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Early Membrane Rupture Events During Neutrophil-Mediated Antibody-Dependent Tumor Cell Cytolysis

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Although cell-mediated cytolysis is a fundamental immune effector response, its mechanism remains poorly understood at the cellular level. In this report, we image for the first time transient ruptures, as inferred by cytoplasmic marker release, in tumor cell membranes during Ab-dependent cellular cytolysis. The cytosol of IgG-opsonized YAC tumor cells was labeled with tetramethylrhodamine diacetate followed by the formation of tumor cell-neutrophil conjugates. We hypothesized that tumor cell cytolysis proceeds via a series of discrete membrane rupture/rescaling events that contribute to marker release. To test this hypothesis, we occluded the fluorescence image of the labeled tumor cells by passing an opaque disk into a field-conjugated plane between the light source and the sample. Multiple small bursts of fluorescent label release from tumor cells could be detected using a photomultiplier tube. Similarly, multiple fluorescent plumes were observed at various sites around the perimeter of a target. These findings support a multihit model of target cytolysis and suggest that cytolytic release is not focused at specific sites. Cytolytic bursts were generally observed at 20-s intervals, which match the previously described reduced nicotinamide-adenine dinucleotide phosphate and superoxide release oscillation periods for neutrophils; we speculate that metabolic oscillations of the effector cell drive the membrane damage of the target. The Journal of Immunology, 1999, 162: 3188–3192.

Materials and Methods

Neutrophil preparation

Peripheral blood was obtained from healthy individuals by venipuncture using heparinized tubes. Neutrophils were isolated from venous blood using Ficoll-Hypaque solutions (Sigma, St. Louis, MO) and density gradient centrifugation (13). Cells were washed twice by centrifugation and then resuspended in HBSS (Life Technologies, Grand Island, NY). Trypan blue staining indicated that 95–99% of the cells were viable.

Latex bead samples

Fluorescent latex beads (Fluoresbrite YG, diameter = 1.14 μm) were obtained from Polysciences (Warrington, PA). To minimize diffusional displacement of the beads during imaging studies, beads were mixed with 2% fluid-phase gelatin at ~40°C (type B; Sigma). The mixture was placed on a microscope slide, a coverslip was added, and the gelatin was allowed to harden at room temperature before observations.

YAC tumor cells

YAC cells were grown in suspension culture using RPMI 1640 medium (Life Technologies) containing 10% FCS and 10 μg/ml penicillin-streptomycin in a humidified CO2 incubator at 37°C (14). YAC cells were thoroughly washed by centrifugation and then resuspended in HBSS. The cells were >95% viable as judged by trypan blue staining.
YAC cell labeling

YAC cells were labeled with tetramethylrhodamine diacetate (TMR) (Molecular Probes, Eugene, OR). Briefly, stock solutions of TMR were prepared by mixing 1.5 mg of TMR with 200 μl of DMSO and then diluting with 50 ml of HBSS. Next, YAC cells were labeled with a 1/10 dilution of the stock solution for 1 h at 37°C. Cells were thoroughly washed by centrifugation (14).

Opsonization

Labeled YAC cells were opsonized with a rabbit anti-mouse thymocyte IgG fraction (adsorbed) (Accurate Chemicals, Westbury, NY) as described previously (14). Next, the cells were washed three times with HBSS. The opsonized and labeled YAC cells were mixed with neutrophils at an E:T ratio of 1:2 and then incubated at 37°C for 30 min.

Optical microscopy

Cells were examined using an axiovert inverted fluorescence microscope (Carl Zeiss, New York, NY) with mercury illumination interfaced to a Percecepts Biovision workstation (Knoxville, TN) (15, 16). The fluorescence images were collected by an intensified charge-coupled device camera (ICCD) (model XC-77; Hamamatsu Photonics, Bridgewater, NJ). A narrow bandpass discriminating filter set was used with excitation at 540/20 nm and emission at 590/30 nm (Omega Optical, Brattleboro, VT). A long-pass dichroic mirror was used at 560 nm. To occlude the bright cytoplasm of labeled tumor cells, a special adapter (Zeiss) for the fluorescence light source was employed. An opaque disk on a glass substrate held on an aluminum slider was placed into a field-conjugated plane of the adapter. Differential interference contrast photomicrographs were collected using Zeiss polarizers and a charge-coupled device camera (Model 72; Dage-MTI, Michigan City, IN). The images were stored on hard disk and photographed using a freeze-frame video recorder (Polaroid, Boston, MA).

Results

In the present study, we tested the hypothesis that membrane rupture and consequent cytoplasmic leakage are early and transient events in tumor cell cytology. Inasmuch as preliminary studies suggested that the fluorescence of cytolysis labels released from cells at early timepoints was a small fraction of the total cellular fluorescence, it was necessary to increase the relative contrast of the released label. To do this, we occluded the bright fluorescence of the target cells. Occlusion is an optical strategy employed by astronomers to visualize the corona of the sun, planetary oblateness, etc. (e.g., Ref. 17). Thus, an opaque disk on a transparent substrate was placed in a field-conjugated plane near the mercury epifluorescence lamp. With the bright cell occluded, the ICCD camera operates at maximal gain, thus allowing detection of the weak fluorescence leakage in real time. To confirm the rigor of the approach, we imaged fluorescent latex beads in the presence and absence of an occultation disk. Fig. 1A shows a fluorescence micrograph of latex beads using conventional illumination. In Fig. 1B, this same field of beads is shown again after the occultation disk is in place. As this figure illustrates, illumination was effectively blocked for a circular region in the image plane corresponding to the disk in the field-conjugated plane of the adapter without a loss in overall image quality. During quantitative intensity measurements, nonspecific background fluorescence (from other cells or prior membrane ruptures) was reduced by reducing the diameter of the field diaphragm (illustrated in Fig. 1C). Thus, we minimize background fluorescence while maximizing the contrast of cytoplasmic marker release using pericellular illumination.

Single-cell marker-release studies

IgG-coated and TMR-labeled YAC cells were incubated with human neutrophils at a 1:2 E:T ratio for 30 min at 37°C and then transferred to a microscope slide and held at 37°C using a heated microscope stage. Target to effector conjugates were selected and then aligned with the occultation disk by adjusting the stage and/or the slider holding the disk. The intensity of released pericellular fluorescence was monitored continuously using a photomultiplier tube and was recorded by a computer. Fig. 2 shows several representative kinetic profiles of pericellular fluorescence from tumor cells in neutrophil-tumor cell conjugates. Neutrophil-tumor cell conjugates were observed at 37°C. The fluorescence of the tumor cell was optically occluded, and the pericellular fluorescence intensity was recorded using a photomultiplier tube apparatus, amplifier, and computer. In these experiments, the fluorescence intensity (ordinate) is plotted against time (abscissa). Trace a shows a representative kinetic profile of pericellular fluorescence intensity over a 7.5-min period. Note the onset and termination of oscillatory marker-release events. Traces b–g show six additional quantitative marker-release studies for different effector cell to tumor cell conjugates. These traces illustrate the heterogeneity of the responses observed. In general, at least three to eight pulses of marker release can be resolved. Similar results were obtained on 15 different days with a total of 113 kinetic traces.

FIGURE 1. Illustration of the imaging technique. To exclude the bright fluorescence of labeled tumor cells while retaining the ability to detect pericellular fluorescence, a “doughnut-like” pattern of concentric circles was created on the sample. A. A field of fluorescent beads is shown. B. The same field of beads is shown again after inserting an opaque disk into a field-conjugated plane between the sample and the lamp. C. The extent of perimeter detection was limited by the field diaphragm. Magnification is ×500.

FIGURE 2. Quantitative fluorometry of pericellular fluorescence of tumor cells in neutrophil-tumor cell conjugates. Neutrophil-tumor cell conjugates were observed at 37°C. The fluorescence of the tumor cell was optically occluded, and the pericellular fluorescence intensity was recorded using a photomultiplier tube apparatus, amplifier, and computer. In these experiments, the fluorescence intensity (ordinate) is plotted against time (abscissa). Trace a shows a representative kinetic profile of pericellular fluorescence intensity over a 7.5-min period. Note the onset and termination of oscillatory marker-release events. Traces b–g show six additional quantitative marker-release studies for different effector cell to tumor cell conjugates. These traces illustrate the heterogeneity of the responses observed. In general, at least three to eight pulses of marker release can be resolved. Similar results were obtained on 15 different days with a total of 113 kinetic traces.
resentative quantitative marker-release experiments. In Fig. 2, trace a, a long timebase was used to show an entire time course of early cytolysis, including background levels before and after marker release. As this illustrates, a series of intensity spikes was observed. Fig. 2, b–g, shows the results of similar single-cell marker-release experiments depicting the heterogeneity of the responses. In general, between three and eight marker-release spikes were observed. The background level of pericellular fluorescence, which increases during marker release, decreases following the last spike. As illustrated in the kinetic studies of Fig. 2, marker release was often observed at 20-s intervals.

To ascertain the spatial characteristics of marker release, we acquired images of marker release using an ICCD camera. Images were captured as the fluorescent marker diffused into the pericellular illumination region. Fig. 3 shows several representative qualitative micrographs of marker release during neutrophil-mediated ADCC. Plumes of label released from the cells were observed. A variety of plume shapes were observed. This may be due, for example, to membrane ruptures near the edge of the occultation disk (excitation cone of light) vs membrane ruptures near the apical or basal surfaces, which would be out of the focal plane, dimmer, and more diffuse. Interestingly, no preferred orientation of the plume was found relative to the position of the effector cell; a similar observation was made previously for ADCC of E (18). Thus, the marker was released as pericellular plumes of fluorescence that occur randomly around the perimeter of a target.

We subsequently confirmed the relationship between the quantitative intensity measurements and the qualitative imaging results. Fig. 4 shows a time-correlated intensity and imaging experiment of a neutrophil-tumor cell conjugate. In this experiment, ~50% of the light was directed to a photomultiplier tube (thus accounting for the slight reduction in the signal/noise ratio in Fig. 4A), whereas the remainder was sent to an ICCD camera. Fig. 4A shows a quantitive kinetic measurement of fluorescence intensity vs time. The timepoints labeled 1, 2, and 3 correspond to the images shown in Fig. 4, B, C, and D, respectively. Thus, the bursts of fluorescence intensity illustrated in Fig. 4A correspond to separate plumes of marker release. For example, the burst of intensity labeled as 1 in Fig. 4A corresponds to the appearance of a bright plume labeled as 1 in Fig. 4B. Similar relationships hold for bursts 2 and 3. These data also illustrate the longitudinal features of individual marker-release events. For example, the bright burst labeled as 1 in Fig. 4B dissipates within several seconds as shown in Fig. 4C and cannot be observed in Fig. 4D. Because the rate of rupture at these early times is faster than diffusion of the marker from the illumination area, the background level of fluorescence increases during the observations. In some cases, this can begin to obscure the individual events (e.g., Fig. 2).

FIGURE 3. A gallery of photomicrographs illustrating marker release. Labeled target cells were occluded during ADCC as described above. A total of 18 examples of fluorescent marker-release experiments are shown to illustrate the heterogeneity of the responses observed. Marker release is associated with a plume of fluorescence intensity, presumably associated with a point of cytolytic membrane rupture. The site of marker release did not correlate with the position of the effector cell. Similar results were obtained on 8 different days; a total of 80 different cells were photographed. Magnification is ×500.

FIGURE 4. Time-correlated fluorometric and imaging experiments. A, Kinetic single-cell fluorescence tracing showing pulses of marker release from labeled tumor cells during neutrophil-mediated ADCC. The fluorescence intensity (photocurrent) is listed at the ordinate, whereas time (in seconds) is given at the abscissa. Multiple temporally distinct marker-release events are shown. Optical micrographs of fluorescence were acquired at three points in time (labeled 1, 2, and 3) in A. B–D correspond to the timepoints labeled 1, 2, and 3 in A. Note that the bursts of marker release illustrated quantitatively in A correspond to distinct marker-release plumes at different positions in B, C, and D, respectively. Similar results were obtained on 3 separate days. For B–D, magnification is ×585.
Discussion

The quantitative analysis of immunological cytolysis can be traced to the work of Brunner et al. (19), who demonstrated that Ag-specific T lymphocytes promote the cytolytic release of $^{51}$Cr from target cells. Early studies also showed that the cytolytic process can be divided into distinct phases. For example, the release of cytoplasmic $^{86}$Rb and small molecules (e.g., ATP and nicotinamide) precedes that of $^{51}$Cr, which is bound to proteins (20, 21). The label used in the present study, TMR, is a small molecule. Because our data represent the earliest detectable TMR release, it seems likely that the cytolytic plumes observed (Figs. 3 and 4) are early marker-release events. Moreover, this early cytolytic damage occurs at roughly the same time as the first committed cellular steps in cytolytic programming (20) and may contribute to subsequent membrane damage (e.g., colloid-osmotic effects). Cytolysis, the release of cytoplasmic contents, consequently begins with multiple small bursts of cytoplasmic content release around the perimeter of a target.

Although cytolysis represents a major form of immunological effector function, its details have remained obscure in nucleated cells due to an inability to detect individual cytolytic events. Previous microscopic studies have observed individual cytolytic events during Ab-dependent lysis of lipid vesicles and sheep E (18, 22). Lysis in both of these systems demonstrated a catastrophic breakdown of the target membrane and a complete loss of cytoplasmic contents. A previous report has indirectly studied cytolysis by following the cytoplasmic calcium concentration of a tumor cell target; this concentration presumably increases due to calcium influx and CTL contact (23). However, cytolytic events were not photographed, and release kinetics were not quantitated. In contrast, our approach is to occlude the fluorescence of bright tumor cells, thereby allowing the weak fluorescence leaking from the cell to be detected. The strategy demonstrated pericellular cytolytic plumes of fluorescent marker release. Our kinetic studies of tumor cell cytolysis indicate that relatively small marker-release events take place without catastrophic membrane rupture at the timepoints examined. This finding is consistent with the fact that conventional imaging techniques cannot detect cytolytic plumes around tumor cells. Thus, in contrast to other cytolytic systems, tumor cell cytolysis is not an all-or-none biological response.

Another key finding in the present study is that multiple marker-release events take place, as illustrated by the multiple fluorescence intensity peaks associated with each target cell (Fig. 2). Again, this observation contrasts sharply with the single marker-release events of liposomes and E (18, 22). Nonetheless, it is consistent with prior indirect studies of complement-mediated lysis of nucleated target cells that supported a multifhit hypothesis (24). Because cytolysis is defined as cytoplasmic marker release, we interpret the multiple marker-release events as corresponding to multiple cytolytic events. Each cytolytic event comprises membrane rupture and rescaling events, as suggested by their finite duration. Hence, each cytolytic event is composed of a membrane rupture event that allows the fluorescent label to leak from a cell and accumulate as a pericellular plume of fluorescence followed by a rescaling event and dissolution of the label into the environment.

Our quantitative kinetic experiments (Fig. 2) show that cytolytic burst peak intensities occur at $\sim 20$-s intervals. This time interval corresponds to that observed for oscillations in other neutrophil functions and properties such as actin assembly, shape and velocity changes, receptor oscillations, and reduced nicotinamide adenine dinucleotide phosphate oscillations (reviewed in Ref. 25). Moreover, the oscillatory release of reactive oxygen metabolites and pericellular proteolysis take place at 20-s intervals (16). Several functional properties of neutrophils appear to be driven by their oscillatory metabolism (15, 16). Furthermore, reactive oxygen metabolite release has been linked to tumor cell cytotoxicity (26–28). Thus, both oscillatory enzymatic and oxidative capacities of neutrophils could contribute to the timing of the oscillatory cytolytic bursts observed in the present study. In either case, it seems likely that oscillatory metabolite concentrations within the effector cell drive the oscillatory cytolytic damage of the target cell.

Studies of the time-dependent formation and dissipation of cytolytic marker-release plumes show their occurrence at multiple sites around the perimeter of tumor cells (Fig. 4, B–D). This suggests that there is no unique membrane site associated with cytolysis. Hence, cytolytic marker release is not necessarily found at sites of effector-to-target cell contact. We have reported similar findings for neutrophil-mediated E lysis (18). The contact zone between target and effector cells may, in fact, be stabilized by links and cross-links among cytoskeletal components, membrane Ags, IgG, and Fc receptor complexes. Furthermore, macrophages form exclusionary zones around sites of contact with IgG-opsonized targets (29). Thus, it may be difficult to damage a contact zone, and such zones may impede the diffusion of cytosolic labels into the extracellular environment.

In addition to providing the first direct evidence regarding the subcellular mechanism of tumor cell cytolysis and dramatic evidence in support of the multihit hypothesis, our studies also provide a new tool for dissecting the temporal elements of tumor cell destruction. It should become possible to image later events during cytolysis by taking advantage of green fluorescent proteins, microscope flow chambers (13), and ICCDs possessing greater dynamic ranges. Moreover, when emerging tools in biology such as imaging spectrophotometry and extracellular fluorescence labels that report their chemical environment (e.g., Ref. 16) are used, it should become possible to temporally correlate well-defined chemical reactions with membrane rupture. Thus, it may be possible to mechanistically associate the molecules participating in host defense with specific cytolytic plumes. It should also be possible to examine additional cell types that may display different cytolytic release mechanisms.

References


