THE RELATIVE IMPORTANCE OF THE CLASSICAL AND ALTERNATIVE COMPLEMENT PATHWAYS IN SERUM BACTERICIDAL ACTIVITY AGAINST *ESCHERICHIA COLI*

A. P. ROBERTS AND RUTH PHILLIPS

*Department of Medical Microbiology, Charing Cross Hospital Medical School, London
W6 8RF*

SUMMARY. The relative importance of the classical and alternative complement pathways in serum bactericidal activity against *Escherichia coli* strains of the common urinary O-serogroups has been assessed with strains that differ widely in their sensitivity to normal human serum. With most promptly serum-sensitive strains, rapid killing occurred, mediated by the classical pathway and, when this pathway was eliminated, delayed killing by alternative-pathway activity occurred. However, one strain of serogroup O1 was affected by the classical pathway only and a strain of serogroup O9 was killed rapidly by the alternative pathway. Strains with delayed sensitivity to normal human serum were largely, and in some cases exclusively, affected by the classical pathway.

When added to heat-inactivated serum, some strains showed no significant growth whereas the viable numbers of other test strains increased more than 50-fold in 3 h. Whether this variation is due to differences in nutritional requirements or sensitivity to some non-complement-dependent bacteriostatic mechanism remains to be determined.

INTRODUCTION

We have shown recently that both the classical and the alternative complement pathways are involved in serum bactericidal activity against most urinary isolates of *Escherichia coli* of serogroup O6 (Roberts and Phillips, 1981). It was considered important to establish whether this is also true for other O-serogroups of *E. coli* commonly associated with urinary-tract infections.

In the present study we assessed the relative importance of classical and alternative complement pathways in serum bactericidal activity against urinary isolates of *E. coli* of serogroups O1, O2, O4, O7, O9, O18 and O75. For each O-serogroup, strains that differed widely in sensitivity to normal serum were studied.

MATERIALS AND METHODS

Bacteria. All strains of *E. coli* were isolated from cultures of suprapubic aspirates of urine. The methods of identification and O-serogrouping have been reported previously (Roberts *et al.*,

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1975). At the time this study began, 10–20 isolates of each of the O-serogroups O1, O2, O4, O7, O9, O18 and O75 were available to us and their sensitivity to normal serum was known. Because the range of sensitivity to serum varies with O-serogroup (Taylor, 1974), three strains of each were selected by the following criteria: (i) the most sensitive strain—the strain with the lowest percentage of survivors after 1 h of the assay in normal human serum; (ii) a strain showing delayed sensitivity to serum—the strain showing the least change in the viable count after 1 h of the assay provided that it was subsequently sensitive to serum; and (iii) the most resistant or least sensitive strain—the strain with the highest percentage of survivors at the end of the assay. For convenience, strains selected under (i) will be referred to as promptly serum-sensitive, under (ii) as delayed serum-sensitive, and under (iii) as serum-resistant. Results will also be given for three strains of serogroup O6, selected by the above criteria from our previous results (Roberts and Phillips, 1981).

Reagents. A solution of 100 mM ethyleneglycoltetra-acetic acid (EGTA; Sigma London Chemical Co. Ltd, Poole, Dorset, BH17 7NH), supplemented with 100 mM magnesium chloride (MgEGTA), in normal saline (NaCl, 0.9% w/v), was prepared as described by Fine *et al.* (1972) and sterilised by filtration through a 0.22- μ m membrane filter (type GS, Millipore Ltd, 11–15 Peterborough Road, Harrow, Middlesex HA1 2BR).

Assay of serum bactericidal activity. The preparation of serum and the assay of bactericidal activity have been described previously (Roberts and Phillips, 1981). Briefly, to 1.6 ml of fresh human serum was added 0.2 ml of normal saline or MgEGTA and 0.2 ml of a bacterial suspension containing $c. 2 \times 10^5$ actively-dividing bacteria in normal saline. Serum inactivated by heating at 56°C for 30 min plus saline or MgEGTA served as controls. Viable counts were made by the pour-plate technique at the start and after incubation for 1, 2 and 3 h at 37°C. The response of each strain was assessed on the same day in normal human serum plus saline (NHS), in NHS + MgEGTA, in heat-inactivated serum plus saline (HIS) and in HIS + MgEGTA. Two strains were assayed on each day and for each strain all tests were done three times on different days.

Distinction between classical and alternative complement pathways. The addition of EGTA to serum chelates calcium ions sufficiently to inhibit classical-pathway activity, but leaves available sufficient magnesium ions for the alternative pathway to function (Fine *et al.*, 1972). In bactericidal tests, however, the remaining available magnesium concentration is too low for active cell division and normal bactericidal activity is suppressed unless the chelator is supplemented with magnesium (Roberts and Phillips, 1981). The difference between bactericidal activity in NHS and in NHS + MgEGTA was, therefore, taken as a measure of classical-pathway activity. When no significant difference was apparent between results in NHS and in NHS + MgEGTA, any anti-bacterial activity in NHS alone was attributed to alternative pathway activation.

RESULTS

The results of assays on promptly serum-sensitive strains are given in table I. Incubation in NHS reduced the viable counts of all these strains to <10% of the inoculum within 1 h. Comparison of results in NHS and in NHS + MgEGTA shows that the prompt killing of all but one of these strains by serum was largely a function of the classical complement pathway. Any remaining, alternative-pathway, activity shown by comparison of the results in NHS + MgEGTA with those in HIS + MgEGTA, was delayed or virtually absent, as with the O1 isolate, no. 470. The one exception to this was that prompt killing of the O9 isolate no. 282 appeared to be unaffected by elimination of the classical pathway.

The results obtained with strains of delayed sensitivity to serum are shown in table II. Only one strain, the O9 isolate no. 422, showed any reduction in viable count during the first hour of incubation in NHS. However, with all but one of the strains, the O18 isolate no. 469, the count was reduced to <10% of the inoculum by the end of

TABLE I

The effect of MgEGTA on the viable count of promptly serum-sensitive strains of E. coli incubated in normal human serum or heat-inactivated serum

Strain no.	Sero-group	Percentage* of inoculum at indicated time in											
		normal human serum						heat-inactivated serum					
		+saline			+MgEGTA			+saline			+MgEGTA		
		1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
470	O1	4	<1	<1	127	508	199	291	1149	2079	171	424	390
508	O2	2	<1	<1	105	52	9	112	288	430	136	287	399
478	O4	7	<1	<1	124	30	6	186	615	841	160	350	441
439	O6	<1	<1	<1	73	5	<1	132	480	986	126	346	611
435	O7	<1	<1	<1	85	15	2	278	640	1454	218	261	260
282	O9	<1	<1	<1	<1	<1	<1	101	222	347	116	110	102
297	O18	<1	<1	<1	78	15	<1	312	433	1224	164	212	329
482	O75	<1	<1	<1	207	394	110	307	1316	5607	304	1126	4490

* Mean of three determinations.

the third hour. Elimination of the classical pathway (NHS + MgEGTA) prevented practically all of the killing of these strains. Comparison of these results with those in HIS + MgEGTA revealed no alternative-pathway activity against the O18 isolate no. 469 or the O1 isolate no. 434 although growth of the latter may have been slightly retarded. The alternative pathway appeared to have some antibacterial activity against the remaining strains, but the count at the end of the third hour was reduced significantly below that of the inoculum only with the O9 isolate no. 422, and this strain showed a slight reduction in count even in HIS.

Four of the eight "serum-resistant" isolates (table III)—the O1 isolate no. 417, the O2 isolate no. 366, the O7 isolate no. 517, and the O75 isolate no. 456—were not really resistant in that a reduction in viable count occurred in the third hour of incubation in

TABLE II

The effect of MgEGTA on the viable count of delayed serum-sensitive strains of E. coli incubated in normal human serum or heat-inactivated serum

Strain no.	Sero-group	Percentage* of inoculum at indicated time in											
		normal human serum						heat-inactivated serum					
		+saline			+MgEGTA			+saline			+MgEGTA		
		1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
434	O1	263	28	2	288	414	489	333	1354	5694	284	456	696
424	O2	174	4	2	226	596	394	308	920	2859	277	781	811
436	O4	149	67	5	132	159	96	165	446	602	142	220	317
504	O6	102	43	<1	152	137	82	113	482	840	108	292	418
412	O7	103	4	<1	196	494	145	364	1090	4235	271	738	509
422	O9	79	2	1	109	66	39	115	108	80	94	101	83
469	O18	234	79	26	170	349	547	230	825	2487	167	257	338
461	O75	343	87	3	222	115	84	327	1708	6253	122	385	410

* Mean of three determinations.

TABLE III

The effect of MgEGTA on the viable count of serum-resistant strains of E. coli incubated in normal human serum or heat-inactivated serum

Strain no.	Sero-group	Percentage* of inoculum at indicated time in											
		normal human serum						heat-inactivated serum					
		+ saline			+ MgEGTA			+ saline			+ MgEGTA		
		1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
417	O1	245	576	147	182	444	457	216	549	1228	155	354	441
366	O2	270	271	64	220	558	434	210	640	940	228	434	450
519	O4	241	684	744	269	546	720	180	787	1996	130	184	245
401	O6	210	754	1545	200	717	1675	126	730	1358	111	347	406
517	O7	215	233	7	182	356	231	205	618	2083	148	199	372
310	O9	307	858	1942	256	600	451	321	1043	2963	294	698	1511
441	O18	342	1680	4910	436	977	2656	341	1186	5729	356	758	1213
456	O75	184	180	21	237	666	935	252	1066	2104	271	539	698

* Mean of three determinations.

NHS. With the O7 isolate <10% of the inoculum remained. Little, if any, bactericidal activity against these four strains was apparent after elimination of the classical-pathway (NHS + MgEGTA). The four remaining strains all grew in NHS throughout the three hours of the assay with the final viable counts showing increases from the initial inoculum that ranged from more than 7-fold for the O4 isolate no. 519, to almost 50-fold for the O18 isolate no. 441. In comparison with their behaviour in NHS, these four strains grew to a similar or greater extent in HIS and less well in HIS + MgEGTA. Three of these four strains also grew to a similar extent in NHS and in NHS + MgEGTA. However, the O9 isolate no. 310, showed some reduction in viable count during the third hour in NHS + MgEGTA although it grew well in NHS and in HIS + MgEGTA. This could indicate that it was sensitised to alternative-pathway activity by MgEGTA.

DISCUSSION

In our previous study of the effect of MgEGTA on the bactericidal activity of human serum (Roberts and Phillips, 1981) we found that promptly serum-sensitive strains of *E. coli* serogroup O6 were killed rapidly by the classical pathway and that, when this pathway was eliminated by the chelator, delayed killing occurred by the alternative pathway; this was comparable with the findings of Traub and Kleber (1976) for strains of *Serratia marcescens*. In the present study of other O-serogroups common amongst urinary isolates of *E. coli*, most strains again responded in a similar way but further patterns of response to serum have emerged; the O1 isolate, no. 470, showed little if any susceptibility to alternative-pathway activity and the killing of the O9 isolate no. 282 appeared to be unaffected when MgEGTA was added to NHS. This latter finding may indicate that the rapid killing in NHS was a function of alternative-pathway activity. However, the possibility that the classical pathway has the same effect in NHS as the alternative pathway has in NHS + MgEGTA cannot be

ruled out and this shows the importance of developing a reliable method of assessing each pathway independently.

Bactericidal activity against the strains of delayed serum sensitivity in this study was largely a function of classical-pathway activity. This is contrary to the findings of Traub and Kleber (1976) with delayed serum-sensitive strains of *S. marcescens* which were mainly affected by the alternative pathway. In our earlier study of *E. coli* O6 strains (Roberts and Phillips, 1981) three of the seven delayed serum-sensitive strains were affected by the alternative pathway and four by the classical pathway. It may be significant that the *S. marcescens* strains and the three *E. coli* O6 strains affected by the alternative pathway showed a reduction in viable count within the first hour of incubation in NHS whereas the four O6 strains and seven of the eight strains in the present study that were affected by the classical pathway all showed an increase in viable count at the end of the first hour. We suggest that a distinction should be made between these two patterns of response. If the term "promptly serum-sensitive" is used for strains in which the viable count is reduced below 10% of the inoculum within 1 h, "delayed serum-sensitive" should refer to strains with first hour counts from 10–100% and strains showing an initial increase in viable count with a subsequent fall could be designated "late serum-sensitive".

If such a distinction is made, then four of the "resistant" strains in the present study should be classified as late serum-sensitive and would fit the pattern of such strains in being mainly affected by the classical pathway. The remaining four strains grew in NHS which, at most, retarded growth when compared with HIS, although one strain, the O9 isolate no. 310, appeared to be sensitised to the alternative pathway by MgEGTA.

The multiplicity of patterns of response to the two complement pathways by different bacterial strains indicates the difficulty of interpreting results of studies on serum bactericidal activity in which the part played by each pathway is not defined. This is particularly so for studies in which sensitivity to serum is changed by mutation or conjugation (Taylor, 1975) or by the introduction of plasmids (Taylor and Hughes, 1978; Binns, Davies and Hardy, 1979; Timmis, Moll and Danbara, 1979). The present work shows that a particular change, conferring, for example, protection against the classical pathway, could convert one promptly serum-sensitive strain to complete resistance, a second only to delayed sensitivity and have no apparent effect on a third.

The marked differences in the growth of the strains in HIS may be important; it varied from no significant growth of the O9 isolate no. 422 to an increase on the initial inoculum of more than 60-fold in three hours for the O75 isolate no. 461. It is interesting that growth was particularly poor with two of the three O9 isolates, both of which have the A type of K-antigen whereas isolate no. 310, which grew well in HIS, does not. It may also be significant that each of the three isolates of groups O1, O7 and O18 grew well. These differences could be a reflection of nutritional requirements or of the effectiveness of a non-complement-dependent bacteriostatic mechanism (Traub and Kleber, 1976). The generally reduced growth when MgEGTA is added to HIS has been noted before for *E. coli* O6 strains (Roberts and Phillips, 1981) and probably indicates that MgEGTA chelates other divalent cations important for growth.

In this study we have attempted to assess the relative importance of the classical and alternative complement pathways in serum bactericidal activity against *E. coli* strains of the common urinary O-serogroups. It is clear that more strains need to be

studied in order to make a clear distinction between strains which are promptly serum-sensitive, delayed serum-sensitive, late serum-sensitive or serum-resistant. There are indications in these results that strains of serogroup O1 are largely unaffected by the alternative pathway whereas this pathway may be particularly important for serogroup O9. It would be interesting to know if the promptly serum sensitive O9 strain, which is rapidly killed even after elimination of the classical pathway, is unique or if similar strains exist in this or other O-serogroups.

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