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Digital fluorescence imaging of fusion of influenza virus with erythrocytes

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Fusion of influenza virus with human erythrocytes at pH 5.2 was followed by fluorescence microscopy using a cooled slow-scan CCD camera. The high sensitivity of the CCD permits repetitive digital imaging of the same cells with minimal photobleaching. The experimental conditions were such that only a small number of virus particles were adsorbed per cell. Quantitative analysis of the data indicated that for most cells only a single fusion event took place. This was, however, sufficient to cause haemolysis within 30 min at 20–22°C for about 60% of cells. There was a highly variable time lag between fusion and haemolysis. The lateral diffusion coefficient of virus particles on the cell surface when bound at pH 7.4 was $< 2 \times 10^{-13}$ $cm^2 \cdot s^{-1}$. The technique should be of value for more detailed studies of the dynamics of viral and other membrane fusion events.

Influenza virus; Fusion; Digital fluorescence microscopy; Charge coupled device; Lateral diffusion

1. INTRODUCTION

The fusion of influenza virus and related enveloped viruses with cells has been the subject of intensive investigation [1–7]. Various fluorescence assays have been described which monitor the intermixing of components of the viral membrane with the membrane of the target cells [8–11]. A particularly simple and effective method introduced by Hoekstra et al. [8] consists of incorporating the fluorescent probe octadecylrhodamine B (R_{18}) in the viral membrane at such high concentrations that its fluorescence is quenched. When fusion occurs, dilution of the probe in the target membrane results in relief of quenching and thus an increase in fluorescence intensity which can be conveniently measured in a fluorimeter.

We are currently interested in the application of charge coupled devices (CCDs) to fluorescence microscopy. Cooled slow-scan CCDs are solid-state imaging devices with exceptional sensitivity, low noise, and wide dynamic range [12,13]. They are thus particularly well suited to low-light-level imaging. Moreover, the digital image is strictly proportional to the original photon flux, thus facilitating accurate quantitative image analysis. Here we investigate the potential of the CCD for following fusion of influenza virus at the single cell level in the fluorescence microscope. The approach is complementary to that employing Nomarski differential interference contrast microscopy with digital image processing which has very recently been employed by Bächi in morphological studies of viral budding and fusion [14].

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2. EXPERIMENTAL

Influenza virus, strain X47, grown in embryonated chicken eggs was generously provided by Professor C. Pasternak (St. Georges Hospital Medical School) and stored frozen in liquid nitrogen until used. The virus was labeled with R_{18} (Molecular Probes Inc.) essentially as described by Hoekstra et al. [8].

Erythrocytes were prepared from fresh human blood and diluted 1 to 10 in 0.1–0.2 M Na phosphate-citrate buffer at either pH 5.2 or 7.4. Subsequently, 10 μl of this suspension were placed on a glass microscope slide and left for 60 s after which time the slide was gently rinsed with the same buffer. Suf-

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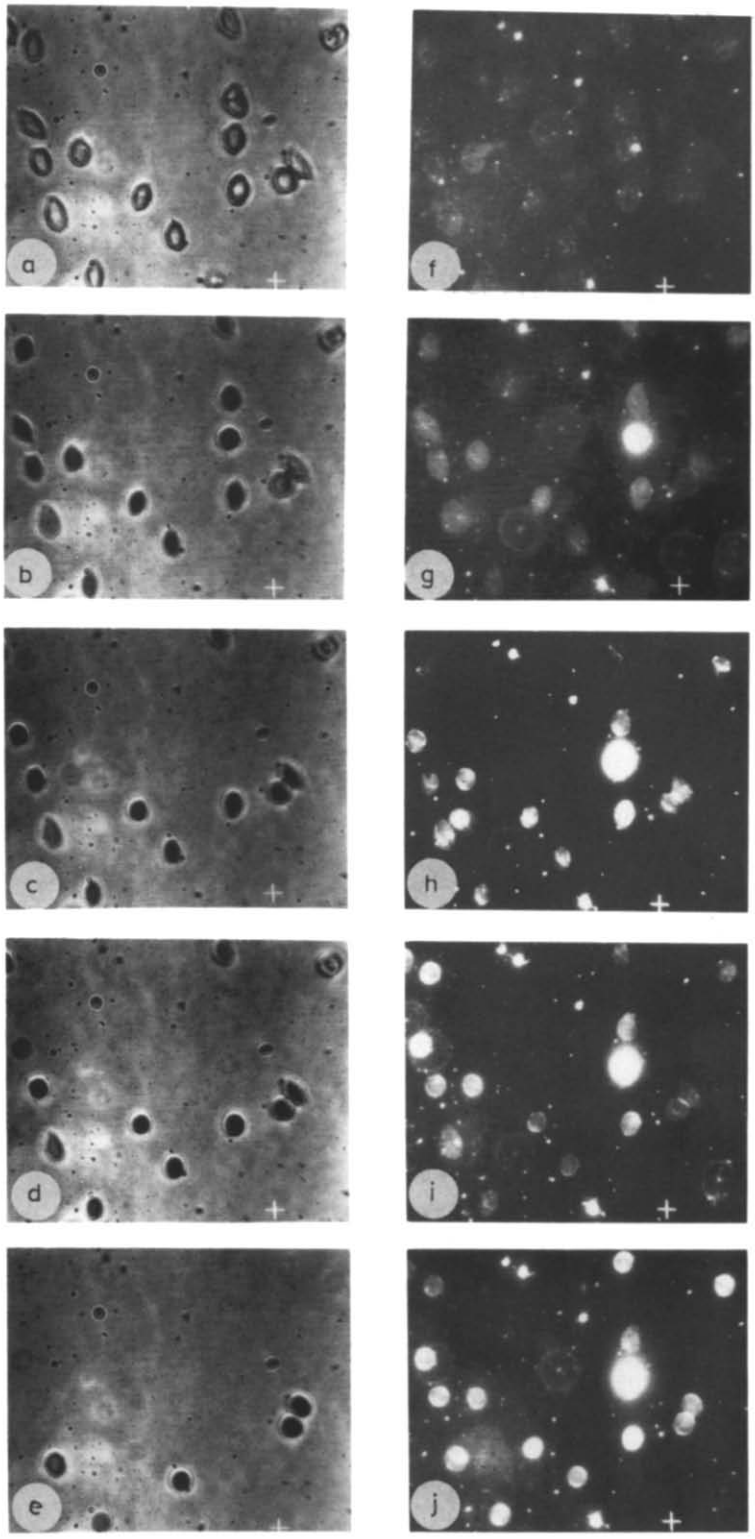
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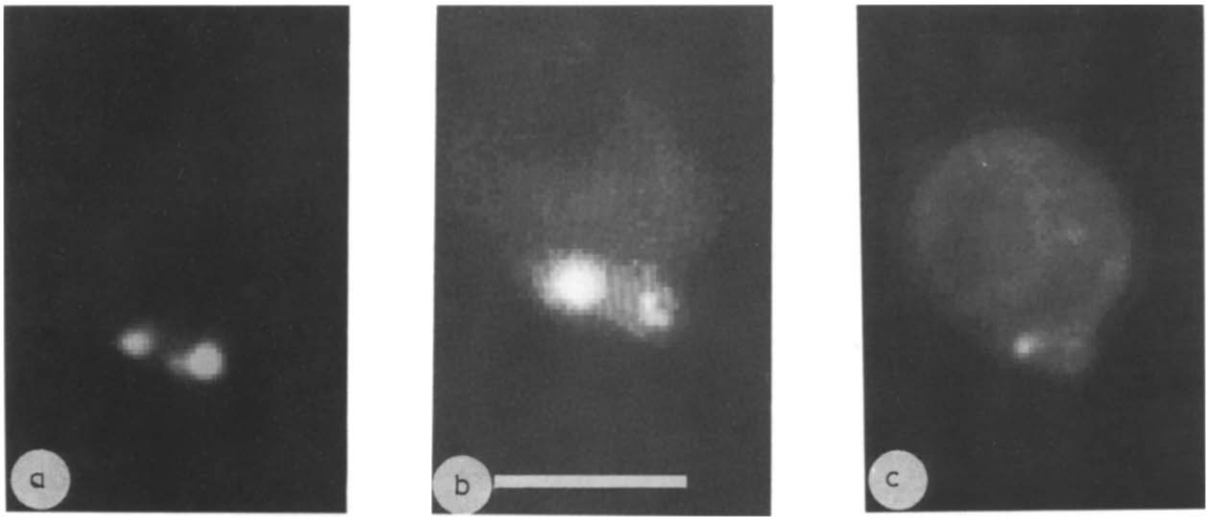

Fig.2. Enlarged display of viral fusion with a single erythrocyte. Times after mixing are (a) 6 min, (b) 12 min, (c) 31 min. Bar = 5.8 μm.

ficient cells remained attached to the slide by this procedure; no advantage was found in precoating the slide with polylysine. The cells were overlaid with 10 μl of the R_{18}-labelled virus (~3 × 10^4 HAU/ml) suspended in the same buffer after which a coverslip with a silicone grease seal was placed over the sample and the slide transferred to the microscope. All measurements were made at room temperature (20–22°C).

Fluorescence microscopy was performed with a Nikon Diaphot epifluorescence inverted microscope equipped with appropriate filters. Illumination was at 514 nm by a 2 W coherent argon ion laser run at low power and incident at a glancing angle on the top of the microscope slide at an intensity of ~10^{-6} W/m^2. A Wright Instruments model 1 CCD camera was mounted on the video port of the microscope. The camera consisted of an EEV P8603 CCD (385 × 576 pixels) mounted in a liquid nitrogen cryostat and operated at ~150 K. An image display board and software for camera control, image display and image analysis was also supplied by Wright Instruments and used in conjunction with a CAS PC-AT1 microcomputer.

3. RESULTS

A sequence of fluorescence images of a group of cells incubated with virus at pH 5.2 is shown in fig.1. Fusion events are readily detectable by the spread of fluorescence from virus to cell. Note that virus adsorbs to both cells and glass slide and that weak autofluorescence/residual light scattering is just detectable from cells prior to fusion. Corresponding bright-field images are also shown in order to follow haemolysis of the cells. In fig.2 an enlarged display of a single cell is presented. The results of control experiments performed at pH 7.4 are shown in fig.3.

Quantitative analysis of the experiment shown in fig.1 is illustrated in fig.4. For this analysis an area of 10 × 10 pixels (2.5 × 2.5 μm) free of virus was selected on each cell. The fluorescence intensities of these areas were normalised for variation of excitation intensity and for photobleaching by averaging the intensities of 50 virus particles which had attached to the glass surface and remained in position for the duration of the experiment. Local background was determined by choosing the nearest possible off-cell area and subtracted from the measured fluorescence intensities. Fig.4a and b illustrates the time course of the fluorescence change for two cells. Fusion of virus occurred in both cells but only one underwent haemolysis during the period of observation. Fig.4c plots the time of haemolysis after virus addition against the final fluorescence for 19 cells which were analysed.

Fig.1. Bright-field (a–e) and fluorescence (f–j) images of viral fusion with erythrocytes at pH 5.2. Times after mixing are (a) 3 min, (b) 8 min, (c) 11 min, (d) 14 min, (e) 30.5 min, (f) 2.5 min, (g) 7.5 min, (h) 10.5 min, (i) 13.5 min, (j) 30 min. Diameter of cursor is 5.8 μm.

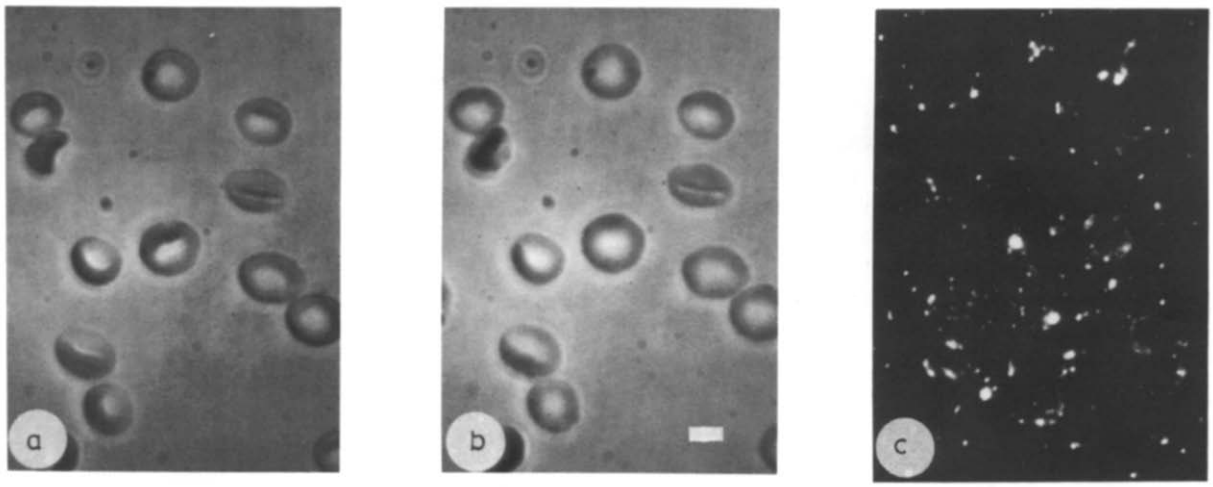

Fig.3. Control experiment showing no viral fusion or haemolysis at pH 7.4. Times after mixing are (a) 12 min, (b) 87 min for bright-field images and (c) 87.5 min for fluorescence. Bar = 5.8 μm.

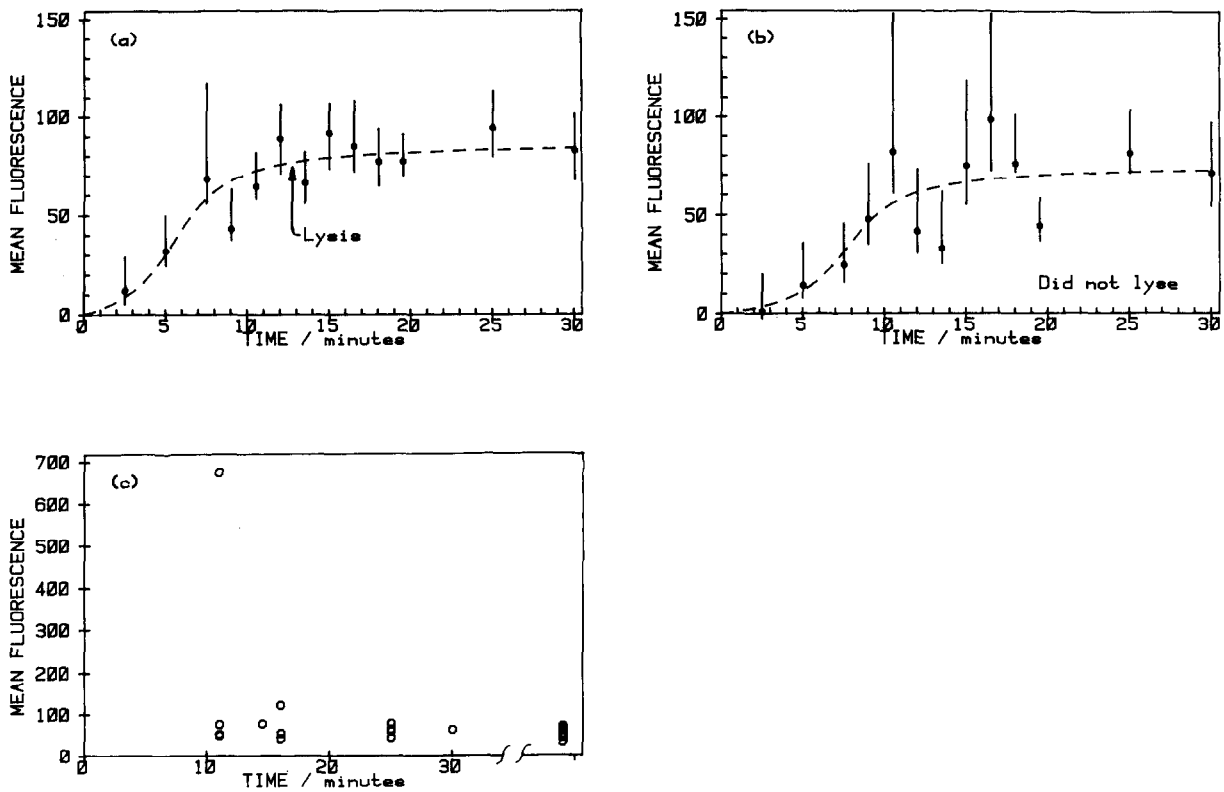

Fig.4. Quantitative analysis of data for individual cells. (a) Cells exhibiting viral fusion and haemolysis; the dashed line is a sigmoid fit. (b) Cell exhibiting viral fusion but no haemolysis within 30 min. (c) Time at which haemolysis occurred plotted against final fluorescence intensity, obtained from the sigmoid fit, for 19 cells. Mean fluorescence is the intensity of a 2.5 × 2.5 μm area on the cell and the error bar is the range of individual pixel intensities. In c the group of 7 cells at the far right did not undergo haemolysis within the 30 min experiment.

4. DISCUSSION

The results shown in figs 1–3 clearly demonstrate the ability of the CCD camera to image viral binding and fusion with cells. For quantitative studies involving sequential imaging, it is essential that extensive photobleaching does not occur during individual exposures. We estimate that photobleaching was less than 3% for each exposure under the imaging conditions of fig.1 and that at least 15 images can be obtained from the same area of the sample. This is a consequence of the exceptional sensitivity of the device which permits images to be obtained with relatively low excitation intensities. Micrographs of fluorescent vital particles have been presented previously using an image intensifier with either photographic or video recording [15,16], although the degree of photobleaching was not stated. CCDs are inherently superior for quantitative analyses because of their linear response over a dynamic range of greater than 10^4. Moreover, the ability to improve signal to noise by time-integrating on the device itself is simpler than the frame-averaging procedures required for video cameras.

The main purpose of this communication is to demonstrate the potential of the imaging method rather than to provide a detailed analysis of the results, which will require more extensive data than we have so far obtained. Nevertheless, some preliminary observations can be made. It is clear from figs 1 and 4, for example, that there may be a considerable time lag between fusion and haemolysis. Individual cells in which fusion is indicated by the spread of fluorescence rapidly round up but haemolysis frequently does not occur until several minutes later. In some cells, no haemolysis occurred during the period of observation (e.g. fig.4b). The results are consistent with a permeability change which subsequently leads to lysis by the colloid osmotic mechanism [3,4,7]. The time lag implies that haemolysis is an unsatisfactory way of monitoring the kinetics of the fusion event.

The amount of virus added to the cells in these experiments was adjusted so that only a small number of virus particles were adsorbed per cell. The quantitative analysis illustrated in fig.4 suggests that in most cases only 1 virus particle fused per cell. This may be deduced from the observation that normally only a single step increase in fluorescence intensity was seen (cf. fig.4a and b) and that the final fluorescence intensities of the cells (fig.4c) were grouped in a rather narrow range. (One cell in fig.1 is exceptional in showing a very large increase, possibly due to fusion of a viral aggregate.) It thus appears that fusion of only one virus particle is sufficient to cause haemolysis of an individual erythrocyte. As shown in fig.4c, however, there is considerable variation in the time taken for haemolysis to occur which is uncorrelated with the time taken for fusion (less than 10 min for essentially all cells). It is thought that leakiness of the viral envelope is responsible for haemolysis [3,11,17]. The data suggest that this is a very variable quantity.

The enlarged images of fig.2 enable the fate of individual virus particles to be more readily followed. The first picture taken just before the onset of fusion shows that the cell has two attached virus particles which are clearly visible. The second image shows one of the virus particles in the process of fusion. In the final image, fusion of this particle is complete and the cell is distorted although haemolysis has not yet occurred. Fusion of the second virus particle has not occurred. Images such as these should permit quantitation of the rate at which molecules introduced at a specific location on the cell membrane become randomised over the cell surface.

Images obtained at pH 7 (fig.3) confirm that no fusion occurs at this pH over a time of at least 90 min. The measurements also confirm that there is no exchange of probe into the erythrocyte membrane in the absence of fusion. The results of this experiment can be used to ascertain whether the virus remains fixed in the position at which it binds to the cell or whether it can migrate over the cell surface. The latter is possible because the neuraminidase present in the viral membrane could cleave the sites of attachment. We noted the coordinates of 37 virus particles attached to cells and found that their mean displacement over the time course of the experiment was less than 0.6 μm, which is of the order of the resolving power of the microscope. This implies that the lateral diffusion coefficient $D_L < 2 \times 10^{-13}$ cm$^2 \cdot$s^{-1}. The present result is consistent with studies by Henis using fluorescence recovery after photobleaching (FRAP) in which an upper limit of D_L of 5 $\times$

10^{-12} $cm^2 \cdot s^{-1}$ was obtained for Sendai virus adsorbed to erythrocytes [15].

In conclusion, the approach described here can readily be extended to nucleated cells and to other viruses or vesicles. As previously emphasised by Aroeti and Henis [20] measurements of viral interaction with single cells are an important alternative to methods which evaluate the average response of a cell population. In a broader context, direct observation of the redistribution of newly inserted components into the plasma membrane of cells is of considerable interest in relation to membrane biosynthesis.

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