Complement-mediated lysis of pigeon erythrocyte ghosts analysed by flow cytometry

Evidence for the involvement of a 'threshold' phenomenon

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1. Flow-cytometric analysis of complement-mediated lysis of antibody-coated pigeon erythrocyte ghosts containing fluorescein was carried out to determine whether lysis involved a gradual release of fluorescein or a 'threshold' release from individual cells. 2. Antibody-coated ghosts were comprised of three subpopulations identified by fluorescence and scatter (size). These were: (a) highly fluorescent, medium scatter, (b) medium fluorescence, high scatter, and (c) low (or zero) fluorescence, low scatter. 3. Lysed ghosts and isolated nuclei were identified by fluorescence microscopy and scanning electron microscopy. 4. Fluorescence distributions analysed by flow cytometry indicated that, after complement attack, those ghosts remaining intact retained all their fluorescent label. 5. A time course of changes in ratios of the three subpopulations indicated that once lysis of an individual ghost was initiated, release of label was complete within 1 min; no stages of intermediary fluorescence appeared, and those ghosts remaining at the end of the experiment retained the same fluorescence intensity as control ghosts. 6. The results supported the hypothesis that complement-mediated cell lysis is a 'threshold' phenomenon; a submaximal response by a cell population representing a complete response by only some of the cells rather than a partial response by all of the cells.

Conventional biochemical analyses, which are carried out on samples from isolated cells or tissues, produce a mean estimation from thousands, if not millions, of cells. A major limitation of this type of analysis is the inability to take into account any heterogeneity in the cell population. There are many reasons for believing that characterization of this heterogeneity is vital if further understanding of tissue regulation is to be achieved. For example, cells may be heterogeneous with respect to their position in the cell cycle (Lloyd *et al.*, 1982) or because of chemical gradients within the tissue. Furthermore it has been proposed that many types of cell activation, particularly those mediated by an increase in intracellular Ca^{2+} , involve 'threshold' responses in individual cells (Campbell, 1983). In order to characterize such threshold responses, 'single-cell analysis' is required. Chemiluminescence (Campbell *et al.*, 1983) and flow cytometry (Mendel-

sohn, 1980; Kruth, 1982) have the necessary sensitivity to achieve this.

Here we report the application of flow cytometry to investigate the heterogeneity within a cell population to formation of the membrane-attack complex of complement (C5b-9). Some years ago, Mayer (1961) proposed the 'one hit' hypothesis for complement-mediated cell lysis, based essentially on a mathematical analysis of the kinetics of lysis (Rapp, 1964; Kitamura *et al.*, 1976). Considerable controversy still exists as to the validity of the 'one hit' hypothesis. In particular it is not clear how this relates to the stoichiometry of the membrane-attack complex and the molecular mechanism by which it causes lysis (Frank *et al.*, 1970; Roualt *et al.*, 1978; Biesecker *et al.*, 1979; Esser *et al.*, 1979; Bhakdi & Tranum-Jensen, 1981). Furthermore there are inconsistencies between the number of membrane lesions predicted from haemolysis data and those

revealed by electron microscopy. In order to resolve these problems it is essential to quantify lysis of individual cells. We have used flow cytometry to analyse the complement-mediated lysis of pigeon erythrocyte ghosts containing fluorescein. These ghosts have the advantage not only of containing a sensitive fluorescent indicator to detect lysis, but also retain their nuclei, enabling both intact and lysed cells to be detected and quantified by light-scattering and microscopy.

Experimental

Preparation of sealed pigeon erythrocyte ghosts

Sealed pigeon erythrocyte ghosts containing fluorescein were prepared by modification of the 'pre-swell' method of Rechsteiner (1975) as described previously (Hallet & Campbell, 1982). Erythrocytes were washed three times in medium A [2 mM-MgCl₂/140 mM-NaCl/5 mM-KCl/10 mM-Tes-(2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulphonic acid), pH 7.4] and then diluted to an equal volume with medium A diluted 1:1 (v/v) with distilled water. After a pre-swell incubation for 10 min at 0°C the suspension was centrifuged for 10 s in an Eppendorf micro-centrifuge and the supernatant discarded. The pellet was diluted to an equal volume in 10 mM-Tes, pH 7.4 (Chelex-treated; Ashley & Campbell, 1979) and incubated for 10 min at 0°C. After this, 0.4 mM-6-carboxy-fluorescein (final concn.) was added, followed by incubation for 10 min at 0°C. Sealing of the ghosts was achieved by the addition of spectroscopically pure 150 mM-KCl (final concn.) and incubation at 37°C for 30 min. Sealed ghosts were washed three times in medium A and used within 6 h of preparation.

Antisera

Antisera to pigeon erythrocytes were prepared in guinea pigs by subcutaneous injection of pigeon erythrocytes as described previously (Campbell *et al.*, 1979). Sera were heat-inactivated by heating to 56°C for 30 min to inactivate complement (Lachmann, 1975) and portions subsequently stored at -20°C. Coating of erythrocytes was achieved by incubating 3-5 µl of antiserum/ml of ghosts (diluted 1:100 with medium A containing 1 mM-CaCl₂) for 5 min at 37°C.

Complement

Portions of normal human serum were stored at -70°C and used as a source of complement. All dilutions were performed in medium A containing 1 mM-CaCl₂. Incubations were at room temperature (18°C).

Flow cytometry

Analysis of ghosts was achieved with a Becton Dickinson FACS III flow cytometer, fitted with a 50 µm nozzle. Distributions of the green fluorescence emitted by entrapped fluorescein (520 < λ < 540 nm; excitation at 488 nm) and scatter distributions were accumulated in a multi-channel (127 channels) pulse-height analyser. Sorting and counting of selected populations of ghosts was carried out at flow rates adjusted to analyse 3000 cells/s.

Fluorescence measurements

Fluorescein release was measured after centrifugation of ghost suspensions for 10 s in an Eppendorf micro-centrifuge. The fluorescence in the supernatant was measured in a Perkin-Elmer 204-A fluorescence spectrophotometer; the excitation wavelength was 490 nm and the emission wavelength was 520 nm. Total entrapped fluorescein was measured after the addition of 0.1% (v/v, final concn.) Triton X-100. This volume of detergent was also added to all samples before the fluorescence measurement.

Microscopy

Sorted ghost subpopulations were analysed by phase-contrast and fluorescence microscopy. For scanning electron microscopy, ghosts were fixed for 2 h at 0°C in 2% glutaraldehyde (diluted medium A containing 1 mM-CaCl₂) followed by 1 h in 1% OsO₄. Initial dehydration was through an ascending ethanol series (20-100%, v/v, ethanol in water) with samples equilibrated for 20 min at each stage. Substitution with amyl acetate was accomplished by repeating this procedure with successively higher concentrations of amyl acetate in ethanol. Samples were then dried by the critical-point method (Anderson, 1951; Hayat & Zirkin, 1973), sputtered with gold and viewed with an ISA Super IIIA scanning electron microscope.

Results

Scatter and fluorescence distributions of sealed pigeon erythrocyte ghosts containing fluorescein

In the flow cytometer, cells pass in single file through a sensing zone in which light-scattering or fluorescence emission may be measured. The signals generated may be viewed as a 'scatter plot' (Fig. 1a, where each dot represents the measurements obtained from individual cells until the screen becomes saturated), or more conveniently as frequency-distribution histograms (Figs. 1b and 1c). Analysis of fluorescein-containing pigeon erythrocyte ghosts coated with anti-(pigeon erythrocyte) antibody is shown in Fig. 1. Scatter-versus-fluorescence plots indicated that the majority of ghosts were highly fluorescent (Fig. 1a), but other subpopulations could be distinguished. Separate

Fig. 1. Fluorescence and scatter distributions of pigeon erythrocyte ghosts obtained by flow cytometry

A suspension of antibody-coated pigeon erythrocyte ghosts containing fluorescein, in medium A containing 1 mM-CaCl₂, was analysed by flow cytometry. (a) shows a plot of fluorescence against scatter of each ghost analysed. Representation of these data as frequency distribution histograms is shown for (b) scatter and (c) fluorescence as number of cells versus amount of scattered light (size) or fluorescence intensity.

scatter and fluorescence distributions assisted identification of these types; scatter distributions showed one major peak, with the suggestion of one or two shoulders (Fig. 1b), whereas fluorescence distributions (Fig. 1c) clearly showed three populations of low (or zero), medium and high fluorescence.

Further characterization of the ghost populations was achieved after cell sorting. Selection of suitable windows indicated that ghosts of medium fluorescence (Fig. 2a) were virtually twice the size (assuming a linear relationship between scatter and cell size) of the highly fluorescent ghosts (Fig. 2b). The subpopulation with low or zero fluorescence also had the lowest scatter (Fig. 2c). The proportions of high fluorescent/medium fluorescent/low (or zero) fluorescent subpopulations were usually about 6–8:1:1 respectively after coating with antibody.

Fluorescence and scatter distributions of ghosts incubated in the presence and absence of complement

Control samples (antibody-coated) diluted in medium A containing 1 mM-CaCl₂, at time zero

Fig. 2. Characterization of subpopulations of pigeon erythrocyte ghosts

A suspension of antibody-coated ghosts was analysed by flow cytometry as described in the legend to Fig. 1. Windows were selected around the three fluorescent subpopulations, and scatter distributions were obtained for each of these. (a), (b) and (c) are scatter distributions of the ghost subpopulations having medium, high and low fluorescence respectively. Markers and numbers refer to channel numbers (127 channels full scale).

and after 30 min had scatter (Fig. 3a) and fluorescence (Fig. 3b) distributions as indicated. Fluorescence distributions indicated three subpopulations of high, medium and low (or zero) fluorescence, whereas scatter distributions discerned two components, which corresponded to the low-fluorescent population and the highly fluorescent population (see Fig. 2). After 30 min incubation with a 1:20 dilution of normal human serum, only one major peak was observed in scatter distributions (Fig. 3c), which corresponded to the low- or zero-fluorescent subpopulation (Fig. 3d). However, at high sensitivity (upper trace in Fig. 3d) it was seen that of those cells retaining their fluorescence after

Fig. 3. Scatter and fluorescence distributions of pigeon erythrocyte ghosts before and after complement attack. Antibody-coated ghosts were incubated in medium A containing 1 mM-CaCl₂ (control, *a*, *b*) or in the same containing a 1:20 dilution of normal human serum (*c*, *d*) for 30 min. (*a*) and (*c*) are scatter frequency distributions, whereas (*b*) and (*d*) are fluorescence frequency distributions. In (*d*), the lower trace was recorded at the same sensitivity as (*b*), while the upper trace was recorded at higher sensitivity.

complement attack, fluorescence intensity per ghost was virtually identical with that of control suspensions. This implies that although only about <5% of the original population of highly fluorescent ghosts remain, they have retained >95% of their fluorescent contents.

Morphology of ghost subpopulations

Examination of typical ghost preparations by phase and fluorescence microscopy confirmed the variations in fluorescence intensities (Figs. 4*a* and 4*b*). Whereas most of the ghosts were highly fluorescent, some had low (arrowed) or no detectable (broken arrow) fluorescence. After cell sorting, the high-scatter medium-fluorescence subpopulation was seen to consist of swollen, mis-shaped ghosts (Fig. 4*c*). Scanning electron micrographs of ghosts, however, showed that, before complement attack, the low-(or zero)-fluorescent subpopulation appeared to be comprised mainly of shrunken, mis-shaped structures (Fig. 4*e*) compared with the total ghost preparation (Fig. 4*d*), whereas after complement attack, this subpopulation comprised mainly empty ghosts containing nuclei (Fig. 4*f*); some free nuclei were also observed.

Time course of changes in numbers of ghosts during complement attack

In order to further analyse the 'threshold' lysis of ghosts by complement, a time course of changes in

subpopulation numbers was performed by flow cytometry.

A suspension of fluorescein-entrapped ghosts was coated with antibody as described in the Experimental section and divided. To one portion (control) an equal volume of medium A containing 1 mM-CaCl₂ was added, whereas to the other an equal volume of a 10-fold dilution of normal human serum in the same medium was added. Windows were selected (as in Fig. 2) around the three subpopulations and relative numbers were obtained at time intervals.

In the control sample, the highly fluorescent ghosts decreased from 74 to 54% of the total over about 1 h. The swollen ghosts with medium fluorescence increased in numbers slightly over this period (from 7 to 12%), whereas those with low (or zero) fluorescence increased in numbers from 18 to 35% of the total (Fig. 5*a*).

In the presence of complement, the decrease in numbers of the highly fluorescent ghosts (73–18%) was paralleled by an increase in the numbers of the subpopulation with low (or zero) fluorescence (from 19 to 75%). Virtually no change in the numbers of the swollen ghosts was observed (Fig. 5*a*).

Released fluorescein, measured in supernatants after centrifugation of suspensions, increased in the control from 20 to 35% and in the presence of complement increased from 17 to 84% (Fig. 5*b*).

Fluorescence distributions of antibody-coated ghost suspensions in the presence and absence of complement at time zero were identical (Fig. 6*a*). In the presence of complement the relative numbers of ghosts of high fluorescence gradually decreased with time (Figs. 6*b* and 6*c*), but the fluorescence intensity of these remaining ghosts was unaltered. Thus, although the actual numbers of highly fluorescent ghosts was decreasing during complement attack, those that remained intact retained *all* their fluorescent content.

Fluorescent distributions of the control suspension changed very little over the time course of the experiment (Fig. 6*d*).

Closer examination of scatter distributions of the highly fluorescent ghosts in another experiment showed that, in the presence of complement, the peak channel increased from 64 to 84 (127 channels full scale) during the course of the experiment (Fig. 7). These changes probably reflect swelling of ghosts before lysis by complement (Bauer & Valet, 1983). No significant changes were seen in control suspensions.

Discussion

When a population of cells responds to a stimulus or a pathogen the question arises, does a 50% change in a particular parameter being measured

Fig. 4. *Optical and scanning electron microscopy of pigeon erythrocyte ghosts*

(a) shows a suspension of pigeon erythrocyte ghosts containing fluorescein viewed under phase contrast; (b) shows the same field viewed by fluorescence microscopy; (c) shows a suspension of the high-scatter medium-fluorescence subpopulation, collected after cell sorting by flow cytometry, viewed under phase contrast; (d) shows a scanning electron micrograph of a ghost preparation. Scanning electron micrographs are shown of the subpopulation with low scatter/low (or zero) fluorescence, obtained by cell sorting after a 30 min incubation in the absence (e) or presence (f) of a 1:20 dilution of normal human serum. The bar marker represents 5 μ m.

represent a 50% change in all of the cells or a 100% change in 50% of the cells (Campbell, 1983)? The results presented here show that flow cytometry offers one approach to answer this question, provided that a suitable fluorescent probe can be selected (Shapiro, 1981; Watson, 1981).

In the present study we have demonstrated that, in a situation where complement attack results in about 80% release of entrapped fluorescein, this appeared to represent total release by 80% of the ghosts; the remaining ghosts retain all their fluorescent labels (Figs. 5 and 6). No states of partial lysis were observed which would have been detected as

ghosts with intermediary fluorescence intensities. Thus, once lysis is initiated, release of contents is rapid and total; states of partial lysis may only be detected as transients. Since the accumulation time of distributions by flow cytometry was about 1 min and no intermediary states of lysis were observed, the time from initiation to completion of lysis must be less than 1 min. These results are consistent with the hypothesis that complement-mediated cell lysis represents a 'threshold' response within individual cells.

Pigeon erythrocyte ghosts prepared by the method described here readily entrap fluorescein

Fig. 5. Changes in numbers of individual ghost populations and fluorescein release during complement attack

(a) shows numbers of ghosts as detected by flow cytometry. $\circ, \Delta, \square$, Control samples (antibody-coated ghosts diluted with medium A containing 1 mM- CaCl_2); $\bullet, \blacktriangle, \blacksquare$, as above with a 1:20 dilution of normal human serum. $\circ, \bullet$, Highly fluorescent; $\Delta, \blacktriangle$, low or zero fluorescence; $\square, \blacksquare$, medium fluorescence. (b) fluorescein release by ghosts: $\circ$, control samples; $\bullet$, after the addition of 1:20 dilution of normal human serum. Windows were selected around the fluorescent subpopulations to allow quantification of ghosts, and the values quoted are expressed as a percentage of the total number of cells analysed.

(about 10% of the total added); at the concentration of fluorescein used, these ghosts are stable for several hours on ice. Observation of typical ghost preparations by fluorescence microscopy (Fig. 4) revealed variations in fluorescence intensities. Flow cytometry discerned three subpopulations (Figs. 1 and 2). Highly fluorescent ghosts constituted about

Fig. 6. Fluorescence distributions of pigeon erythrocyte ghosts before and after complement attack

Fluorescence frequency distribution histograms are shown of ghosts incubated: (a) in the presence of medium A containing 1 mM- CaCl_2 (control), or in the same containing a 1:20 dilution of normal human serum, at time zero (identical distributions); (b) for 26 min with normal human serum; (c) for 51 min with normal human serum (distributions recorded at two sensitivities); (d) control sample after 65 min. Bar markers indicate channels 16 and 50 (127 channels full scale).

Fig. 7. Scatter distributions of pigeon ghosts during complement attack

Scatter distributions of the fluorescent ghosts are shown after: (a) 0 min; (b) 3 min; (c) 14 min; (d) 35 min; (e) 60 min incubation with 1:20 dilution of normal human serum. Bar markers and numbers indicate peak channel numbers.

80% of the total population, whereas subpopulations with low scatter/low or near zero fluorescence, together with high scatter/medium fluorescence were present in about equal ratios. Before complement attack the low-scatter/low-(or near zero)fluorescence fraction was mainly comprised of shrunken ghosts, whereas after complement attack empty ghosts predominated. The ghosts of medium fluorescence and high scatter appeared as swollen, mis-shaped structures. Identification of these fractions was confirmed by scanning electron microscopy (Fig. 4).

In spite of the fact that the 'one hit' hypothesis for complement-mediated cell lysis is more than 20 years old (Mayer, 1961, 1973), there remains much controversy as to its validity. The results reported here are consistent with the essence of this hypothesis, namely that cell lysis requires a critical

condition in each cell, presumably dependent on the number and composition of the membrane-attack complexes (C5b-9) on the individual cell. Thus complement-mediated cell lysis belongs to the group of cellular responses classified as 'threshold' (Campbell, 1983). The mechanism of this 'threshold', and in particular whether the rise in intracellular Ca^{2+} induced by complement (Campbell *et al.*, 1979, 1981) has any role to play in provoking this 'threshold' (Campbell & Luzio, 1981), remains to be established. The application of fluorescent monoclonal antibodies to C9 (Morgan *et al.*, 1983), combined with flow cytometry will enable the relationship between lysis and the number of C9 molecules per cell to be determined. It has been argued that a complete understanding of complement action requires a system in which functional activity, electron-microscopical appearance and immunochemical characteristics can be monitored concomitantly (Boyle & Borsos, 1980). Our results using flow cytometry show that it is essential that all of these parameters are assessed in individual cells.

Flow cytometry provides a method for establishing the existence of 'threshold' cell responses under both physiological and pathological conditions, as an essential preliminary to understanding the molecular basis of cell activation and cell injury.

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