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# STUDIES ON THE IMMUNE BACTERIOLYSIS

# XIV. REQUIREMENT OF ALL NINE COMPONENTS OF COMPLEMENT FOR IMMUNE BACTERIOLYSIS[1]

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SUMMARY Using highly-purified preparations of C'3, C'5, C'6, C'7, C'8 and C'9 and BAC' 1a, 4, 2a cells, which were prepared by reaction of sensitized bacteria (BA) and the complement reagent lacking classical third component (R3) fortified with partially purified C'2, it was demonstrated that lysozyme could act on its substrate to form serum spheroplasts only after BAC'1a, 4, 2a, 3, 5, 6, 7, 8, 9 cells had been formed.

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## INTRODUCTION

Immune hemolysis has been extensively analyzed by many investigators and is known to result from sequential reactions of sensitized erythrocytes (EA) with nine components of complement, now designated as C'1, C'4, C'2, C'3, C'5, C'6, C'7, C'8 and C'9 (INOUE and NELSON, 1966).

Among the other immune reactions, in which complement is known to be necessary, both immune adherence (NISHIOKA and LINSCOTT, 1964) and immune phagocytosis (NELSON, 1962) requires four early reacting components of complement, C'1, C'4, C'2 and C'3. For chemotaxis, either C'3 fragment or C'5, C'6 and C'7 is known to be responsible, after activation by the immune complex and early reacting complement components (WARD, 1967). Anaphylatoxin was found to be derived from either C'3 or C'5 molecules (COCHRANE and MÜLLER-EBERHARD, 1968).

DOZOIS and coworkers demonstrated that all the four classical complement components known at the time of his work were necessary for the immune-bactericidal reaction (1943). Later it was found, in this laboratory, that lysozyme activity was essential for the immune bacteriolysis (serum spheroplast formation) but not for the immune-bactericidal reaction (INOUE *et al.*, 1959).

The step in the complement reaction responsible for immune bacteriolysis has not been determined because highly-purified prepara-

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tions of various components have not been available simultaneously. This paper shows that all nine components and also lysozyme, are necessary for immune bacteriolysis.

## MATERIALS AND METHODS

1. *Bacteria*

A streptomycin-resistant mutant, strain 004, of *Escherichia coli* B, Hershey was maintained on Y medium agar (INOUE *et al.*, 1968). For induction of β-D-galactosidase and growth Lac-ρ-medium was used, as described in the previous paper (INOUE *et al.*, 1968).

2. *Diluent, antiserum (lysozyme-free), egg white lysozyme and assay methods for lysozyme and for β-D-galactosidase*

These were as described in the previous paper (INOUE *et al.*, 1968).

3. *Complement reagent lacking the activity of classical third component (R3)*

R3 was prepared from fresh guinea pig serum according to the description of MAYER (1961), and then repeatedly adsorbed on bentonite to remove serum lysozyme (INOUE *et al.*, 1968).

4. *Purified preparations of components of guinea pig complement*

These were prepared by the methods outlined in previous papers (INOUE and NELSON, 1965, 1966; INOUE *et al.*, 1967; YONEMASU and INOUE, 1968). The hemolytic reactivities of the preparations used are shown in Table 1.

5. *Partially purified second complement component (C'2)*

This was prepared by the method of Borsos and coworkers (1961).

## RESULTS

### *Identification of the intermediate step where lysozyme acts in forming spheroplasts*

An overnight culture of *E. coli* B/Sm strain 004 in Lac-ρ-medium was harvested by centrifugation. The bacteria were washed twice with cold isotonic phosphate-buffered saline containing 0.1% gelatin, 0.0005 M $MgCl_2$ and 0.00015 M $CaCl_2$, pH 7.2 (GPB-saline), and resuspended in the same buffer at a concentration of $1.0 \times 10^9$ bacteria/ml. The bacterial suspension was mixed with an equal volume of 1/100 antiserum (lysozyme-free) and incubated at 37°C for 30 min. After incubation the sensitized bacteria (BA) were centrifuged, washed three times with GPB-saline, and resuspended in the same buffer at a concentration of $1.0 \times 10^9$ cells/ml. A portion of the BA suspension was warmed at 30°C for 15 min and then added to a mixture of an equal volume of 1/10 dilution of R3 in GPB-saline and 0.3 volume of stock solution of C'2, at 30°C. After incubation at

TABLE 1 *Hemolytic reactivities of the preparations of purified components of guinea pig complement employed.*

| Preparation | Approximate $C'H_{50}$ units per ml[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | C'2 | C'3 | C'5 | C'6 | C'7 | C'8 | C'9 |
| C'2 #020668 | 3,000 | 0 | 0 | 0 | 6,600 | 0 | 512 |
| C'3 #031668 | 0 | 19,700 | 0 | 0 | 0 | 0 | 0 |
| C'5 #030468 | 0 | tr | 59,000 | 0 | 8 | 0 | 4 |
| C'6 #120367 | 0 | 0 | 0 | 2,200 | 0 | 3 | 0 |
| C'7 #030868 | 0 | 0 | 0 | 0 | 19,700 | 0 | 0 |
| C'8 #021768 | 0 | 0 | 0 | 18 | 0 | 65,500 | tr |
| C'9 #030968 | 0 | 0 | 0 | 0 | 0 | 0 | 32,000 |
| R3 #121867 | 2,500 | 4 | tr | tr | 1,000 | 72,900 | 24,300 |

*a* Titrated by Microtiter methods (Yonemasu and Inoue, 1968).
" tr " Indicates traces amount detected.

30°C for 20 min, the bacteria (BAC'1a,4,2a) were centrifuged in the cold, washed twice with cold GPB-saline and resuspended in the same buffer at a concentration of $1.0 \times 10^9$ bacteria/ml.

The reaction mixtures shown in Table 2 were prepared in centrifuge tubes in an ice bath. The bacterial suspension (BA or BAC'1a,4,2a) was added last.

The tubes were incubated successively in water baths at 30°C and 37°C for 45 min each with continuous gentle shaking. Then morphological changes in stained preparation from each reaction mixture were examined microscopically and the reaction mixtures were centrifuged in the cold. The supernatant was titrated for released β-D-galactosidase using *o*-nitrophenyl β-D-galactoside as substrate. Activities are expressed as optical densities at 420 mμ (Expt. 031868).

As shown in Table 2, only in tube K were spheroplasts formed and a significant amount of enzyme released. Therefore lysozyme can act on the bacterial cell wall only after all the nine components of complement have reacted on the antigenic site combined with antibody.

## DISCUSSION

As shown in this paper, immune bacteriolysis requires all nine components of complement before lysozyme attacks the rigid structure of the bacterial cell wall. It is still obscure how erythrocytes or bacteria are lyzed by the complex of complement components, which has been activated by reactions consequent on formation of the antigen-antibody complex on the cell surface. Both reactions require the same components, so the mechanisms responsible for the "hole" or "channel" formation in both the erythrocyte membrane (BORSOS *et al.*, 1964, SCOTT *et al.*, 1966) and bacterial cell wall (INOUE *et al.*, 1968) must be the same. Com-

TABLE 2 *Identification of the intermediate step where lysozyme acts in forming spheroplasts* (Expt. 031868)

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GPB-saline | ml 4.0 | ml 1.0 | ml — | ml 4.0 | ml 3.0 | ml 2.4 | ml 1.8 | ml 1.2 | ml 0.6 | ml 0.3 | ml — | ml 3.4 | ml 2.8 | ml 2.2 | ml 1.6 | ml 1.3 | ml 1.0 |
| 1 : 2 C'3 | — | 0.6 | 0.6 | — | — | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| 1 : 2 C'5 | — | 0.6 | 0.6 | — | — | — | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | — | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| 1 : 2 C'6 | — | 0.6 | 0.6 | — | — | — | — | 0.6 | 0.6 | 0.6 | 0.6 | — | — | 0.6 | 0.6 | 0.6 | 0.6 |
| 1 : 2 C'7 | — | 0.6 | 0.6 | — | — | — | — | — | 0.6 | 0.6 | 0.6 | — | — | — | 0.6 | 0.6 | 0.6 |
| 1 : 2 C'8 | — | 0.3 | 0.3 | — | — | — | — | — | — | 0.3 | 0.3 | — | — | — | — | 0.3 | 0.3 |
| 1 : 2 C'9 | — | 0.3 | 0.3 | — | — | — | — | — | — | — | 0.3 | — | — | — | — | — | 0.3 |
| 25 μg/ml lysozyme | — | — | 1.0 | — | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | — | — | — | — | — | — |
| $1 \times 10^9$/ml BA | 1.0 | 1.0 | 1.0 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| $1 \times 10^9$/ml BAC' 1a, 4, 2a | — | — | — | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Incubated at 30°C for 45 min, and then at 37°C for 45 min | | | | | | | | | | | | | | | | | |
| Morphological changes[a] | — | — | — | — | — | — | — | — | — | — | + | — | — | — | — | — | — |
| β-D-galactosidase activity ($OD_{420}$) | .031 | .040 | .055 | .015 | .046 | .039 | .035 | .054 | .050 | .051 | .138 | .022 | .020 | .034 | .028 | .028 | .053 |

*a* + : about a quarter of the bacteria were converted to spheroplasts.
— : no morphological change was observed.

parative studies should be done on this.

Nelson has shown that ingestion by immune phagocytosis requires the early-reacting complement components, C'1, C'4, C'2 and C'3 while later-reacting components do not enhance the degree of phagocytosis (1962). Phagocytized bacteria are digested by various hydrolyzing enzymes derived from leukocytes, which contain a relatively large amount of intracellular-lysozyme (AMANO *et al.*, 1954). Gram-negative bacteria cannot be attacked by lysozyme alone. However, they become susceptible to this and other hydrolyzing enzymes after treatment with antibody and all nine components of complement, as shown in this and previous papers (INOUE *et al.*, 1968). Therefore it is suggested that, when gram-negative bacteria are phagocytized, their intracellular digestion requires all nine components of complement fixed on the bacteria, although their ingestion by these cells needs only four early-reacting components.

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