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THE BACTERICIDAL ACTION OF IMMUNOGLOBULIN A

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It is widely believed that IgA in the external body secretions has an important protective role against viral and bacterial infections (Smith, Bellanti and Chanock, 1967; Mouton *et al.*, 1970). An apparent obstacle to the concept of the antibacterial role of IgA is the absence of complement in the external secretions. It has also been shown that IgA is unable to fix the first component of complement (Ishizaka *et al.*, 1966), that it does not sensitise red cells to agglutination by anti-complement globulin sera, and that IgA haemagglutinins do not lyse red cells in the presence of complement (Adinolfi *et al.*, 1966*a*). Colostral IgA can, however, lyse *Escherichia coli* in the presence of lysozyme and a heat-labile serum factor assumed to be complement (Adinolfi *et al.*, 1966*b*). The experiments to be described were performed as an attempt to resolve this conflicting evidence.

MATERIALS AND METHODS

A strain of *Escherichia coli* (E458/64) supplied by Dr Joan Taylor was used. Colostral IgA (sIgA) and serum IgA were prepared by the method of Newcomb, Normansell and Stanworth (1968). Reagents deficient in one or more components of complement, E (endpiece), M (midpiece), R3, R4, and RH, were prepared by standard methods from fresh human serum (Kabat and Mayer, 1961). They were used at a dilution of 1 in 2 with respect to the serum from which they were prepared, to ensure an adequate concentration of bactericidal factors. All the reagents were shown to be non-haemolytic before use in bactericidal experiments by testing with an equal volume of 2 per cent. sheep red cells sensitised with anti-sheep cell serum. Reagents R1 and R2 were also prepared by the addition of RH to E and M respectively. They were used at a dilution of 1 in 5 for the detection of C'1 and C'2 in serum fractions prepared by ion-exchange chromatography.

A reagent (DE) deficient in several complement components was prepared by a method derived from that of Vroon, Schultz and Zarco (1970). Twenty ml of fresh human serum was diluted with 80 ml of 0.005M sodium phosphate buffer, pH 7.5. The precipitate was discarded. The supernate was dialysed against the same phosphate buffer containing 0.04M NaCl, and then applied to a column of DEAE cellulose (DE52, Whatman) packed in a column made from a 25-ml nylon syringe. The effluent was discarded and the column washed with buffer. Bound protein was eluted with 0.005M phosphate buffer, pH 7.5,

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containing 0.10M NaCl. The eluate DE was dialysed against normal saline and concentrated by ultrafiltration to 10 ml. In later experiments this procedure was performed on a larger column with 200-ml samples of serum and monitored on a Uvicord spectrophotometer (LKB, Sweden). The eluate was collected in 50-ml fractions coded DE1, DE2, etc., and these were concentrated 10-fold by ultrafiltration before use in bactericidal experiments.

The eluates DE from two of the small columns were pooled and fractionated on Sephadex G-200 (Pharmacia, Uppsala, Sweden) in 0.14M NaCl, 0.005M phosphate buffer, pH 7.5. Six fractions, G1 to G6, were collected and were concentrated 5-fold. All reagents were prepared at 4-10°C.

Bactericidal reaction mixtures contained egg-white lysozyme (Sigma) at a final concentration of 100 µg per ml, and 0.3 ml CFT buffer (Oxoid). The source of antibody was 0.2 ml of heated serum (RH), sIgA or IgA. Serum factors were provided by R reagents, serum fraction DE, or Sephadex G-200 fractions, usually in 1-ml volumes. The inoculum was 0.1 ml of a 10^7 per ml suspension of *E. coli* prepared from an overnight culture on DST Agar (Oxoid). The total volume was made up to 2.0 ml with saline. The mixtures were incubated for 1 hr at 37°C before viable counts of surviving organisms were made.

RESULTS

Experiments in which reagents E, M, R3, R4 and RH were a source of serum factors showed that E and R4 were bactericidal without the addition of further antibody. When sIgA was added, M became bactericidal, but R3 and RH remained inactive. Analysis of the immunoglobulin content of these reagents showed that they all contained IgM and IgG, and that IgA was absent from M but present in the other reagents. It was concluded that E, M and R4 contained the additional serum factors necessary for the bactericidal action of IgA, and that R3 and RH were deficient in these. The results of an experiment using the reagent M are shown in table I. The antibody source was sIgA or RH,3 which is serum absorbed with zymosan and then heated. The reagents M, sIgA and RH,3 were not bactericidal when used alone, but the combination of sIgA or RH,3 with M killed more than 90 per cent. of the inoculum.

TABLE I
*Bactericidal activity of various mixtures of sIgA, RH,3 and M**

Reagent mixture	Viable count per ml†
0.2 ml saline+1.0 ml M	500,000
0.2 ml sIgA +1.0 ml M	25,000
0.2 ml sIgA +1.0 ml saline	460,000
0.2 ml RH,3+1.0 ml M	15,000
0.2 ml RH,3+1.0 ml saline	645,000

* The mixtures were made up to 2 ml with lysozyme, buffer, saline and bacterial inoculum as described in the text.

† After incubation for 1 hr at 37°C.

In the next experiment (table II), the bactericidal effect of sIgA and RH,3,4 in the presence of decreasing amounts of M were compared. RH,3,4 is serum treated with hydrazine, absorbed with zymosan and heated. RH,3,4 supported bacterial killing with small volumes of M, whereas sIgA did so only in the

presence of a larger amount of M. It was concluded that M is relatively deficient in a heat-stable factor that is resistant to hydrazine, is not absorbed by zymosan

TABLE II
*Bactericidal activity of various mixtures of sIgA, RH,3,4 and M**

Reagent mixture	Viable count per ml†
0.2 ml saline + 1.2 ml M	500,000
0.2 ml sIgA + 1.2 ml M	85,000
0.2 ml RH,3,4 + 1.2 ml M	15,000
0.2 ml sIgA + 1.0 ml M	120,000
0.2 ml RH,3,4 + 1.0 ml M	15,000
0.2 ml sIgA + 0.7 ml M	220,000
0.2 ml RH,3,4 + 0.7 ml M	15,000
0.2 ml sIgA + 0.4 ml M	230,000
0.2 ml RH,3,4 + 0.4 ml M	15,000

* Mixtures were made up to 2 ml with lysozyme, buffer, saline and bacterial inoculum as described in the text.

† After incubation for 1 hr at 37°C.

and is present in RH3,4 together with antibody. Since heated serum alone is not bactericidal, a heat-labile factor must also be present in M.

TABLE III
*Comparison of the sensitivity of the complement-dependent bactericidal action and the lysis of sensitised sheep red cells**

Reagent mixture containing 0.1 ml RH and 1.0 ml of	Viable count per ml	Percentage lysis of sensitised red cells
saline	1,095,000	0
complement 1 in 5	185,000	100
complement 1 in 10	440,000	100
complement 1 in 20	570,000	100
complement 1 in 50	740,000	100
complement 1 in 100	805,000	50
complement 1 in 150	960,000	25
complement 1 in 200	995,000	<25
complement 1 in 300	1,060,000	0
complement 1 in 500	1,100,000	0

* The complement source was human serum that had been absorbed with heat-killed *E. coli*. Mixtures were made up to 2.0 ml with lysozyme, buffer, saline and 0.1 ml of bacterial suspension or sensitised sheep red cells.

When a more sensitive test for complement was used, i.e., the substitution of sensitised sheep red cells for the bacterial inoculum in the reagent mixtures, 50 to 75 per cent. of the cells were lysed. This indicated that trace amounts

of complement were present. However, an experiment designed to compare the sensitivity of haemolysis of sensitised sheep cells under these conditions

TABLE IV
*Bactericidal activity of mixtures of sIgA or RH and reagent DE**

Reagent mixture (ml)	Viable count per ml
0.2 ml sIgA + 1.0 ml saline	435,000
0.2 ml RH + 1.0 ml saline	425,000
0.2 ml saline + 1.0 ml DE	95,000
0.2 ml sIgA + 1.0 ml DE	70,000
0.2 ml RH + 1.0 ml DE	70,000

* Mixtures were made up to 2.0 ml with lysozyme, buffer, saline and bacterial inoculum.

with that of the complement-dependent bactericidal reaction showed that complement concentrations sufficient to lyse 50 per cent. of red cells had little bactericidal activity (table III). Therefore the bactericidal activity of sIgA

FIG. 1.—Chromatography on DEAE cellulose of human serum equilibrated with 0.04M NaCl in 0.005M sodium-phosphate buffer at pH 7.5. Stepwise elution with (a) 0.10M NaCl in 0.005M sodium-phosphate buffer, pH 7.5, (b) 0.14M NaCl in 0.005M sodium-phosphate buffer, pH 7.5, and (c) 0.84M NaCl in 0.005M sodium-phosphate buffer, pH 7.5.

and M could not be accounted for by the trace amounts of complement present in M. Furthermore, M contains IgM, and if the residual complement in M was sufficient to support a complement-dependent bactericidal reaction it would be apparent without the addition of sIgA.

DE was not lytic for sensitised sheep red cells. The results of an experiment (table IV) showed that it was nevertheless bactericidal. Bacterial killing occurred without the addition of IgA or RH. Reagent DE therefore contained heat-labile factor, heat-stable factor and IgA. Fig. 1 shows the protein distribution, monitored at a wavelength of 254 nm of human serum fractionated

TABLE V
*Bactericidal activity of mixtures of IgA or RH with serum fractions prepared by ion-exchange chromatography**

Reagent mixture	Viable count per ml
0.2 ml RH+1.5 ml saline	675,000
0.2 ml IgA+1.5 ml saline	665,000
0.2 ml RH+1.5 ml DE4	535,000
0.2 ml IgA+1.5 ml DE4	535,000
0.2 ml RH+1.5 ml DE5	120,000
0.2 ml IgA+1.5 ml DE5	285,000
0.2 ml RH+1.5 ml DE6	5,000
0.2 ml IgA+1.5 ml DE6	255,000
0.2 ml RH+1.5 ml DE7	5,000
0.2 ml IgA+1.5 ml DE7	295,000
0.2 ml RH+1.5 ml DE8	90,000
0.2 ml IgA+1.5 ml DE8	285,000
0.2 ml RH+1.5 ml DE9	135,000
0.2 ml IgA+1.5 ml DE9	445,000
0.2 ml RH+1.5 ml DE10	205,000
0.2 ml IgA+1.5 ml DE10	475,000
0.2 ml RH+1.5 ml DE11	375,000
0.2 ml IgA+1.5 ml DE11	680,000
0.2 ml RH+1.5 ml DE12	345,000
0.2 ml IgA+1.5 ml DE12	680,000
0.2 ml RH+1.5 ml DE13	505,000
0.2 ml IgA+1.5 ml DE13	655,000

* Mixtures were made up to 2.0 ml with lysozyme, buffer and bacterial inoculum.

by chromatography on DEAE-cellulose. The elution position of certain complement components recorded in the figure have not been confirmed, and are from the paper by Vroon *et al.* The bactericidal activity of fractions DE1 to DE13 is recorded in table V. Fractions DE6 and DE7 when mixed with RH killed more than 99 per cent. of the inoculum, but only about 60 per cent. when mixed with IgA. It was concluded that fractions DE6 and DE7 contained heat-labile factor, but were somewhat deficient in heat-stable factor, which is contributed by heated serum RH. A pool prepared from fractions DE6 to DE10 from a similar experiment was tested for the presence of C'1 and C'2. Reagents R1 and R2 alone were not lytic for sensitised sheep cells but, when mixed, rapidly produced 100 per cent. lysis. The pool DE6-10, which was

bactericidal, did not produce lysis of sensitised sheep cells either alone or when mixed with R1 or R2. None of these reagents was anticomplementary. The heat-labile factor in DE6-10 was therefore not C'1 or C'2.

FIG. 2.—Protein distribution of reagent DE fractionated on a column of Sephadex G-200 (85³cm × 3.4 cm). Filtration was performed with 0.14M NaCl in 0.005M sodium-phosphate buffer, pH 7.5.

The total protein distribution of reagent DE fractionated by gel filtration on Sephadex G-200 is shown in fig. 2. The distribution of IgM, IgA, IgG,

TABLE VI

*Bactericidal activity of mixtures of IgA or RH with serum fractions prepared by ion-exchange chromatography and gel filtration**

Reagent mixture	Viable count per ml
0.2 ml RH + 1.5 ml saline	660,000
0.2 ml RH + 1.5 ml G1	630,000
0.2 ml RH + 1.5 ml G2	635,000
0.2 ml RH + 1.5 ml G3	610,000
0.2 ml RH + 1.5 ml G4	565,000
0.2 ml RH + 1.5 ml G5	445,000
0.2 ml RH + 1.5 ml G6	120,000
0.2 ml IgA + 1.5 ml saline	620,000
0.2 ml IgA + 1.5 ml G6	155,000
0.2 ml saline + 1.5 ml G6	655,000

* Mixtures were made up to 2.0 ml with lysozyme, buffer and bacterial inoculum.

siderophilin and albumin is also recorded. The bactericidal activity of the six fractions is shown in table VI. When mixed with RH, fraction no. 6 killed

80 per cent. of the inoculum, and fraction no. 5 showed only slight bactericidal activity. Fraction no. 6 alone was not bactericidal, but when IgA was added more than 75 per cent. of the inoculum was killed. It was concluded that fraction no. 6 contained heat-labile factor and heat-stable factor but was deficient in IgA. The absence of IgA from fraction no. 6 was confirmed by gel-diffusion analysis. The filtration position of the heat-stable and heat-labile factors indicates that they have molecular weights of less than 100,000.

DISCUSSION

The experiments described, especially those in which serum fractions prepared by ion-exchange chromatography and gel filtration were used, show that IgA is bactericidal in the absence of the complete complement system. Secretory IgA has previously been reported to lyse *E. coli* (Adinolfi *et al.*, 1966*b*). It is now apparent that serum IgA is also bactericidal, and this may be important in the extravascular spaces from which IgM is largely excluded.

The interpretation of the earlier experiments with R reagents presents certain difficulties because the results reported here probably depend on their use at a dilution of 1 in 2, instead of the more usual dilution of 1 in 5. The possibility of residual complement activity must therefore be considered. The R reagents were not haemolytic when tested with an equal volume of a sensitised sheep red cell suspension, but when a more sensitive test was used, i.e., the substitution of sensitised sheep red cells for the bacterial inoculum in an identical reagent mixture to that used for the bactericidal experiment, there was partial haemolysis. The experiment recorded in table III shows that complement giving a comparable degree of haemolysis killed only about 20 per cent. of the inoculum, which is much less than the bactericidal activity of reagent M. A further indication that the bactericidal properties of M were not dependent on complement is that IgM present in reagent M should be sufficient to promote a bactericidal reaction if complement was also present. As the results in table I show, reagent M was bactericidal only if IgA or heated serum was added. Because of the difficulties in interpretation of these results, it was considered necessary to find other ways of inactivating the complement system. The reagents prepared by chromatography fulfil this requirement because they are completely without haemolytic activity as a result of their multiple deficiencies in complement components.

Two factors essential for the bactericidal activity of IgA are postulated to explain the results obtained. The first is a heat-labile factor found mainly in the euglobulin fraction of serum. It is resistant to hydrazine and is removed from serum by absorption with zymosan, and by absorption with heat-killed *E. coli*. It is distinguished from all complement components except C'1 and C'2 by its heat-lability. Its molecular weight is much lower than that of the C'1 complex, and it differs from C'2 in being a euglobulin. The reagent DE6-10 contained the heat-labile factor, but C'1 and C'2 could not be detected in this reagent. On this evidence it is concluded that the heat-labile factor is distinct from all known complement components.

The heat-stable factor resists heating at 56°C for 1 hr and is found mainly in the pseudoglobulin fraction of serum. It is resistant to hydrazine and is not removed from serum by absorption with zymosan. The elution position of the heat-stable factor on ion-exchange chromatography has not yet been precisely located, but it is eluted after the heat-labile factor in a position intermediate to the peak containing C'3, C'5, C'6 and C'7 and that containing C'4 and C'9. Components C'6 and C'7 are eluted before C'3 and C'5. The heat-stable factor is therefore unlikely to be C'6 or C'7 but must be distinguished from C'3 and C'5. Unlike C'3 it is a pseudoglobulin and its molecular weight is lower than that of C'3 and C'5. Furthermore it is not absorbed from serum by zymosan. It is not C'4 because it resists treatment with hydrazine, and it is of lower molecular weight. These observations on the nature of the heat-stable factor are, however, tentative, because no attempt has been made to confirm which complement components are present in the fractions prepared by chromatography. Such information would not help to establish the nature of the heat-stable factor until a greater degree of purification has been achieved.

The evidence at present available does not therefore exclude the possibility that the heat-stable factor is a known complement component. If the IgA system utilised a single factor involved in the complement reaction, one would expect an antigen-antibody reaction initiated by IgA antibody to be potentially able to use at least part of the complement sequence and terminate in lysis of sensitised red cells. The lysis of red cells by IgA antibody has not been demonstrated (Adinolfi *et al.*, 1966a; Adinolfi *et al.*, 1966b), and this can be interpreted as support for the view that the IgA system proposed here and the complement system are independent of one another, and that the factors involved in the two systems are not interchangeable.

SUMMARY

Bactericidal activity of serum IgA and secretory IgA in the presence of serum fractions deficient in several components of complement has been demonstrated. Two new serum factors independent of the complement system are postulated to explain the bactericidal action of IgA.

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