*S. Ahlstedt, B. Kaijser*

# Synergistic Protective Effect of Antibodies and Ampicillin in Mice Infected Intraperitoneally with Escherichia coli

*Summary:* The possibility of a synergistic effect between immune factors and ampicillin against bacterial infections has been investigated in mice infected intraperitoneally with *Escherichia coli.* Following active immunization against the O antigen, synergy between the specific immune factors arising and ampicillin was observed provided that the animals were not infected until the antibodies were formed, i. e. four days after immunization. If the animals were infected three days after immunization, i. e. before the antibodies were detectable, no such synergistic effect could be seen. Injection of mice with rabbit *E. coli* O antibodies four hours prior to infection and subsequent treatment with ampicillin resulted in enhanced protection of the animals, compared to that observed with antibodies or ampicillin alone. Furthermore, one rabbit antiserum out of three raised against the capsular K antigen also showed a synergistic protective effect with ampicillin. The results suggest that antibodies may have a beneficial effect on the treatment of infections with a bactericidal drug such as ampicillin, but that the biological significance of these antibodies has to be further elucidated. In contrast, stimulation of the immune defence system with serologically unrelated *E. coli* bacteria without increase of homologous antibodies, does not appear to enhance bacterial killing by ampicillin.

*Zusammenfassung: Synergistische Schutzwirkung von Antikörpern und Ampicillin bei intraperitoneal mit Escherichia coli infizierten Mäusen.* Zur Beurteilung eines möglichen synergistischen Effektes von Immunfaktoren und Ampicillin wurden intraperitoneal mit *Escherichia coli* infizierte Mäuse als Modell benutzt. Aktive Immunisierung gegen das O-Antigen ergab einen gewissen Synergismus der gebildeten Immunfaktoren mit Ampicillin, vorausgesetzt, die Tiere wurden erst nach der Bildung von Antikörpern, d. h. vier Tage nach Immunisierung, infiziert. Wurden die Tiere drei Tage nach der Immunisierung infiziert, d. h. bevor Antikörper meßbar nachweisbar waren, so war keine derartige synergistische Wirkung festzustellen. Wurden *E. coli*-O-Antikörper vom Kaninchen Mäusen vier Stunden vor der Infektion und der Penicillinbehandlung gegeben, so ergab sich eine gesteigerte Schutzwirkung im Vergleich zu der Wirkung je von Antikörpern oder Ampicillin allein. Ferner zeigte eines von drei Kaninchen-Antiseren gegen das K-Kapselantigen ebenfalls einen synergistischen Effekt mit Ampicillin. Aus diesen Ergebnissen war zu schließen, daß Antikörper eine günstige Wirkung auf die Behandlung von Infektionen mit Bakteriziden wie z. B. Ampicillin haben könnten, daß aber die biologische Funktion dieser Antikörper näher geklärt werden müßte. Eine Stimulierung des Immunabwehrsystems mit serologisch nichtverwandten *E. coli*-Stämmen ohne die Erzeugung homologer Antikörper dürfte dagegen die Bakterizidie durch Ampicillin nicht verstärken.

## Introduction

Recurrent bacterial infections are still a clinical problem despite the availability of a wide range of antibacterial agents. In order to improve therapy, increasing importance is being attached to the evaluation of the protective capacity of immune factors, especially of those which may function synergistically with antibacterial drugs. Recently it was shown that the $(Fab)_2$ fragment of antibodies against *Pseudomonas aeruginosa* endotoxin had a synergistic effect on the protection by gentamicin in compromised as well as in normal mice with experimental *Pseudomonas* infections (1). Also, immunoglobulin potentiated the protective effect of dibekacin against corneal ulcers in mice (2).

Antibodies against an invading organism can be directed against various virulence antigens, such as cell wall structures, enzymes or toxins. The antibodies against these different structures have differing protective capacities (3). Unspecific stimulation of the defence mechanisms in various ways, (i. e. BCG, levamisole) is also known to result in increased resistance against infections. When investigating the possibility of obtaining synergy between immune factors and antibacterial agents, the occurence of antibodies against different bacterial components should be taken into consideration as well as unspecific stimulation of the defence system.

In this study we present some data indicating that antibodies against the O and K antigen of *Escherichia coli* bacteria may act synergistically with ampicillin, but that unspecific stimulation with serologically unrelated *E. coli* bacteria does not mediate such effects.

### Materials and Methods

*Animals:* The animals used were CBA female mice, 20–22 g body weight in the immunization experiments and NMRI female mice, 20–22 g body weight (Anticimex, Stockholm, Sweden) in the experiments on passive immunization by transfer of rabbit antisera (see below).
Antiserum for passive immunization was produced in New Zealand rabbits of both sexes, 2–2,5 kg body weight.
*Bacteria:* E. coli bacteria of serogroups O6:K2a,2c:H1, O6:K13:H1 and O22:K13:H1 (WHO designations Bi 7458/41, Su 4344/41 and E 14a) were used.
*Immunizations:* Mice were injected intraperitoneally with $10^8$ formalin-killed *E. coli* O6:K13:H1 bacteria. Serum was taken

Dr. *S. Ahlstedt,* Research and Development Laboratories, Astra Läkemedel AB, S–151 85 Södertälje, Sweden; Dr. *B. Kaijser,* University of Göteborg, Institute of Medical Microbiology, Guldhedsgatan 10, S–413 46 Göteborg, Sweden.
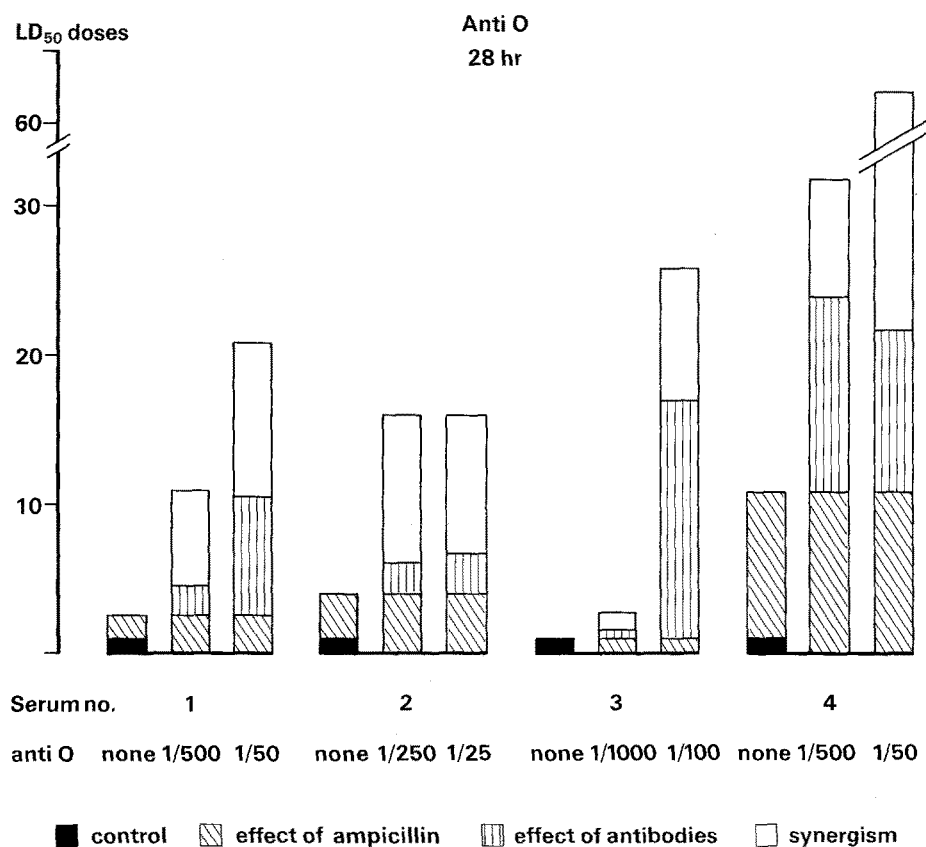
*Figure 1:* Protective effect of rabbit antiserum against *E. coli* O6 antigen with or without 25 mg/kg body weight ampicillin in intraperitoneally infected mice recorded after 28 h. The antisera were diluted as indicated. The reproducability in the experiments was ± 25%.

three and four days after immunization by retro-orbital venous plexus bleeding. The serum samples were stored at −20°C until used. Four rabbits were immunized with $10^6$ to $10^8$ formalin-killed. *E. coli* O6:K13:H1 bacteria as described by *Ahlstedt* et al. (4) to obtain antibodies specific against the O6 antigen. Two sera from rabbits immunized once (serum 1 and 2) or repeatedly (serum 3 and 4) were used. For inducing antibodies against the K13 antigen three rabbits were hyperimmunized according to *Holmgren* et al. (5) using formalin-killed and later live *E. coli* O22:K13:H1 bacteria. Only hyperimmune sera from these animals were used. Sera were stored at −20° C until used.

*Protection experiments:* For infections *E. coli* O6:K2a,2c:H1 or O6:H13:H1 bacteria were used. The microorganisms were cul-

tured over night in tryptose phosphate broth (Difco) substrate and then recultured for 3 h in the same substrate to obtain rapidly growing virulent bacteria. The optical density at 620 nm was 0.30–0.36 extinction units corresponding to about $2 \times 10^8$ cells/ml. The bacterial suspension was then diluted 1:4 stepwise with the substrate giving inocula ranging from concentrated to 1:256 the *E. coli* O6:K2a,2c:H1 strain and from 1:4 to 1:1024 for the *E. coli* O6:K13:H1 strain. 0.5 ml of each suspension was used to infect each mouse intraperitoneally.

The actively immunized animals were infected using the *E. coli* O6:K2a,2c:H1 strain three or four days after vaccination Sixteen animals received the same infective dose, and eight were given ampicillin (Astra Läkemedel AB, Södertälje, Sweden) 25 mg/kg
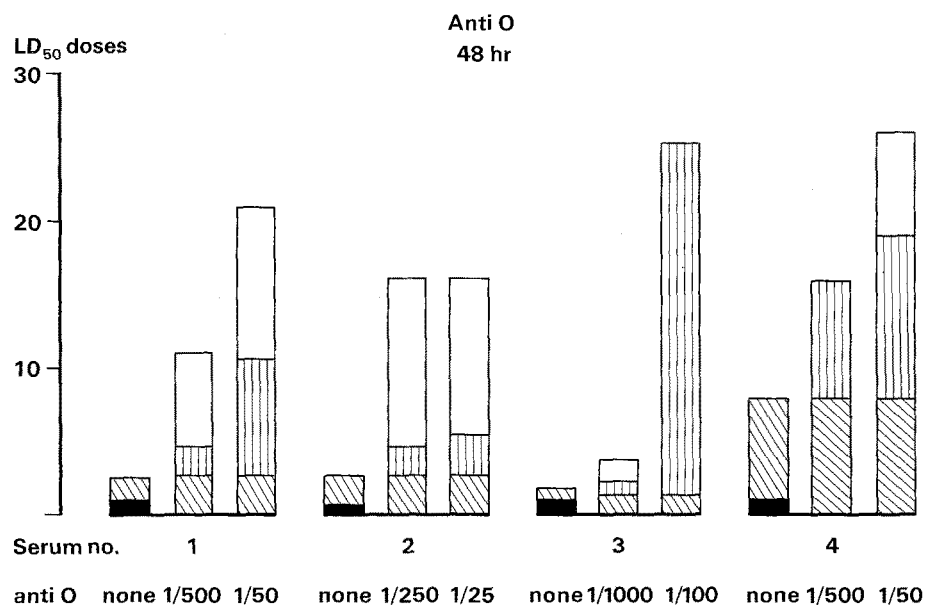


*Figure 2:* Protective effect of rabbit antiserum against. *E. coli* O6 antigen with or without 25 mg/kg body weight of ampicillin in intraperitoneally infected mice recorded after 48 h. The antisera were diluted as indicated. The reproducability in the experiments was ± 25%. The symbols are as in Figure 1.

body weight subcutaneously subsequent to the infection which was given intraperitoneally.

The passively immunized animals were given antiserum against serotype O6:K13:H1 for studies of the O6 antibodies and against serotype O22:K13:H1 for studies of the K13 antibodies. The antisera were first diluted in saline as shown in Figures 1 and 2 and then administered intraperitoneally. After four hours the animals were infected intraperitoneally with serotype O6:K2a,2c:H1 for studies of O6 antibodies and with serotype O6:K13:H1 for studies of K13 antibodies. Sixteen animals per serum dilution received each of the bacterial inocula. All these animals were also concomitantly treated with ampicillin, 25 mg/kg body weight for the O6:K2a,2c:H1 bacteria and 10 mg/kg body weight for the O6:K13:H1 bacteria. These concentrations of ampicillin were established in pilot experiments to give minute protection alone against the two different strains. The mortality was recorded after 28, 48 and 72 h. From 48–72 h no changes could be seen in the mortality rate, so the results from the latter time were omitted. The results were expressed as $LD_{50}$ doses of the controls. The synergistic effect of antibodies on the protection by ampicillin was calculated as the difference in resistance expressed as $LD_{50}$ doses compared to controls between the animals both immunized and treated with ampicillin and those given single treatment only.

*Antibody determinations:* The antibody levels in the sera were recorded using the enzyme-linked immunosorbent assay (ELISA) originally described by *Engvall* and *Perlmann* (6) according to the modifications by *Ahlstedt* et al. (7), using specific anti-mouse IgG and IgM as well as anti-rabbit IgG and IgM antisera (Nordic labs, Tilburg, The Netherlands). The antibody titers were recorded as the extinction value ($OD_{405}$) after 100 minutes of enzyme reaction at serum dilution 1:100.

## Results

### Active Immunization

Active immunization of mice with the *E. coli* O6 antigen resulted in enhanced resistance against intraperitoneal infection with *E. coli* bacteria of the same O group. As shown in Table 1 both the immunization and ampicillin treatment separately resulted in protection. A combination of immunization and ampicillin treatment increased the resistance of the mice very little above that seen for ampicillin alone. This was verified by repeated experiments. In the serum from these mice very little antibodies against the O6 antigen were recorded as could be expected so short time after immunization (Table 2).

Increase of the time periods up to four days between immunization and infectious challenge gave different re-

*Table 1:* Resistance of mice after immunization with *E. coli* O6:K13:H1 against infection with *E. coli* O6:K2a,2c:H1 three days later expressed as $LD_{50}$ values compared to untreated controls

| Groups | Hours after infection | | |
| | 28 | 48 | |
| --- | --- | --- | --- |
| Control | 1 | 0.5 | |
| Ampicillin | 32 | 2.8 | |
| Immunization | 19 | 19 | |
| | — | — | |
| | 51 | 22 | theoretical additive effect |
| Immunization + Ampicillin | 37 | 22 | no synergism |

*Table 2:* *E. coli* antibody titers after active immunization of mice with *E. coli* O6:K13:H1. The antibody determinations were made with the ELISA

| Time after immunization | Antibody level | |
| | IgG | IgM |
| --- | --- | --- |
| 3 days | neg. | 0.20* |
| 4 days | neg. | 0.72 |

* Optical density at 405 nm after 100 min of enzymatic reaction obtained with serum dilution 1:100. Background levels at 0.10 extinction units.

*Table 3:* Resistance of mice after immunization with *E. coli* O6:K13:H1 against infection with *E. coli* O6:K2a,2c:H1 four days later expressed as $LD_{50}$ values compared to untreated controls

| Groups | Hours after infection | | |
| | 28 | 48 | |
| --- | --- | --- | --- |
| Control | 1 | 0.5 | |
| Ampicillin | 6 | 1 | |
| Immunization | 3 | 2 | |
| | — | — | |
| | 9 | 3 | theoretical additive effect |
| Immunization + Ampicillin | 12 | 6 | weak synergism |

sults. After this period a slightly potentiated resistance was found with the combination of immunization and ampicillin treatment as compared to that of mono therapy (Table 3) indicating synergy. In addition antibody production could be demonstrated in this experiment (Table 2).

### Passive Immunization

Injections of antibodies directed against the O6 antigen of the infecting strain also protected the animals. The combination of antiserum and ampicillin treatment potentiated the protection as compared to that seen for treatment with antiserum or ampicillin alone. This was found for all four sera analysed, indicating synergy between antibodies and the antibiotic. However, the effect was less accentuated after 48 h as compared to 28 h (Figures 1 and 2). The dilutions of antisera to be used were determined in pilot experiments. The antibody titers in the sera are shown in Table 4. No obvious relation could be found between protection or synergistic effects as compared to the antibody titer demonstrated.

Passive administration of antibodies directed against the K13 antigen of the infecting strain resulted in protection for all three sera analysed. However, a synergistic effect of antibodies on the protective effect with ampicillin was only seen for one (no. 6) out of the three sera tested (Figures 3 and 4). The antibody titers in the sera are shown in Table 4. These did not explain why only serum (no. 6) gave synergy and not the others.
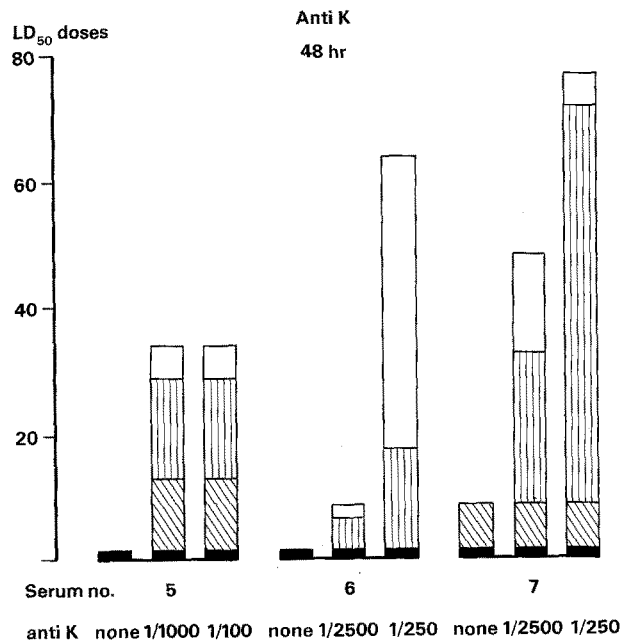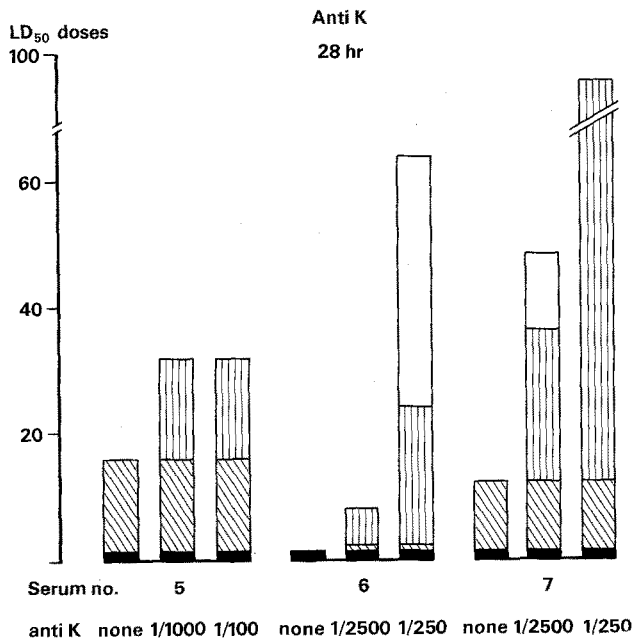
*Figure 3:* Protective effect of rabbit antiserum against. *E. coli* K13 antigen with or without 10 mg/kg body weight of ampicillin in intraperitoneally infected mice recorded after 28 h. The antisera were diluted as indicated. The reproducability in the experiments was ± 25%. The symbols are as in Figure 1.

*Figure 4:* Protective effect of rabbit antiserum against *E. coli* K 13 antigen with or without 10 mg/kg bodyweight of ampicillin in intraperitoneally infected mice recorded after 48 h. The antisera were diluted as indicated. The reproducability in the experiments was ± 25%. The symbols are as in Figure 1.

## Discussion

Recently a number of studies have been reported dealing with a possible combined effect of antibacterial agents and the host defence against infections. Some antimicrobial drugs may have a negative (8, 9, 10) and others a positive (1, 2, 11) effect on the defence against an infection.

In the present study we have shown some synergy between ampicillin and antibodies particularly against *E. coli* somatic (O) but also against the capsular (K) virulence antigens. These findings are in agreement with recent observations showing synergy between antibodies against the endotoxin of *P. aeruginosa* and gentamicin in the protection of im-

*Table 4:* Antibody titers in rabbit antisera used for passive immunization. The antibody determinations were made with the ELISA

| Rabbit No. | Antibody level against | | | |
| | O6 | | K13 | |
| | IgM | IgC | IgM | IgC |
| --- | --- | --- | --- | --- |
| 1 | 0.50* | 0.10 | | |
| 2 | 0.58 | 0.15 | | |
| 3 | 0.80 | 0.55 | | |
| 4 | 0.88 | 0.74 | | |
| 5 | | | 0.23** | 0.34 |
| 6 | | | 0.21 | 0.20 |
| 7 | | | 0.20 | 0.76 |

\* Optical density at 405 nm after 100 min enzyme reaction obtained with serum dilution 1:100. Background levels at 0.05 extinction units.
\*\* Background levels at 0.01 extinction units.

munologically compromised mice (1) and also with results showing synergy between immunoglobulin and dibekacin in experimentally infected mice (2).

The mechanisms for such synergistic effects are not obvious. Ampicillin is a bactericidal drug interfering with the cellwall synthesis. Bactericidal antibodies against the *E. coli* O antigen have previously been shown (12). Such antibodies and ampicillin may function synergistically. *Haranaka* et al. (1) showed, however, that (Fab)₂ fragments, which scarcely possess bactericidal activity also acted synergistically with gentamicin.

The occurrence of bactericidal antibodies against the *E. coli* K antigen is still controversial (12). The antibody response against the K antigens shows much individual variation. A similar individual variation might explain why only one antiserum out of three gave synergistic protection with ampicillin in spite of good protective activity of all the antisera. The *E. coli* K antibodies have been shown to promote phagocytosis (13). It seems very unlikely, however, that such a mechanism could explain the observed synergy. Furthermore, activation of macrophages by immunization following early infection when very few specific antibodies had been formed gave no synergistic effect on the protection although this resulted in increased resistance per se, with the doses employed in this study. However, preliminary data suggest a potentiated effect when the dose for stimulation as well as the infecting dose are increased 10-fold.

In conclusion, antibodies against the *E. coli* O antigens in particular but also against the K antigens may act synergistically with ampicillin thereby increasing its protective effects. This may have an important consequence in that cure

rates will be lower for those patients who have some kind of dysfunction in their immune defence mechanisms against a particular organism following administration of certain antibacterial agents. It also implies that the effect of a certain drug against a certain organism should be tested in vivo and that in vitro testings may not be sufficient. The mechanism for the described synergy may be mediated by bactericidal effects of the antibodies, which are more accentuated for the O antibodies than for the K antibodies. In contrast moderate stimulation of the macrophages shows no such synergistic effect. Additional so far unexplained immune factors must, however, also be taken into account.

### Literature

1. *Haranaka, K., Sugane, K., Mashimo, K.:* Combination therapy of anti-endotoxin antibody and gentamicin in the immunosuppressed mice with *Pseudomonas aeruginosa* infection. Jpn. J. Exp. Med. 45 (1975) 207–213.
2. *Kawaharajo, K., Homma, J. Y.:* Synergistic effect of immune gammaglobulin fraction on protection by antibiotic against corneal ulcers in ecperimental mice infected with *Pseudomonas aeruginosa.* Jpn. J. Exp. Med. 46 (1976) 155–165.
3. *Kaijser, B., Ahlstedt, S.:* Protective capacity of antibodies against *Escherichia coli* O und K antigens. Infect. Immun. 17 (1977) 286–289.

4. *Ahlstedt, S., Holmgren, J., Hanson, L-A.:* Protective capacity of antibodies against *E. coli* O antigen with special reference to the avidity. Int. Arch. Allergy 46 (1974) 470–480.
5. *Holmgren, J., Eggertsen, G., Hanson, L-Å., Lincoln, K.:* Immunodiffusion studies on *Escherichia coli.* I. Identification of O, K and H antigens in an O6 strain. Acta Pathol. Microbiol. Scand. 76 (1969) 304–318.
6. *Engvall, E., Perlmann, P.:* Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-leballed anti-immunoglobulin in antigen-coated tubes. J. Immunol. 109 (1972) 129–135.
7. *Ahlstedt, S., Carlsson, B., Hanson, L-Å., Kaijser, B., Mattson-Balzer, I., Sohl-Åkerlund, A.:* The application of the ELISA for determination of immunoglobulin class specific bacterial antibodies. Scand. J. Immunol. Suppl. (1978) in press.
8. *Forsgren, A., Gnarpe, H.:* Tetracyclines and host-defence mechanisms. Antimicrob. Agents Chemother. 3 (1973) 711–715.
9. *Majeski, J. A., Alexander, J. W.:* Evaluation of tetracycline in the neutrophil chemotactic response. J. Lab. Clin. Med. 90 (1977) 259–277.
10. *Webster, L. T., Butterworth, A. E., Mahmond, E. N., Mngola, Warren, K. S.:* Suppression of delayed hypersensitivity in schistosome-infected patients by niridazole. N. Engl. J. Med. 292 (1975) 1144–1147.
11. *Reynard, A. M., Dutcher, B. S.:* Antibiotic-complement synergy in killing rough and smooth *E. coli* (Abstract). 17th Intersc. conference on Antimicrobial Agents Chemother., New York, 1977.
12. *Olling, S.:* Sensitivity of gram-negative bacilli to the serum bactericidal activity: a marker of the host-parasite relationship in acute and persisting infections. Scand. J. Infect. Dis. vol. 9 pp 1–40, Suppl. 10 (1977).
13. *Howard, C. J., Glynn, A. A.:* The virulence for mice of strains of *Escherichia coli* related to the effects of K antigens on their resistance to phagocytosis and killing by complement. Immunology 20 (1971) 767–777.

---

## Discussion

*Mattie:* The thigh muscle infection model we described was also used for studies of host factors and, during the years, we noticed that the slope of the dose effect response changed a little, indicating that even in this inbred strain of SPF-mice some host factors had changed. So in studies of synergism between host factors and antibiotics it is very important to use a well-defined range.

*Ahlstedt:* We had to reduce the ampicillin doses otherwise we could not see any synergistic effect at all.

*Sabath:* What was the variation in LD5$^0$ that you would find with ampicillin? I noticed that some of your changes, where you showed

synergy, might have been twofold, and I wonder if some of them might be explanable as experimental variation?

*Ahlstedt:* Only with the anti-K serum No. 7 in Figures 3 and 4. Otherwise we calculated the usual experimental variation to be ± 25%.

*Allison:* In relation to children with absent opsonization, more or less constant antibiotic cover is needed to prevent infection. When these children are treated with whole serum, rather than immune serum globulin, antibiotic cover can be withdrawn altogether and the time needed for treating occasional infections is also reduced. Thus serum factors can prevent most infections and work in synergy with antibiotics to resolve any infections that do occur.