Investigations in animals and humans with acquired or induced immunodeficiency states have clearly demonstrated that antimicrobial agents are most effective when host immune responses are unimpaired. Synergistic interactions which occur between the host immune system and antimicrobial agents may contribute to the successful outcome of antimicrobial chemotherapy. Ideally, therefore, antimicrobial agents should potentiate or at least not depress immunological responses. However, in practice this may not always be the case since many commonly used antimicrobial agents have been reported to inhibit cellular and humoral immune reactivity. Although such immunosuppressive effects are unlikely to influence the short-course antimicrobial chemotherapy of acute infections in immunologically normal individuals, they may complicate responses in immunocompromised patients. The absence of immunosuppressive properties may be an important factor to be considered in the selection of antimicrobial agents for therapeutic and prophylactic use in immunocompromised individuals. More research is therefore required into the effects of antimicrobial agents on immune responses in vivo.

We have previously reported that erythromycin increases the random and leuco-attractant-induced migration of human polymorphonuclear leucocytes (PMNLs) in vitro. Ingestion of erythromycin stearate (4 x 250 mg tablets daily) by normal adult volunteers was not associated with increased PMNL migration in vivo. However, it has recently been reported that the procedures involved in processing PMNLs for in vitro experiments cause the removal of cell-associated drugs. We have therefore repeated and extended our previous investigations using an increased dose of erythromycin stearate as well as higher serum concentrations in the PMNL migration assay. With this system we have found that ingestion of erythromycin stearate is associated with enhanced PMNL migration in vitro.

Material and methods
Healthy adult volunteers were used to assess the effects of ingestion of a single oral dose of 500 mg erythromycin stearate on PMNL migration, phagocytosis and antimicrobial activity. These PMNL functions were tested before erythromycin intake and 90 minutes and 1 week after ingestion of the antibiotic.

Erythromycin
Erythromycin base was used for in vitro experiments; it was dissolved in the appropriate cell-suspending medium and its effects on the various PMNL functions investigated at concentrations ranging from 2.7 x 10^-6 M to 5 x 10^-4 M (2 - 370 µg/ml).

PMNL functions — studies of cell motility
PMNLs were obtained from heparinized venous blood (heparin 5 U/ml) and resuspended to a final concentration of 6 x 10^7/ml in N-2-hydroxy-ethylpiperazine-N'-2-ethane-sulfonic acid (Sigma Chemical Co., St Louis, Mo.) — buffered Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY) supplemented with 0.05% bovine serum albumin (BSA) and 5% heat-inactivated autologous serum after hypotonic lysis of residual erythrocytes with 0.84% ammonium chloride. Serum was included in the cell-suspending medium so it enhances the solubility of erythromycin. Endotoxin-activated fresh autologous serum (EAS) was used as the leuco-attractant. The serum was activated with bacterial endotoxin (Escherichia coli 0127:B8; Difco Laboratories, Detroit, Mich.) 500 µg/ml, which was diluted eightfold for in vitro studies. In random migration systems the leuco-attractant was replaced with an equal volume of HBSS. The erythromycin was
added to the PMNLs and remained with the cells in the chemotaxis chamber throughout the incubation period. The assays of motility were performed in modified Boyden chambers with membrane filters (5 μm pore size). The chambers were incubated for 90 minutes and the results expressed as the number of cells which completely traversed the filter (in cells per high-powered microscope field) as an average of results for triplicate filters.

For in vitro studies only migration to EAS was investigated before and after ingestion of erythromycin.s streake. To stimulate in vitro conditions and compensate for loss of cell-associated erythromycin, the PMNLs were resuspended in 20% time-matched autologous serum and the EAS concentration was increased to 25%. Incubation times of 60 minutes and 90 minutes were used.

**Leucotactic potential of erythromycin**

The intrinsic leucotactic potential of erythromycin was assessed using the checkerboard filter assay of Zigmond and Hirsch. Migration stimulatory concentrations of the antibiotic were placed above and below the filter to provide positive and negative gradients and chemokinetics systems in the absence of a leuco-attractant. The leucotactic potential of erythromycin was also studied by an orientation assay as described below, using positive concentration gradients of the antibiotic in the absence of a known leuco-attractant.

**Effects of erythromycin on the leuco-attractant-induced orientation of PMNLs**

The orientation assay was performed according to the method of Zigmond using a 3 mm thick perspex microscope slide with two wells 1 mm deep and 4.5 mm wide separated by a 1 mm bridge across the width of the slide. The two wells were filled with HBSS solution, the one being supplemented with BSA (0.05%) and the other with 12.5% EAS.

Untreated control of erythromycin-treated PMNLs adherent to glass coverslips were placed on the bridge between the two wells and the orientation chamber was incubated at 37°C on a heated microscope stage. PMNLs in the centre of the bridge were observed by interference contrast microscopy between 10 and 15 minutes after chamber assembly. The direction of orientation was judged on morphological examination. A minimum of 100 polarized PMNLs were scored. These results are expressed as the percentage of PMNLs orientated towards the EAS.

**Complement activation**

The effects of erythromycin at a fixed concentration of 2.5 x 10^{-6} M on complement activation by bacterial lipopolysaccharide were assessed by incubating 0.8 ml fresh normal serum with 500 μg lipopolysaccharide (E. coli 0127:B8) and erythromycin for 30 minutes at 37°C (final volume 1 ml) followed by measurement of the total haemolytic complement activity using the checkerboard filter assay of Zigmond and Hirsch. PMNLs were omitted from the control system. The tubes were incubated on a rotator at 37°C and aliquots of 0.5 ml were transferred into 2.5 ml ice-cold PBS after 5, 15 and 30 minutes of incubation, centrifuged and washed twice (500 g for 5 minutes) to remove non-phagocytosed bacteria; the radioactivity associated with the PMNL pellet was measured in a liquid scintillation spectrophotometer. The results are expressed as the percentage of Staph. aureus phagocytosed after correction for background values.

**PMNL antimicrobial activity**

To measure PMNL antimicrobial activity, 5 x 10^{6} PMNLs were incubated with 1 x 10^{8} Staph. aureus (Cowan I) in a final volume of 1 ml PBS. The bacteria were pre-opsonized using fresh autologous serum. The tubes were rotated for 30, 60 and 90 minutes at 37°C, after which 0.1 ml aliquots were withdrawn and transferred to 0.9 ml sterile distilled water to lyse the PMNLs and release the ingested bacteria. Serial tenfold dilutions were then made and 0.1 ml aliquots transferred to nutrient agar plates, incubated overnight at 37°C and the number of CFUs enumerated. The results are expressed as the percentage killing of Staph. aureus by PMNLs in relation to a control system from which the PMNLs were omitted. The in vitro effects of erythromycin were investigated by adding the antibiotic (2 μg/ml) to both the control and experimental systems and comparing the results with those obtained in the absence of the antibiotic. PMNL staphylocidal activity was also assessed after ingestion of a single oral dose of 500 mg erythromycin stearate.

**Chemiluminescence**

Post-phagocytic oxidative metabolism was investigated by measuring chemiluminescence. PMNLs (3 x 10^{6}) were incubated with Candida albicans (2 x 10^{6}), 10% fresh autologous serum and 10^{-6} M luminol (5-aminio-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St Louis, Mo.) in a final reaction volume of 1 ml HBSS on a rotator at 37°C. After 10 minutes of incubation, which gave maximal chemiluminescence using this system, the emitted light was measured using a Lumac Biocounter (model 2010, Lumac Systems Inc., Titusville, Fl) and recorded as counts per minute (cpm). For measurements of resting chemiluminescence, C. albicans was omitted. PMNL chemiluminescence was measured before and after ingestion of erythromycin stearate by normal individuals.

**Animal studies**

Groups of 16 - 20 2-month-old sex-matched NMRI mice weighing approximately 20 g were infected intraperitoneally containing tritiated thymidine (thymidine-methyl-3H; New England Nuclear Corp., Boston, Mass.) 20 μCi/10 ml nutrient broth. After 18 hours' incubation at 37°C the bacteria were washed three times with sterile PBS and resuspended to 5 x 10^{8} colony-forming units (CFUs) per ml of PBS supplemented with glucose (10^{-4} M), calcium and magnesium (5 x 10^{-6} M). The bacteria were pre-opsonized by incubating 0.3 ml of the bacterial suspension with 1,2 ml of 5% fresh autologous serum in PBS for 30 minutes at 37°C on a rotator, after which the bacteria were centrifuged and washed (2000 g for 15 minutes) and resuspended to 1 x 10^{6} CFUs/ml in PBS. To measure phagocytosis PMNLs and radiolabelled, opsonized Staph. aureus were incubated at a ratio of 1:10 in a reaction volume of 2 ml containing 5 x 10^{6} PMNLs and 5 x 10^{8} bacteria per ml. PMNLs were omitted from the control system. The tubes were incubated on a rotator at 37°C and aliquots of 0.5 ml were transferred into 2.5 ml ice-cold PBS after 5, 15 and 30 minutes of incubation, centrifuged and washed twice (500 g for 5 minutes) to remove non-phagocytosed bacteria; the radioactivity associated with the PMNL pellet was measured in a liquid scintillation spectrophotometer. The results are expressed as the percentage of Staph. aureus phagocytosed after correction for background values.

The effects of erythromycin base on PMNL phagocytosis of Staph. aureus were assessed in vitro at a fixed antibiotic concentration of 2.7 x 10^{-6} M (2 μg/ml). The erythromycin was added during opsonization. PMNL phagocytosis was also investigated after ingestion of erythromycin stearate by adult volunteers using time-matched autologous PMNLs and serum.
with 2 x 10^7 CFUs of C. albicans (strain Y240: CBS 5736, obtained from the South African Institute for Medical Research, Johannesburg). C. albicans were passaged twice in mice, reisolated, cultured in vitro, harvested, washed, enumerated and injected intraperitoneally in a final volume of 0.5 ml PBS. One hour before experimental infection of the mice the animals in the experimental groups were injected intraperitoneally with erythromycin lactobionate 1.5 mg (obtained from Abbott Laboratories (Pty) Ltd, Johannesburg) in a volume of 0.5 ml PBS. Control mice were injected with 0.5 ml PBS containing 0.045 mg benzyl alcohol preservative (contained in the erythromycin lactobionate). The 1.5 mg dose of the antibiotic used in these experiments was selected because it was the highest dose which caused no detectable irritant or toxic effects in the mice. The 60-minute period between injection of erythromycin and induction of the experimental infections was chosen because the intraperitoneal concentration of erythromycin lactobionate at this time (30 μg/ml) was considerably less than the minimal inhibitory concentrations for C. albicans (> 6 mg/ml). Results of these experiments are expressed as mean survival times.

**Serum levels of erythromycin**

These were measured using an erythromycin base standard and *Bacillus subtilis* (strain ATCC 6633) as the test organism.

**Expression and statistical assessment of results**

The results of investigations of PMNL functions are expressed as the mean value with standard error for each series of experiments. Statistical analyses of these results were performed using Student’s t test (paired t statistic).

**Results**

**PMNL migration**

Erythromycin base at concentrations of > 5 x 10^-1 M enhanced PMNL random motility and migration to EAS (Table I). Ingestion of a single oral dose of 500 mg erythromycin stearate was associated with a significant increase in PMNL migration to

### TABLE I. IN VITRO EFFECTS OF ERYTHROMYCIN ON PMNL RANDOM MOTILITY AND MIGRATION TO AUTOLOGOUS EAS

<table>
<thead>
<tr>
<th>Test system</th>
<th>Migration to EAS</th>
<th>Random migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMNL only (control)</td>
<td>164 ± 17</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>PMNL + 5 x 10^-7 M erythromycin</td>
<td>172 ± 18</td>
<td>12 ± 3*</td>
</tr>
<tr>
<td>PMNL + 2.5 x 10^-7 M erythromycin</td>
<td>187 ± 24</td>
<td>13 ± 3*</td>
</tr>
<tr>
<td>PMNL + 5 x 10^-7 M erythromycin</td>
<td>226 ± 22***</td>
<td>15 ± 3**</td>
</tr>
</tbody>
</table>

*P < 0.05.
**P < 0.01.
***P < 0.025.

Results are the means ± SE for 5 subjects, expressed as cells per high-powered microscope field.

### TABLE II. EFFECTS OF INGESTION OF A SINGLE ORAL DOSE OF 500 mg ERYTHROMYCIN STEARATE ON PMNL MIGRATION TO EAS, PHAGOCYTOSIS AND ANTIMICROBIAL ACTIVITY

<table>
<thead>
<tr>
<th>Time of testing</th>
<th>PMNL migration to EAS</th>
<th>Chemiluminescence (cpm x 10^3)</th>
<th>Phagocytosis (%) of Staph. aureus after:</th>
<th>Intracellular killing of Staph. aureus (No. of surviving bacteria x 10^4) after 60 min incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min</td>
<td>90 min</td>
<td>5 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Before ingestion of erythromycin stearate</td>
<td>116 ± 18</td>
<td>169 ± 15</td>
<td>6.6 ± 2.6</td>
<td>73 ± 6</td>
</tr>
<tr>
<td>90 min after ingestion of one oral dose of erythromycin stearate 500 mg</td>
<td>151 ± 13*</td>
<td>212 ± 10**</td>
<td>6.6 ± 2.6</td>
<td>70 ± 6</td>
</tr>
<tr>
<td>7 days after the ingestion of erythromycin stearate</td>
<td>87 ± 8</td>
<td>131 ± 18</td>
<td>8.2 ± 2.7</td>
<td>79 ± 3</td>
</tr>
</tbody>
</table>

*P < 0.005.
**P < 0.025.
***P < 0.05.

Results expressed as mean values ± SE for 8 subjects in PMNLs per high-powered field.

Percentage Staph. aureus ingested by PMNL (8 subjects). Results for 6 subjects. Mean serum erythromycin value for the group = 1.2 ± 0.78 mg/ml.
EAS for both incubation periods, with a return on subsequent testing after 7 days to pre-erythromycin administration levels (Table II). Erythromycin per se was not chemotactic according to results obtained with both the filter and orientation assays (results not shown). The antibiotic at a concentration of 5 x 10^{-4} M (but not at lower concentrations) caused a slight but insignificant increase in the numbers of PMNLs orientated in an EAS gradient. The mean values ± SE for four different experiments were 81 ± 3 % and 83 ± 3 % orientated PMNLs in the control systems and for systems containing 5 x 10^{-3} M erythromycin respectively.

**Complement activation**

Erythromycin at a concentration of 2,5 x 10^{-4} M had no effect on the extent of complement activation mediated by bacterial lipopolysaccharide. The mean values with SE for three different experiments were 158 ± 5, 155 ± 5, 76 ± 11, and 77 ± 7 CH50 units respectively for untreated serum, serum treated with erythromycin only, serum treated with bacterial lipopolysaccharide only, and serum treated with both erythromycin and lipopolysaccharide.

**Phagocytosis**

Although slight potentiation of phagocytosis of erythromycin-treated Staph. aureus by PMNLs was observed after 5 minutes incubation, these effects were not significant. No difference between the control systems and systems containing erythromycin was observed after 15 minutes and 30 minutes of incubation (Table III). Likewise, ingestion of erythromycin was not associated with detectable effects on PMNL phagocytosis of Staph. aureus in vitro.

**Antimicrobial activity**

Erythromycin at the concentration tested potentiated PMNL killing of Staph. aureus (Table III). Ingestion of erythromycin by normal individuals was also associated with significantly enhanced PMNL bactericidal activity for Staph. aureus (Table II).

**Animal studies**

Pretreatment of mice with erythromycin lactobionate 1,5 mg significantly (P < 0,005; t statistic for two means) improved the mean survival times of the mice on subsequent challenge with C. albicans. Control mice (N = 20) had a mean survival time (± SD) of 36,4 ± 9,4 h after intraperitoneal infection with C. albicans, while the 20 pretreated mice had a mean survival time (± SD) of 60,8 ± 2,2 h. Since these micro-organisms are resistant to the antimicrobial activity of erythromycin, it is likely that the protective effects of the antibiotic observed in these studies are related to nonspecific immunopotentiating properties of the antibiotic.

A series of control experiments were included to test the validity of this conclusion. We observed for C. albicans that inclusion of erythromycin 30 µg/ml in the culture medium did not affect: (i) the rate of growth of C. albicans; or (ii) the rate of phagocytosis of C. albicans in comparison with untreated control C. albicans. Furthermore, the mean survival times of mice infected with untreated C. albicans or C. albicans grown in medium containing erythromycin 30 µg/ml were 38,6 ± 8,5 h and 41,3 ± 6,7 h respectively. These results are not significantly different and show that the increased survival times of erythromycin-treated mice are due to the effects of the antibiotic on the host immunodefence system (and not directly on the microorganism).

**Discussion**

Forsgren and Schmeling have previously reported that erythromycin does not affect PMNL migration in vitro. However, we have found that erythromycin at concentrations considerably greater than those attainable in vivo enhances the random and leuco-attractant-induced migration of human PMNLs in vitro. The difference in results between the two studies is probably related to differences in the concentrations of erythromycin which were used. In this study we have confirmed our previous finding that erythromycin increases the movement of human PMNLs in vitro. The 5 x 10^{-4} M concentration of the antibiotic (equivalent to 370 µg/ml) at which maximal stimulation of PMNL migration was observed was 200-fold greater than the serum levels of erythromycin attained during antimicrobial chemotherapy (Bio-availability Records, Abbott Laboratories, 1975). These findings therefore seem to be of questionable in vivo significance. Furthermore, we have previously reported that ingestion of erythromycin stearate (4 x 250 mg tablets daily for 4 days) by normal adult volunteers was not associated with any detectable effect on PMNL migration. However, the concentrations of other agents such as levamisole and dapsone which enhance PMNL motility in vivo are considerably less than those required to achieve the same effect in vitro. Detection of immunopharmacological effects in vitro may also be dependent on the presence of adequate concentrations of autologous serum in the PMNL migration assay system to compensate for the loss of cell-associated drug which occurs during cell processing. For these reasons we repeated the investigation of the in vivo effects of erythromycin on PMNL migration using a 500 mg dose of erythromycin stearate and increased the concentrations of autologous serum used in the assay system. Using these modifi-

<table>
<thead>
<tr>
<th>TABLE III. EFFECTS OF A FIXED CONCENTRATION OF 2,7 x 10^{-4} M (2 µg/ml) ERYTHROMYCIN BASE ON PMNL PHAGOCYTOSIS OF STAPH. AUREUS AND STAPHYLOCCIDAL ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test system</td>
</tr>
<tr>
<td>PMNL only (control)</td>
</tr>
<tr>
<td>58 ± 9*</td>
</tr>
<tr>
<td>PMNL + 2,7 x 10^{-4} M erythromycin base</td>
</tr>
</tbody>
</table>

*P < 0.005.

1Results expressed as the mean values ± SE for 3 subjects (% of Staph. aureus ingestion by PMNLs).

2Results expressed as the mean values ± SE for 8 subjects.
cations we have found that ingestion of erythromycin was associated with increased PMNL migration to autologous EAS. The mean serum concentration for the group of 9 adult volunteers was 1.2 μg/ml and the stimulation of PMNL migration was 30% and 25% after 60 minutes' and 90 minutes' incubation respectively. The serum concentrations of the antibiotic may not accurately reflect the cellular concentration of the antibiotic in vitro since erythromycin concentrates 13-fold in human PMNLs and 20-fold in rabbit alveolar macrophages.

The mechanism of enhanced PMNL migration mediated by erythromycin in vitro is related to the anti-oxidant activity of the antibiotic by protection of the cell from auto-oxidation mediated by the myeloperoxidase/hydrogen peroxide/halide system. In the present investigation we have found that erythromycin per se is not leucotactic, does not affect the generation of serum-derived leuco-attractants and does not increase the numbers of cells responsive to EAS as measured by orientation. Enhanced PMNL locomotion is probably due to the ability of erythromycin to sustain cellular migration by reducing the level of leuco-attractant-induced auto-oxidation.

Erythromycin at a therapeutic concentration (2 μg/ml) caused a slight but insignificant increase in the phagocytosis of Staph. aureus by PMNLs in vitro. Ingestion of the antibiotic by the adult volunteers was not associated with potentiation of PMNL phagocytosis. Erythromycin 2 μg/ml significantly enhanced PMNL phagocytosis in vitro and this effect was also observed after ingestion of the antimicrobial agent. Post-phagocytic oxidative metabolism was slightly (7%) reduced after erythromycin intake, an effect which may be related to increased PMNL migration. Since PMNL phagocytosis and post-phagocytic chemiluminescence were not increased by erythromycin either in vitro or in vivo, it is likely that the enhancement of PMNL antimicrobial activity by erythromycin is a consequence of the antimicrobial activity of the antibiotic. The increased PMNL antimicrobial activity following ingestion of erythromycin was observed using a 5% serum concentration containing only 0.06 μg of the antibiotic.

To determine the in vitro significance of erythromycin-mediated increased PMNL migration, the effects of the antibiotic on the mean survival time of mice experimentally infected with lethal doses of C. albicans were investigated. These microorganisms were used since they are resistant to the antimicrobial effects of erythromycin. Pretreatment of the mice with erythromycin lactobionate was associated with a significant increase in the mean survival time in comparison with that of untreated control mice. Since erythromycin is not antimicrobial for C. albicans, the most likely mechanism responsible for the increased survival time of the antibiotic-treated mouse is nonspecific immunopotentiation, possibly by stimulation of PMNL migration.

We have achieved similar results using erythromycin base, thus excluding possible immuno-enhancing effects of lactobionate. We accept that the relationship between erythromycin-mediated increased PMNL migration in adult humans and nonspecific immunopotentiation in the mouse model requires further investigation. Experiments are being performed to determine the minimal concentrations of erythromycin at which the protective effects are evident and to measure the effects of erythromycin on PMNLs and other cellular immune functions such as mononuclear leucocyte proliferation and migration in erythromycin-treated mice.

This study has demonstrated that in addition to antimicrobial activity, erythromycin possesses immunostimulatory properties which are evident in vitro. It is likely that interactions which occur between erythromycin and the host immunodefence system contribute to the successful outcome of antimicrobial chemotherapy with this antibiotic.

REFERENCES