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Visualisation of Nitric Oxide Generated by Activated Murine Macrophages

A. M. Leone,[1] V. W. Furst, N. A. Foxwell,[1] S. Cellek,[1] and S. Moncada[1,2]

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, United Kingdom

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We have visualised the release and approximate diffusion profile of nitric oxide (NO) from activated murine macrophages using a high transmission microscope coupled to a high sensitivity photon counting camera. The images generated by NO were cell-associated and spread over an area of approximately 175 μm from the activated macrophage. The signals obtained were dependent on the presence of exogenous L-arginine in the medium and followed a time course similar to that previously described for the generation of NO by the inducible form of NO synthase. The light signal was attenuated by the inhibitor of NO synthase, N$^{\omega}$-nitro-L-arginine methyl ester. Studies using superoxide-deficient macrophages further confirmed that the signals detected were generated by NO rather than reactive oxygen intermediates.

The L-arginine:nitric oxide (NO) pathway is now known to play a role in many physiological and pathophysiological processes (1,2). The instability (3) and high diffusibility of NO have caused difficulties in the measurement of this novel mediator in biological systems. New techniques for the detection of NO have been developed (4–9), but none of these permits its direct measurement in tissues and, in the absence of this, researchers have attempted to model the diffusion of NO (10,11). In order to gain a better understanding of the properties and mode of action of this atypical mediator we have now developed a method for its visualisation. Using a high transmission microscope coupled to a high sensitivity photon counting camera, we have visualised the release and diffusion profile of NO from activated murine macrophages.

MATERIALS AND METHODS

Preparation of cells. A murine monocytic cell line (J774.16; ATCC TIB 67) was grown to approximately 20% confluence on thin glass coverslips (BDH) placed in 12-well plates (Tissue culture plastic, Falcon/Costar) and maintained in standard RPMI 1640 medium containing 10% foetal calf serum, 10 μg/ml penicillin, 10 I.U./ml streptomycin and 2 mM glutamine, at 37°C in a humid atmosphere containing 5% CO_2. Cells were activated using murine recombinant interferon-gamma (IFN-γ, Genzyme) at 50 units/ml and lipopolysaccharide (LPS, Difco) at 10 μg/ml. At various time points after activation, the cover slips were removed from the cell culture wells, washed gently three times with warm Krebs' solution and placed under the microscope. J774.C3C cells were obtained as a gift from Dr. B. Bloom, Albert Einstein College of Medicine, New York and grown described as above. Both J774.16 and J774.C3C cell lines produce equivalent quantities of NO after activation (30–40 μM per 10^6 cells in 2.5 ml medium per 24 h, n = 6); however, only the former line is able to synthesise and release superoxide, from which other reactive oxygen intermediates (ROIs) are mainly derived (12).

Visualisation protocol. The imaging medium (IM) consisted of 5 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, sodium salt; Sigma) in Krebs' solution containing 15 μM hydrogen peroxide as activator. The IM was made up at least two hours before use to allow the initial burst of chemiluminescence to decay to a stable but significant background level. L-arginine or the NO synthase inhibitor, N$^{\omega}$-nitro-L-arginine methyl ester, (L-NAME, Sigma), was added into the IM immediately before use. After the cells were placed, positioned and focused under the microscope, 100 μl of IM was applied. Photon counting was started immediately, using an Argus 50 photon counting system (Hamamatsu Photonics UK Ltd.) coupled to a Zeiss Axiavert 135 TV inverted microscope (Carl Zeiss, Oberkerken, Ltd.). The camera was mounted directly under the microscope objective, allowing maximum light transmission. The unit was housed in a light-tight enclosure during the imaging. Bright field images were overlaid with pseudo-colourised photon counted images in slice mode, allowing signal intensities to be correlated with colour. Photon counts were also made in gravity mode for quantification and integrated over 45 seconds.

[1] Present address: The Cruciform Project, 140 Tottenham Court Road, London W1P 9LN, UK.
[2] For correspondence, Fax: 0171 209 0470.

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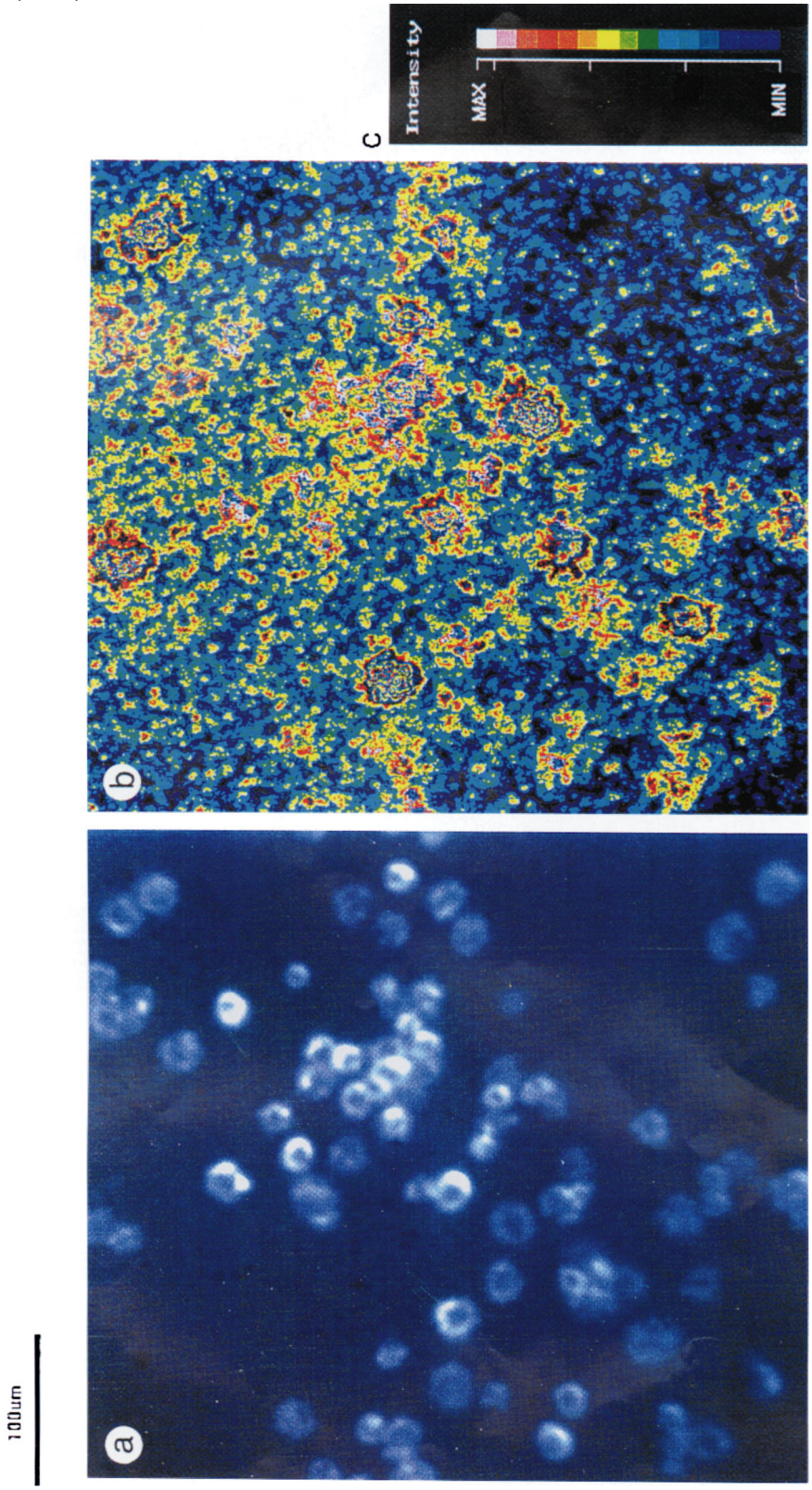

FIG. 1. A typical bright field image from activated J774.16 cells. b: The bright field image has been overlaid with its corresponding photon counted image. c: The relationship between colourisation and signal intensities observed. The image is representative of over one hundred individual visualisations.

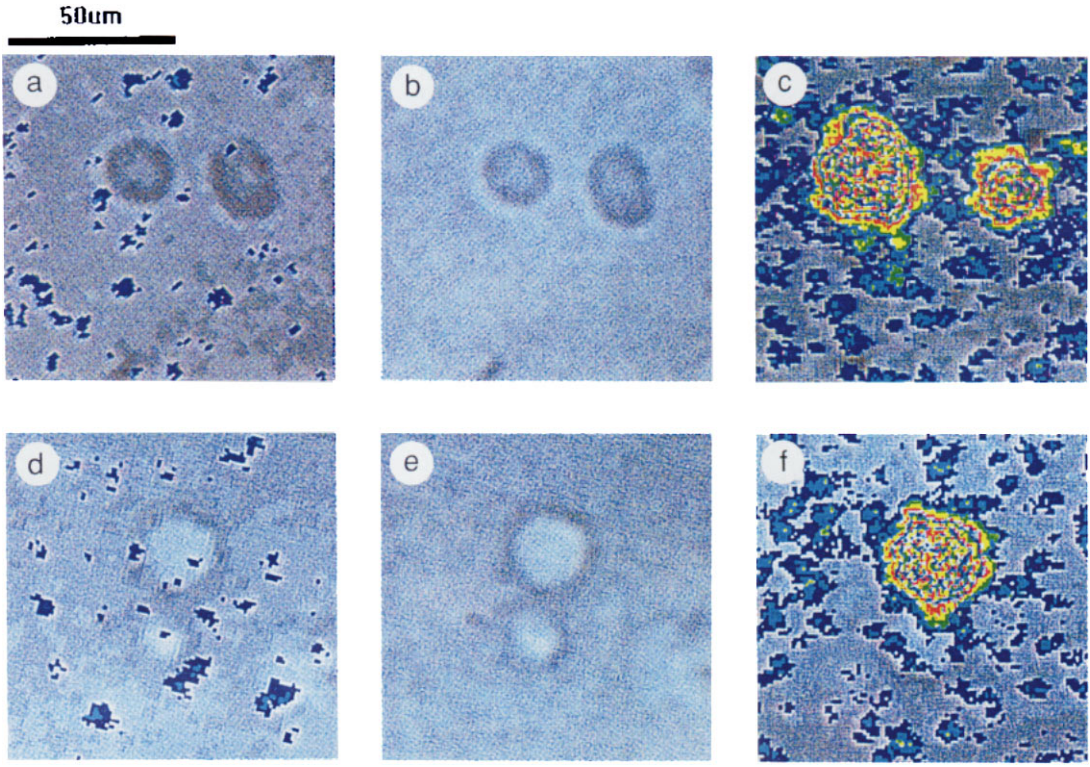

FIG. 3. Chemiluminescence from superoxide-producing J774.16 (a,b and c) and superoxide-deficient J774.C3C (d,e and f) macrophages. a and d: Photon counted images obtained when L-arginine was absent from the IM. b and e: The bright field images obtained from superoxide-competent and deficient cells, respectively. c and f: Photon counted images obtained when L-arginine (400 μM) was present in the IM. The image is a representative of four sets of experiments.

RESULTS

When authentic NO [10 nM to 5 μM final concentrations; prepared as described previously (13)] was added to IM contained in a dark box environment, light was generated in a concentration-dependent fashion ($R = 0.99$).

Almost all of the activated J774.16 murine macrophages in the field gave rise to light signals, although the intensity varied among the cells (Fig 1). A pseudo-three-dimensional image derived from a single macrophage was constructed using the Argus 50 software (Fig 2). Diffusion of NO was measurable up to 175 μm radially from the majority of activated macrophages.

Reduced light emissions were observed from the cells in L-arginine-free IM (Fig 3a and d); peak light intensities in arginine-free IM averaged 360 ± 110 photon counts, whereas they averaged 1200 ± 110 photon counts in normal IM ($p<0.05$). When L-NAME (1.5 mM) was added to L-arginine-containing IM, light intensities were reduced (230 ± 80 photon counts) to near background (100–200 photon counts) light levels (Fig 4a).

Light intensities observed reached a peak 16 hours after the cells were activated, falling by approximately 66% of maximum at 48 hours (Fig 4b). A few cells gave rise to very weak light signals at 2 hours following activation, but these were not significantly greater than those from control, unactivated macrophages.

To demonstrate that the light generated in our studies was derived from NO rather than ROIs, we studied both J774.16 and J774.C3C cells. No significant difference was observed in peak light intensities between the two cell lines (Fig 3).

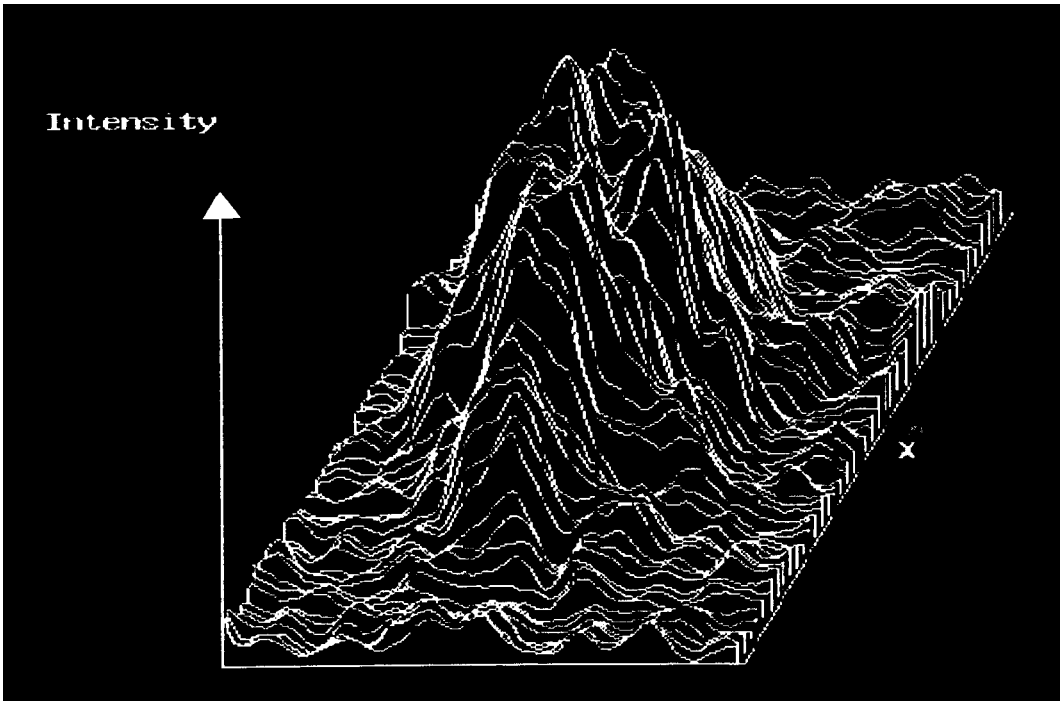

FIG. 2. A constructed "3-D" image of the chemiluminescent signal derived from a single activated murine macrophage.

DISCUSSION

When authentic NO was added to IM in our experiments, light was generated in a concentration-dependent fashion. Since NO does not react with luminol in the absence of hydrogen peroxide (14), it has been argued that NO reacts with hydrogen peroxide to form peroxynitrite (9), which in turn generates light when it reacts with luminol (14). In addition, NO has been reported to react with hydrogen peroxide to form singlet oxygen (15), which is also capable of generating light when it reacts with luminol. Thus the light release observed when NO reacts with the IM may involve either mechanism or, more likely, both. The generation of luminescence in the presence of NO is therefore consequent to an interaction as yet undefined. Although there have been studies quantifying the luminol based NO-induced light signal in a number of cell types (9,12,14), none of these has actually visualised the image.

The possibility that the luminescence observed in our experiments might be resulting from reaction of ROIs with IM is unlikely, since the J774.C3C cell line, which does not produce significant quantities of ROIs (12), gave similar images to those seen with the J774.16 cell line.

The light signal produced by activated macrophages was attenuated in the absence of L-arginine, which is consistent with previous studies which have shown that NO synthesis is markedly reduced in macrophages when exogenous L-arginine is absent (16). The reduction in the chemiluminescent signal by L-NAME further indicates that the observed light was derived from NO synthase.

In our experiments, maximum light intensities were observed 16 hours post-activation, which is coincident with the known time profile for the appearance of NO synthase after activation (17). This observation also indicates that the luminescence generated by these activated macrophages is due to the release of NO rather than ROIs, as has previously been suggested (18), since stimulation of these cells with LPS and IFN-γ has been shown to induce NO synthase over this time-course but not to result in the generation of superoxide under these conditions (17).

The method we have developed is sufficiently sensitive to visualise the release of NO from

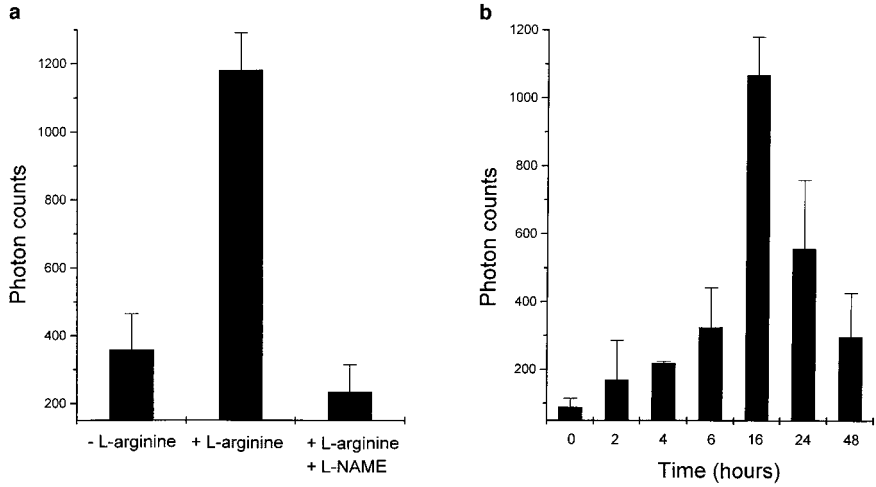

FIG. 4. Photon counts obtained from activated J774.16 cells in the presence and absence of L-arginine (400μM) and in the presence of L-arginine (400μM) + L-NAME (1.5 mM) (n = 5). b: Time-course of maximum light intensities obtained from activated J774.16 cells. (n = 3–6).

individual activated macrophages. Our results show that NO diffuses for some distance from the generating cell. Therefore NO may not only be an intracellular killing mechanism but could contribute to the extracellular killing of some pathogens such as *Schistosoma mansoni* larvae (19). Indeed, it is likely that groups of macrophages strategically placed in tissues might constitute a protective NO-dependent barrier against extracellular micro-organisms or the growth of cancer cells. This may also be true for NO as a physiological mediator, since theoretical predictions suggest that NO, by diffusing distances similar to those observed in our present study, might affect as many as two million neurones in a 3-dimensional space from a point of release (11). If this is the case, then NO might be considered not as a mediator in the classical sense but as the generator of an environment in which biological events occur.

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