PEPTIDE CLEAVAGE IN SHEEP ERYTHROCYTE MEMBRANES DURING THE ACTION OF COMPLEMENT

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1. Introduction

Most publications dealing with the complement system describe the purification of the various components involved, their sequence of reaction, their activation, their physicochemical properties and their split products [1]. In contrast, little biochemical attention has been focussed on the final target of complement action, the cell membrane. The work that does exist concerns the phospholipids of erythrocytes, but at this writing no certain chemical changes in these membrane components have been demonstrated during complement mediated hemolysis [2, 3]. The same is true for complement-sensitive liposomes [4]. Nevertheless, various groups have attributed the micromorphologic consequences of complement action on erythrocyte- and artificial lipid-membranes to changes in the organisation of membrane lipids [5, 6]; these changes are circular holes or depressions, 70–100 Å in diameter, depending on the membrane and complement source and appear already after the action of complement component, C5.

There are no published data on the possible action of complement on membrane proteins, presumably because useful methods for the fractionation of membrane peptides have only recently become available [7, 8]. However, by applying these techniques of electrophoretic molecular sieving to our analysis of complement-induced disruption of erythrocyte membranes we find that important alterations of membrane peptides occur during this event.

2. Methods

We have used standard methods for the lysis of sheep erythrocytes sensitized with anti-sheep-erythrocyte-antibodies by guinea-pig complement, for the preparation of intermediate complexes of various complement components with erythrocytes (EAC 1, 4, 2, EAC 1–8, EAC 1–9 and E*) as well as for the isolation of the reagents (R3-Serum, C3–8 reagent and C9) [9–11]. Erythrocyte ghosts from all species were prepared as in [12] by sequential lysis in 5 mM phosphate, pH 8, but with particular emphasis on the removal of leukocytes and platelets (to prevent protease contamination). For electrophoretic molecular sieving the membranes were dissolved in 1% sodium dodecyl sulfate (SDS), with simultaneous rupture of disulfide bridges in 40 mM dithiothreitol [7]. Peptide separation was in 5.8% polyacrylamide, containing 1% SDS buffered with tris-acetate-EDTA (40 mM/20 mM/2 mM), pH 7.4. Staining for proteins was with Coomassie blue, and with "Stains-All" for glycoproteins [7]. After removal of excess stain as in [7] the gels were scanned at 620 nm in a GILFORD Spectrophotometer (Model 240) with a synchronous scanner (Model 2410-5). Pyronin T was used as tracking dye and gels were calibrated with pure human immunoglobulin G (155,000 daltons), phosphorylase A

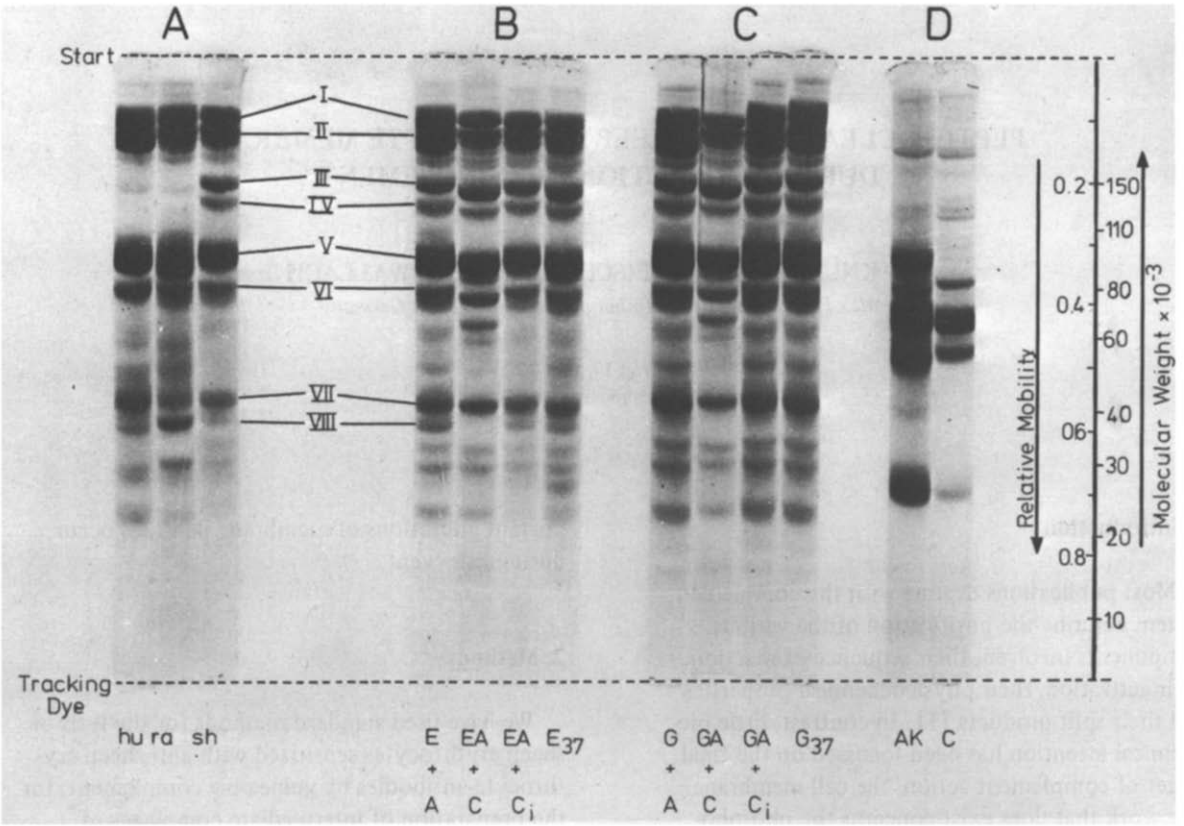

Fig. 1. (A) Molecular weight distributions of the peptides in the normal erythrocyte membranes of different species. (B) Peptide patterns (left to right) of: membranes from sensitized erythrocytes (E+A); membranes from sensitized, complement-lysed erythrocytes (EA+C); membranes from sensitized erythrocytes, treated with inactivated complement ($EA+C_i$); membranes from erythrocytes exposed to standard incubation conditions (E_{37}). (C) Peptide patterns (left to right) of: "ghosts" treated with antiserum (G+A); "ghosts" treated with antibody and complement (GA+C); "ghosts" treated with inactivated complement ($GA+C_i$); "ghosts" exposed to standard incubating conditions (G_{37}). (D) Peptide patterns (left to right) of rabbit anti-sheep-erythrocyte antiserum and of guinea-pig complement.

(92,000 daltons). human-transferrin (71,000 daltons), human serum albumin (69,000 daltons) heavy immunoglobulin chains (55,000 daltons), ovalbumin (45,000 daltons), light immunoglobulin chains (22,000 daltons), chymotrypsinogen (17,800 daltons), myoglobin (17,000 daltons) lysozyme (14,000 daltons) and cytochrome *c* (13,000 daltons). The proportion of protein in various bands was determined from the band areas, measured gravimetrically, assuming equivalent staining for the various peptide species [7]. Membrane proteins were assayed fluorometrically as in [13]. After treatment with antibody and complement or its components as in [9–11] the membranes were washed in 5 mM phosphate, pH 8, by twice sedimenting at 20,000 *g* (Sorval RC2B, rotor SS34).

3. Results

The normal peptide distribution in the erythrocyte membranes of man (hu), sheep (sh) and rabbits (ra) is given in fig. 1A. Our data are of higher resolution than obtained in another comparative study [15], but the major components in the membranes of sheep and rabbit erythrocytes resemble the principal peptides which account for about 75% of the protein mass in human erythrocyte

Table 1
Main peptide pattern in erythrocyte membranes of three species.*

Major peptide	Sheep MW	Sheep %	Human MW	Human %	Rabbit MW	Rabbit %
I	~320,000	~14	~360,000	~18	~340,000	~16
II	~270,000	~14	~340,000	~20	~300,000	~20
III	155,000	7	–	–	–	–
IV	130,000	4	–	–	–	–
V	95,000	30	89,000	30	92,000	25
VI	80,000	7	78,000	8	75,000	9
VII	42,000	5	41,000	4	40,000	6
VIII	38,000	2	36,000	5	25,000	3

* Molecular weights of the peptides I to IV were obtained in gels of 3% and 4.1% acrylamide concentration. This is true also for staining percentages.

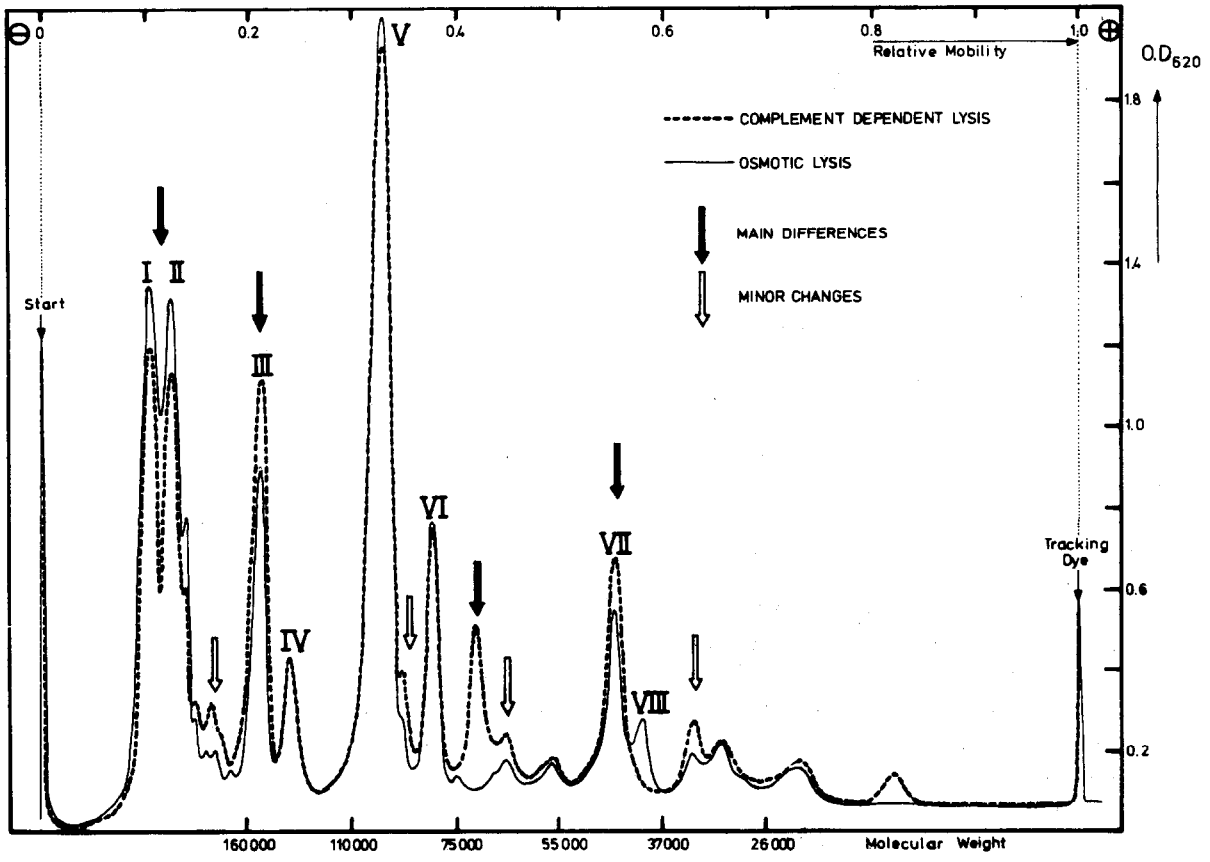

Fig. 2. Spectrophotometric scan (620 nm) of electropherograms of sheep erythrocyte membranes after osmotic or complement-mediated lysis. The complement dilution was 1:50 and the incubation for 40 min at 37°. Equal amounts of protein were applied to each gel.

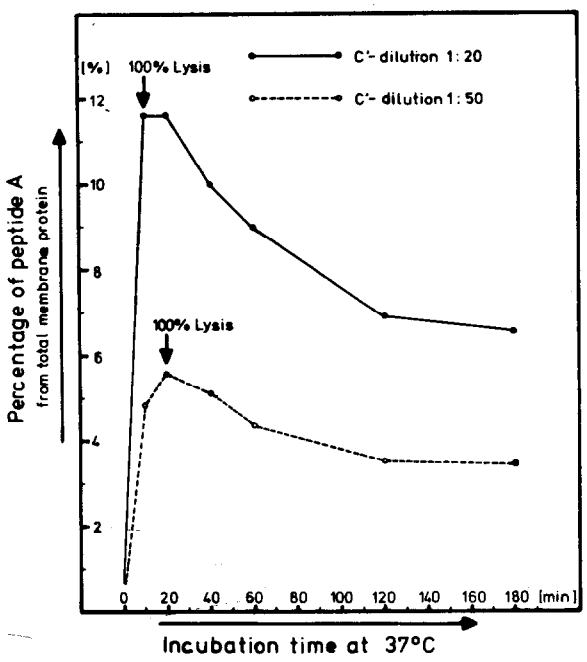

Fig. 3. Changes in membrane peptides after incubating sensitized sheep erythrocytes at 37° with full guinea-pig complement for different periods of time.

membranes. However, there are some significant differences between human and sheep membranes, the latter containing two additional peptides of about 150,000 and 140,000 daltons (fig. 1A and table 1).

The "ghosts" of sensitized sheep erythrocytes, lysed by complement, exhibit the following changes in the membrane peptides (fig. 2):

1. The proportion of peptides I + II diminishes by about 20%.
2. The proportion of protein staining in the region of peptide III (about 155,000 daltons) rises from about 7% to an average of 13%.
3. A new peptide of about 71,000 daltons appears; it accounts for about 10% of the protein staining and is termed peptide A.
4. A second new peptide of about 31,000 daltons emerges; it accounts for about 2% of the protein staining and is termed peptide B. It becomes more sharply defined at polyacrylamide levels greater than 6.5%.
5. Staining in the region of peptide VII increases from about 5% to about 8.5%.

The generation of peptide A is proportional to the complement level used, but diminishes as incubation at 37° is extended; then the proportion of peptide A falls of with time by the same percentage at all complement concentrations employed (fig. 3).

To show that we are dealing with complement-mediated phenomena, rather than manipulative artefacts, we have performed the following controls: (a) exposure of cells and membranes to the usual incubation temperatures and times, but without addition of antibody or complement; (b) osmotic lysis of sensitized erythrocytes; (c) treatment of sensitized cells with heat-inactivated complement, followed by osmotic lysis; (d) exposure of non-sensitized cells to active complement, followed by osmotic lysis; (e) treatment of sensitized cells with complement, inactive due to lack of Ca^{2+} and Mg^{2+}, followed by osmotic lysis. None of these manipulations changed the normal peptide pattern (fig. 1B). However, the action of full complement on sensitized, purified sheep erythrocyte "ghosts" elicited the same alterations observed with intact cells (fig. 1C). Finally, electrophoretic comparisons of membranes, sensitizing antiserum and the complement employed, clearly indicate that the described effects are not due to the absorption and concentration of these reagents on the membranes (fig. 1D).

Human erythrocyte "ghosts", from cells of blood type A, which cross reacts with sheep Forssman antigen, showed no change in their peptide pattern after complement action. However, experiments with intact human type A erythrocytes, sensitized with anti-sheep serum, yielded only partial hemolysis, even at the highest complement levels.

Our experiments show that the action of guinea-pig complement on sensitized sheep erythrocytes, or "ghosts" derived therefrom, effects a significant peptidolysis in the membrane proteins of high molecular weight, producing new electrophoretic components of lower molecular weight. This suggests, but does not prove, that membrane proteins are the final target of complement action. However, we are now testing this possibility, using functionally pure complement components, certain cellular "intermediates" (EAC 1, 4, 2; EAC 1–8; EAC 1–9) and modified membranes. Data already in hand suggest that membrane peptidolysis occurs already early during the action of complement.

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