed by use of a t test for the difference between means (15).

RESULTS

The influence of a saline suspension of zymosan on RE hyperplasia and hyperfunction is presented in table IA. The zymosan-injected rats manifested a failure in body weight gain, whereas a gain was observed in the saline-injected control group. Although liver weight was unaltered, spleen and lung showed an increase in organ weight of 230 and 114 %, respectively. In relationship to body weight, the liver was significantly larger in the zymosan-treated group. No weight alterations were observed in kidney. Associated with organ hyperplasia, which was limited to those organs containing predominantly RE elements, there was a profound increase in phagocytic function as denoted by the decreased half-time (t/2) of the injected colloidal carbon from a mean 6.7 minutes in the control group to 0.6 minutes in the zymosan group (fig. 1).

The K index, a measure of the total phagocytic activity of the RES, and the index α, a measure of the phagocytic activity per unit weight of liver and spleen, were determined (1). The increased α and K indexes indicate that the enhanced RE function is due to increased activity of pre-existing macrophages as well as the formation of new RE cells. These findings are in agreement with previous observations (1, 2).

The influence of the suspending medium, Tween 80, on phagocytic function can be obtained from a comparison of data presented in tables IA and IC. In contrast to the mean t/2 of 6.7 minutes in the saline-injected group, the Tween-injected rats showed a t/2 of 2.3 minutes. Further studies have confirmed the stimulating effect of Tween 80 on RE activity. The marked increase in the K and α indexes associated with normal organ weights suggest that the increased removal rate of colloidal carbon is due to enhanced RE cellular activity.

The fraction, lipid I, obtained from zymosan was devoid of RE stimulatory activity as indicated by the removal rate of colloidal carbon and the α index, (table IB). Likewise, no RE hyperplasia was induced by this fraction. Residue A from which the readily extractable lipid, essentially triglyceride, was removed, manifested the RE stimulatory activity of the original zymosan preparation as denoted by the enhanced removal rate of colloidal carbon and the increased α index. Similarly,
residue B possessed all the RES stimulatory activity of original zymosan preparation. Analysis of residue B by previously described procedures (16) indicated it to be free of any lipid material. These observations demonstrate that the active RE stimulating agent in zymosan is not a lipid.

An investigation of the influence of the polysaccharide complex of zymosan on RE activity was therefore undertaken. The effect of two polysaccharides, glucan and mannan, derived either from yeast or cell walls of yeast, is presented in table 1C. When compared to Tween 80-injected control rats, rats injected with mannan showed no alteration in phagocytic function as denoted by the removal rate of colloidal carbon (fig. 1). Liver, lung and spleen weights were unaltered in the mannan group. In marked contrast, the glucan preparations derived either from yeast cells or from zymosan produced marked hyperplasia of lung and spleen. Phagocytic function denoted either by the removal rate of colloidal carbon or the $\alpha$ index was markedly enhanced in the glucan-treated groups. Analysis of an active glucan preparation demonstrated it to be lipid free.

**DISCUSSION**

The ability of the cell wall from *Saccharomyces cerevisiae* to produce RE hyperfunction and hyperplasia has been amply demonstrated (1-4). This stimulation involves the formation of new RE cells as well as the enlargement and hyperactivity of the pre-existing macrophages (1, 2, 12, 17). Histological studies reveal that zymosan is actively phagocytized by Kupffer cells and within 24-48 hours is broken down or solubilized by RE cells, with the release of an agent that either primarily or secondarily activates the RES. The induced RES hyperplasia was not due to the particulate nature of zymosan since the injection of identical amounts of $\gamma$-iron particles into male rats produced no RE hyperplasia and no alteration in phagocytic function.

A previous investigation of the agent in zymosan that produces RES stimulation led to the conclusion that the active component was a lipid or a mixture of lipids (12). The polysaccharide-protein-ash residues were stated to be inactive (12). The methods employed in the preparation of these materials were not presented by the author, making it impossible to determine whether a loss of polysaccharide activity would result. It is also possible, since certain lipids have been demonstrated to be capable of stimulating the RES (5, 18, 19), that the previous observations (12) may be a reflection of a nonspecific lipid effect.

The finding that an active principle in zymosan is the polysaccharide, glucan, contributes to the understanding of the chemical nature and possible structural requirements of certain RE stimulating agents. Glucan, which is part of the outer membrane of the yeast cell wall (11, 20), has been characterized as a polysaccharide consisting of a chain of glucopyranose units linked by a 1-3 $\beta$-glucosidic linkage, with a molecular weight of approximately 6500 (13). In contrast, mannan, which is associated with a protein in the cell wall (20), has been demonstrated to have a molecular weight of about 100,000 (14) and is composed of a chain of mannose units linked through positions 1 and 6, with a mannopyranose unit linked glycosidically to the second carbon atom of each alternate mannose unit (14). The marked difference in molecular weight indicates that the size of the molecule is not the determining factor in stimulating the RES, but possibly that the unique 1-3 $\beta$ type linkage that characterizes glucan is a requirement. Studies are currently in progress to determine the relationship of the chemical configuration of neutral polysaccharides to their ability to activate the RES.

Since zymosan exhibits many properties similar to endotoxins (1, 3), the relationship between glucan or its degradation products and bacterial lipopolysaccharides that have been demonstrated to produce nonspecific immunity and increased resistance to a number of bacterial infections (21-23) must await further study.

The observations that an RE activator in zymosan is glucan raises the question as to whether the manifold phenomena (1-10, 24) observed in zymosan-injected animals are due solely to the influence of glucan or its degradation products. If glucan can be demonstrated to evoke all of the previously observed alterations in zymosan-injected animals, it is obvious that all of these phenomena will be a manifestation of enhanced RE function. The use of a highly purified chemical agent to produce RE activation should allow further delineation of the physiopathology of the RES.
We are indebted to Miss Jackie Houston and Mrs. Jackie Ann Logue for assistance in the conduct of these experiments, and for the cooperation of Robert Light and Dr. Fred Di Carlo of Standard Brands, Inc., Stamford, Conn., in the preparation of the various fractions of zymosan.

REFERENCES