Department of Bacterial Pathogenicity[1], Department of Virology[2], The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Protective effect of Oxadin® on experimental *Yersinia enterocolitica* infection in rats

H. Najdenski[1], V. Kussovski[1], Y. Michailov[2] and A. Vesselinova[1]

The effect of Oxadin® (a new Bulgarian antimicrobial chemotherapeutic agent) on some parameters of non-specific immune response was investigated in a rat model of infection. After mimicking natural *Yersinia enterocolitica* systemic infection the number and functional activity of blood leucocytes and peritoneal macrophages were compared between groups of animals treated with Oxadin® before and after infection. A significant immunostimulating effect of Oxadin® was found in both experimental groups but was better expressed when administered before *Yersinia* infection. Bactericidal response of peritoneal macrophages (killing ability) and phagocytic activity of polymorphonuclear leucocytes from animals treated with Oxadin® and thereafter infected with *Yersinia enterocolitica* were significantly activated during the first week of study. These findings correlated with the enhanced number of both types of phagocytic cells and the higher glycolytic activity of peritoneal macrophages.

1. Introduction

During the past two decades, *Yersinia enterocolitica* has aroused the interest of clinicians and microbiologists because of its wide range of clinical manifestations and increasing knowledge of the genetic regulation of its virulence factors. Infection with *Y. enterocolitica* causes intestinal lesions including acute enteritis, enterocolitis and mesenterial lymphadenitis [1, 2]. In rare cases, particularly in immunocompromised hosts, *Y. enterocolitica* can induce disseminated infection with abscess formation in the spleen, liver, and other organs [3, 4].

Recommendations of the World Health Organization for antibiotic treatment of yersiniosis include tetracycline, chloramphenicol, gentamycin, and cotrimoxazole [5]. On the basis of more recent results [6, 7], the cephalosporins cefotaxime, ceftazidime, and ceftriaxone; aminoglycosides; imipenem; ticarcillin-clavulanic acid; aztreonam; fluoroquinolones, and trimethoprim-sulphamethoxazole may prove useful in the therapy of zoonotic yersinioses.

Despite the diversity of available antibacterial agents nowadays, *Yersinia* infections are still a serious problem with their unsolved pathogenesis [8]. Moreover, many antibiotics are overused in veterinary practice and thereby considered as the primary cause for multiplication of resistant strains [9]. Hence, the synthesis and characterization of new antibacterial agents have been intensively investigated [10, 11]. Previous studies have established that Oxadin® (a new original Bulgarian antimicrobial chemotherapeutic agent) has a well expressed *in vitro* inhibiting and bactericidal effect on *Y. pseudotuberculosis* and *Y. enterocolitica* [12].

The present study was undertaken to estimate the effect of Oxadin® on some parameters of immunological reactivity in a reproducible rat model of systemic *Yersinia* infection. The influence of Oxadin® on the quantity and functional activity of the effector cells responsible for the natural resistance of a macroorganism – blood leucocytes and peritoneal macrophages – were examined following different schemes for its application.

2. Investigations, results and discussion

The results showing total number of blood leucocytes are given in Fig. 1. On day 1 a significant increase in number of leucocytes was observed only in the group of animals infected with *Y. enterocolitica* (group IV). A week later (day 7) strong leucocytosis was found in groups I (rats treated with Oxadin®) and III (pre-treated with Oxadin® and infected with *Y. enterocolitica*). At the last interval (day 15) significant leucocytosis was observed only in group III. The phagocytic activity of polymorphonuclear leucocytes against *Y. enterocolitica* cells calculated as phagocyte number (PN) and phagocytic index (PI) was not changed during the first day of examination in groups I to IV. During the next two intervals (days 7 and 15) significantly enhanced phagocytic activity in both PN and PI was established in groups I, II, and III (Fig. 2).

In contrast to the leucocyte number, peritoneal macrophages were found in large numbers in groups I, II, and III during day 1 of examination. Gradually the number of peritoneal macrophages was normalized being raised only in group III by day 7 (Fig. 3). A strong killing effect of peritoneal macrophages against *Y. enterocolitica* cells was established as early as day 1 in groups II, III, and IV. During the next two intervals (days 7 and 15) the augmen-

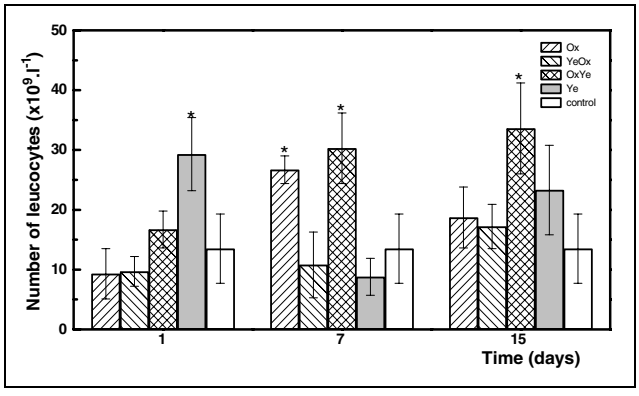

Fig. 1: Number of blood leucocytes from rats. Groups: Ox – Oxadin® [group I – rats treated with Oxadin®]; YeOx – *Y. enterocolitica* + Oxadin® [group II – rats infected intraperitoneally with *Y. enterocolitica* and subsequently treated with Oxadin®]; OxYe – Oxadin® + *Y. enterocolitica* [group III – rats treated with Oxadin® and subsequently infected intraperitoneally with *Y. enterocolitica*]; Ye – *Y. enterocolitica* [group IV – rats infected intraperitoneally with *Y. enterocolitica* control – group V [healthy animals]. Values are +/– S.D.
* – Significantly different from control [$P < 0.01$]

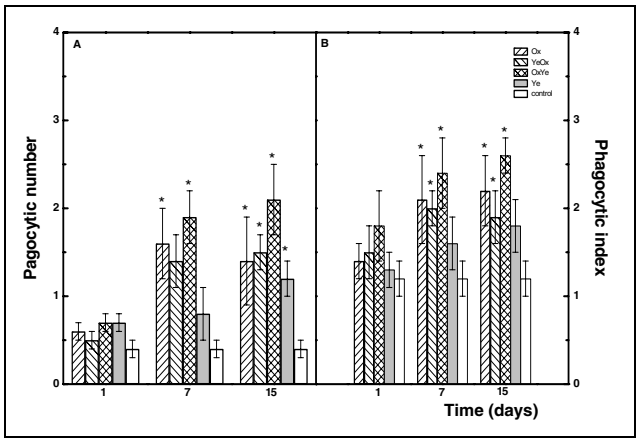

Fig. 2: Phagocytic activity of polymorphonuclear leucocytes from rats. A – phagocytic number; B – phagocytic index. Groups – see Fig. 1. Values are +/– S.D.
* – Significantly different from control [P < 0.01]

ted killing ability of macrophages was retained in group III only (Fig. 4A). Macrophages having higher glycolytic activity were isolated from animals of group III during the whole experimental period (Fig. 4B). Inconsistently raised glycolytic activity of macrophages was detected in group IV (day 1) and group II (days 7 and 15).

Circulating leucocytes and peritoneal macrophages are an important element in host resistance to infection. As the first protective cells against a potential bacterial invader (in our case *Y. enterocolitica*) the assessment of the influence of Oxadin® on their functional activity was of great importance. Evidently, a marked immunomodulatory effect was observed only after Oxadin® pre-treatment of *Yersinia* infected rats.

We found that the bactericidal response of peritoneal macrophages (killing ability) and phagocytic activity of polymorphonuclear leucocytes from rats treated with Oxadin® and infected thereafter with *Y. enterocolitica* (group III) were significantly activated on days 3 and 7 of examination. These findings correlated with the increased number of both types of phagocytic cells. Generally, Oxadin® was shown to induce an influx of peritoneal macrophages with higher glycolytic activity and provoke a more highly enhanced "killing" ability of these cells. This correlation

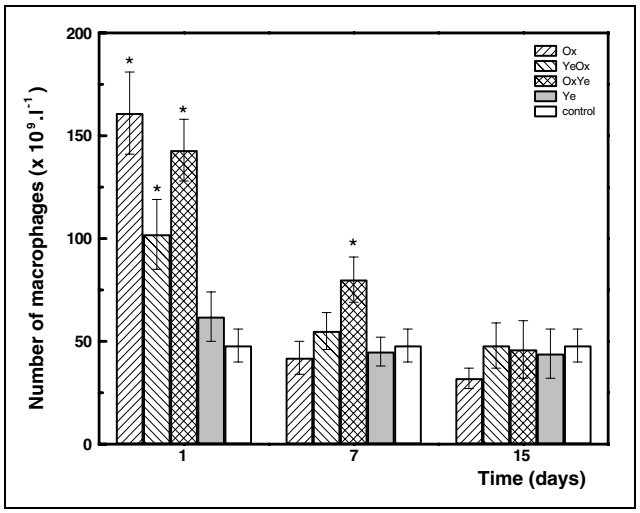

Fig. 3: Number of peritoneal macrophages from rats. Groups – see Fig. 1. Values are mean +/– S.D.
* – Significantly different from control [P < 0.01]

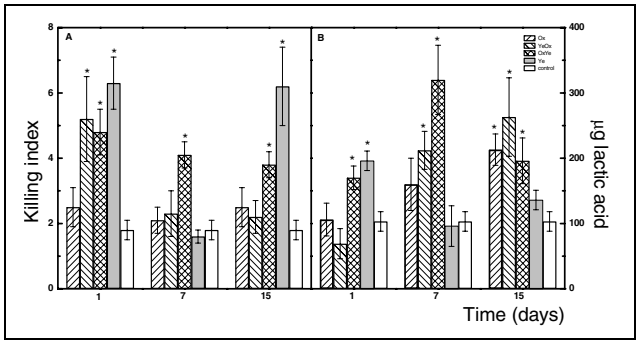

Fig. 4: Killing ability presented as killing index against *Y. enterocolitica* cells [A] and glycolytic activity [B] of peritoneal macrophages from rats. Groups – see Fig. 1. Killing index ratio of bacterial number at zero time and bacterial number after 2 h incubation with macrophages. Values are mean +/– S.D.
* – Significantly different from control [P < 0.01]

confirms the hypothesis that the glycolytic metabolic pathway provides energy for the defense functions of this type of phagocytic cell [16].

Our study suggests that Oxadin® can produce immunostimulation with regard to the early cellular defense mechanisms against invading bacterial pathogens. From previous *in vitro* experiments [12] and from the results of this study Oxadin® may be classified as an effective nonspecific immunostimulator preventing various bacterial infections and their complications, in particular *Y. enterocolitica* infections.

3. Experimental

3.1. Materials

3.1.1. Bacterial strains

The strain IP 8944 of *Y. enterocolitica*, used in this study, belongs to serogroup 0 : 3, biotype 4, pYV+ and is non-enterotoxigenic. This strain had been isolated from a man in Greece and was kindly provided by Dr. E. Carniel (Yersinia Reference Center, Institute Pasteur, Paris, France). The bacteria were cultivated in BHI broth (Difco) at 25 °C for 24 h, then diluted 1 : 15 in fresh BHI broth supplemented with 0.2 M $MgCl_2$ and further cultivated for 4 h at 37 °C.

3.1.2. Antimicrobial agent

Oxadin® is the sodium salt of 2-(4-uracilmetilen)-5-(4-bromphenil)-6-hydroxy-2,3-dihydro-(6*H*)-1,3,4-oxadiazin synthesized by Troya-Farm Company (Bulgaria).

3.1.3. Experimental animals

Thirty nine rats weighing 180.0 ± 10.0 g were divided in 4 groups each of 9 animals and a control group of three animals as follows:

Group I – healthy animals treated intraperitoneally with Oxadin® 10% (0.5 ml of a dose of 50 mg daily, for three consecutive days);

Group II – rats infected intraperitoneally with *Y. enterocolitica* 0 : 3 (1.0×10^9 cfu) and treated after 24 h with Oxadin® as for group I;

Group III – animals treated with Oxadin® as for group I and then infected with *Y. enterocolitica* 0 : 3 as described for group II;

Group IV – rats infected intraperitoneally with *Y. enterocolitica* 0 : 3 (1.0×10^9 cfu) only;

Group V – controls (healthy animals).

At intervals of 1, 7, or 15 days after the last procedure described for each experimental group the animals were sedated by intravenous injection of sodium pentobarbital and killed.

3.2. Methods

3.2.1. Harvesting of polymorphonuclear leucocytes

The leucocytes were separated from heparinized blood (10 U of heparin per ml) using a 3% solution of gelatin and Polysep (Pharmachim, Bulgaria) as separation media following the manufacturer's instructions. Purity of polymorphonuclear leucocytes was ≥86%, and viability determined by the trypan blue exclusion test, was ≥92% in all specimens.

3.2.2. Phagocytosis assay

This was performed in vitro using polymorphonuclear leucocytes according to a method described earlier [13]. The results are presented as phagocytic number (PN, the mean number of bacteria engulfed by 100 leucocytes) and phagocytic index (PI, the mean number of bacteria engulfed from the leucocytes which phagocytosed), counting 3.0×10^2 leucocytes from different sections of the monolayer stained by Giemsa.

3.2.3. Harvesting of peritoneal macrophages

Peritoneal macrophages were obtained after four-fold lavage of peritoneal cavity with 10 ml cold Hank's solution. Viability determined by the trypan blue exclusion test was > 90%. Polymorphonuclear leucocytes and peritoneal macrophages were enumerated and standardized by counting in a Burker's chamber.

3.2.4. Killing ability of macrophages

The Killing test was carried out by the method of Vissar et al. [13]. 1.0×10^6 peritoneal macrophages/ml and 1.0×10^7 bacterial cells/ml were incubated together in a volume of 500 µl with rotation at 37 °C in separate tubes for each time point. At time zero and after 2 h, 550 µl of ice-cold Hanks was added to the suspension to stop phagocytosis. Killing indicated by the decrease in the total number of bacteria (i.e. bacteria associated with macrophages and non-cell-adherent bacteria in the supernatant) was assessed by adding 400 µl samples of the bacterium-cells suspension to 1600 µl of ice-cold distilled water and mixing the suspension for 1 min to lyse the macrophages. The number of viable bacteria was enumerated microbiologically. The "killing index" parameter was determined as a ratio of initial bacterial number at time zero to the bacterial number after 2 h incubation with macrophages.

3.2.5. Glycolytic activity of macrophages

Glycolysis was assayed according to Barker and Summerson with some modifications [15]. Lactic acid production was measured in the reaction mixture with the following components in 2.5 ml: 80 µM of Tris buffer (pH 7.2), 60 µM of glucose, 25 µM of KCN and 2.0×10^7 peritoneal macrophages. After a 2 h incubation at 37 °C without shaking, 0.5 ml of 30% trichloroacetic acid was added to each sample. The amount of lactic acid was determined in protein-free supernatant. In the presence of sulfuric acid and *p*-hydroxybiphenyl, the lactic acid is turned into a purple compound. The intensity of this coloration was measured at a wavelength of 540 nm in a Zeiss Spekol spectrophotometer (Model 11, Jena, Germany). The results are expressed as µg of lactic acid released by 2.0×10^7 cells in 2 h.

3.2.6. Statistical analysis

The results are expressed as the mean value ± standard deviation. To assess the significance of differences within experiments Student's t-test was used. Statistical significance was defined as $P < 0.01$.

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Assoc. Prof. H. Najdenski, Ph.D.
Institute of Microbiology
Bulgarian Academy of Sciences
"Acad. G. Bontchev" Str., Bl. 26
1113 Sofia
Bulgaria
usema@bas.bg