Alveolar Macrophage-Lymphocyte Rosette Formation: Failure of Levamisole to Alter Activity

RICHARD L. ZIPRIN,* STEPHEN R. FOWLER, AND DONALD A. WITZEL
Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, College Station, Texas 77840

Received for publication 14 December 1976

Levamisole, an anthelminthic agent with immunostimulatory properties, does not alter in vitro macrophage-lymphocyte rosette formation between sheep alveolar macrophages and autochthonous lymphocytes.

Levamisole is a low-molecular-weight synthetic compound, (−)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole, used widely in agriculture and veterinary medicine as an anthelminthic agent. It is also capable of stimulating immune responses in a variety of animal species, including man (1, 3, 6, 8), and has been used successfully in immune therapy of malignant tumors (10, 14). Its mode of action on the immune system is unknown. Evidence shows that levamisole stimulates phagocytic activity (7), macrophage Fc receptor activity (11), and in vitro chemotactic activity (7).

Several lines of evidence show that thymus-derived lymphocyte activity is influenced, if not controlled, by macrophage activity. In addition, some lymphocyte functions are dependent upon antigen processing and presentation by macrophages. The precise nature of such macrophage-lymphocyte interaction is currently in question, but the requirement is no longer in doubt (13). Scolay and Lafferty (12) have shown that sheep lymphocytes in the effluent flow from lymph nodes are more reactive in graft-versus-host reactions than those from the afferent limb of the lymphatic circulation. They have postulated that macrophages within the lymph nodes "groom" the lymphocytes as they pass through the lymph node matrix. The studies by Lipsky and Rosenthal (4, 5) of in vitro macrophage-lymphocyte interactions to form macrophage-lymphocyte rosettes are germane. Such rosette formation is antigen independent but is enhanced by antigen. Macrophage-lymphocyte rosette formation is not due to Fc receptor activity, nor is it a form of phagocytosis. Thus, a separate class of membrane receptors is responsible for macrophage-lymphocyte interactions.

The specific physical association between lymphocytes and macrophages may be a mechanism by which macrophages influence lymphocyte activity (5). We have investigated the effects of levamisole on the ability of sheep alveolar macrophages to participate in macrophage-lymphocyte rosette formation.

Alveolar macrophages were obtained by bronchopulmonary lavage of anesthetized adult sheep with 150 ml of sterile isotonic saline. The alveolar macrophages were recovered from the saline by centrifugation, suspended in sterile Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) containing 1.25% sodium citrate, and again recovered from suspension by centrifugation. The cells were then washed once with sterile Hanks balanced salt solution without citrate and resuspended in Eagle minimum essential medium (Grand Island Biological Co.) containing 10% heat-inactivated fetal bovine serum. Antibiotics added to the Eagle minimum essential medium were: polymyxin B sulfate, 100 U/ml; lincomycin HCl monohydrate, 250 μg/ml (both from Grand Island Biological Co.); penicillin G, 100 μg/ml (Nutritional Biochemicals Group, Cleveland, Ohio); and streptomycin sulfate, 100 μg/ml (Sigma Chemical Co., St. Louis, Mo.).

Peripheral venous sheep blood was collected in heparinized Vacutainers (Becton-Dickinson, Rutherford, N.J.), and the lymphocytes were isolated by gradient centrifugation with Lymphocyte Separation Medium (Litton Bionetics, Kensington, Md.). The isolated lymphocytes were suspended in Eagle minimum essential medium, supplemented with fetal bovine serum and antibiotics as above, and kept at 4°C until ready for use, never more than 18 h.

The phenomenon of in vitro macrophage-lymphocyte rosette formation was described originally in papers by Lipsky and Rosenthal (4, 5), which should be consulted for the experimental details required for its demonstration. A 1 ml suspension containing 10⁶ macrophages was applied to each well of Lab-Tek tissue culture chamber/slides (Miles Laboratories, Naperville, Ill.). The slides were then incubated in...
a 5% CO₂ atmosphere at 37°C for appropriate periods of time as dictated by the requirements of specific experiments. The minimum incubation time was 12 h. Shorter incubation times result in greatly reduced macrophage-lymphocyte rosette formation. During this incubation period, the viable macrophages adhered to the glass surface of the slides, and, in our experiments, they were exposed to levamisole, which was incorporated into Eagle minimum essential medium at the concentrations shown in Table 1. The monolayers were washed to remove the nonadherent and nonviable cells. A 1-ml suspension of autochthonous lymphocytes, 10⁶ to 5 x 10⁶/ml, was placed in each culture chamber, over the glass-adherent macrophages, and the culture chamber/slides were placed on a rotating platform (50 rpm) in a 5% CO₂ atmosphere at 37°C for 1 h. This allowed time for the lymphocytes and macrophages to interact. The superstructure of the chamber slides was broken away, and the slides were gently washed with phosphate-buffered saline (pH 7.2, 0.15 M), air-dried, and stained with Wright stain (Anderson Laboratories, Fort Worth, Tex.). Finally, the percentage of rosette formation and the number of lymphocytes attached per macrophage were determined by microscopic examination of the slides. These experiments were manipulated in a manner that assured uniform macrophage cell layer densities and lymphocyte-macrophage ratios among the treatment groups within an experiment. The single variable was levamisole concentration. Each experiment consisted of six treatment groups (Table 1), with four replicates per group. The data collected were analyzed by conventional statistical methods for significant differences between the treatment means and correlation between levamisole treatment and rosette formation.

Table 1 lists results from 10 experiments. The percentage of macrophage-lymphocyte rosette formation and the number of lymphocytes bound per 100 macrophages.

### Table 1. Effect of levamisole HCl on percentage of macrophage-lymphocyte rosette formation and number of lymphocytes bound per 100 macrophages

<table>
<thead>
<tr>
<th>Expt</th>
<th>Timed exposure/h</th>
<th>Antibiotics in culture medium</th>
<th>Conc of levamisole HCl (µg/ml)</th>
<th>F ratio</th>
<th>$\bar{x}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>3.125</td>
<td>6.25</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>P, Po, L, S</td>
<td>54</td>
<td>56</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>121</td>
<td>112</td>
<td>106</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>P, Po, L, S</td>
<td>70</td>
<td>67</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>P, Po, L, S</td>
<td>63</td>
<td>64</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>145</td>
<td>133</td>
<td>127</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>P, Po, L, S</td>
<td>62</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>Po, L</td>
<td>53</td>
<td>51</td>
<td>49</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>Po, L</td>
<td>78</td>
<td>81</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>P, S</td>
<td>57</td>
<td>55</td>
<td>51</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>P, S</td>
<td>142</td>
<td>162</td>
<td>148</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
<td>P, Po, L, S</td>
<td>50</td>
<td>40</td>
<td>52</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>P, S</td>
<td>175</td>
<td>180</td>
<td>167</td>
</tr>
</tbody>
</table>

Summary of rosette formation (%)

|                         | 55.5 | 57.7 | 56.8 | 57.6 | 58.4 | 55.1 | 0.290 | 56.8 |

Summary of no. of lymphocytes/100 macrophages

|                         | 114.7 | 130 | 120.6 | 134.5 | 123.9 | 112.5 | 0.413 | 122.8 |

Ratio of bound lymphocytes to macrophages

|                         | 2.06  | 2.25 | 2.12 | 2.33 | 2.12 | 2.04 |

---

* In experiments 1 through 8, alveolar macrophages used were from animals that were pre-anesthetized with xylazine and atropine and were anesthetized with sodium thiamylal; in experiments 9 and 10, no pre-anesthetics were used. Fluothane (Ayerst Laboratories, N.Y.) was the sole anesthetic agent.

* P, Penicillin; Po, polymyxin; L, lincomycin; and S, streptomycin.

* $F$ ratio from analysis of variance; 95 and 99% confidence limits are 2.77 and 4.25, respectively.

* Percentage of macrophage-lymphocyte rosette formation.

* Number of lymphocytes bound per 100 macrophages.
NOTES

A rosette was defined as a macrophage with one or more adherent lymphocytes.

The data (Table 1) show no dose-dependent relationship between the concentration of levamisole to which the macrophages were exposed and the percentage, or intensity, of lymphocyte adherence. Some values in an experiment (Table 1) appear to be significantly different from others, but no pattern to the appearance of deviant values in the table can be related to dose of levamisole. All results are similar, regardless of the antibiotic treatment, duration of exposure to levamisole, or the anesthetic and pre-anesthetic agents used during bronchopulmonary lavage.

Subsequent to the above series of experiments, one additional experiment was conducted in which the macrophages were incubated and exposed to levamisole for 3 h. The levamisole concentrations were the same as those indicated in Table 1. The lymphocyte concentration was increased to 1 × 10⁶/ml to produce a practical level of macrophage-lymphocyte rosetting. No dose-dependent increase in rosetting was observed.

Rhodes (9) has shown that macrophage F₅-receptor activity increases with the length of time the cells are held in culture. Similarly, the ability of macrophages to rosette syngeneic lymphocytes also increases with time in culture (4). Schmidt and Douglas have shown that treatment of macrophages with levamisole induces an increase in F₅-receptor activity (11); thus, we needed to determine whether levamisole would also increase the receptor activity for macrophage-lymphocyte interactions, because such results might explain, in part, the immunostimulatory mechanisms of levamisole. However, observations presented here show no effect of levamisole on in vitro macrophage-lymphocyte rosette formation.

ACKNOWLEDGMENTS

Levamisole HCl was kindly supplied by R. G. Eggert of American Cyanamid, Princeton, N.J. We thank James H. Johnson, Claudio Castillo, and Richard Wilson for assistance with bronchopulmonary lavage procedures.

LITERATURE CITED


