Cellular pathogens often impose intriguing variations on the script of normal postphagocytic events and control the fusion competence of the vacuoles they occupy. Parasite-containing vacuoles can display phenotypes that restrict or permit fusion, but the pathogen signals that dictate vacuolar phenotypes in most cases have not been identified.

Cells dually infected with different prokaryotic and/or eukaryotic pathogens have rarely been reported in the literature (Box 1). We found that, by a suitable choice of organisms, it is possible to construct 'doubly infected vacuoles' that, at least temporarily, shelter two different organisms. The first doubly infected vacuoles contained the rickettsia Coxiella burnetii and the protozoan flagellate Leishmania amazonensis, organisms that normally live within fusion-permissive vacuoles (Fig. 1). We also observed doubly infected vacuoles in which C. burnetii was associated with Trypanosoma cruzi or with Mycobacterium avium.

**Fusion-prone partner vacuoles**

Coxiella burnetii and L. amazonensis both inhabit fusion-permissive, prelyrosomal or phagolysosome-like vacuoles. It is not known if fusion of these vacuoles with lysosomes is required for survival or is upregulated by molecules released by the pathogens.

Coxiella burnetii is a highly infective, spore-forming bacterium transmitted by aerosol from animals to man. This organism, not yet grown in a pure culture, was responsible for outbreaks of Q fever later traced to infected sheep brought into the laboratory. We have used an attenuated phase II strain of C. burnetii, derived by sequential transfer of infective bacteria in avian eggs. Phase II organisms infect cells in culture but are not virulent for laboratory animals, nor do they revert to phase I. Nevertheless, safety regulations require level 3 containment for both phases of C. burnetii, a stricture that helped to keep the organism as the 'Sleeping Beauty' of intracellular infection. Indeed, among the 652 C. burnetii entries in Medline in the last 30 years, only 24 deal, and often peripherally, with the biology of C.-burnetii-infected cells.

Leishmania amazonensis and C. burnetii have common features that make them useful partners in dually infected cells. (1) Different cell types can be efficiently infected in vivo and develop large vacuoles containing numerous organisms. (2) Infected, non-replicating cells survive for several days or more, and cell lines can be persistently infected. (3) The organisms thrive within acidified hydrolase-rich vacuoles that avidly fuse with secondary lysosomes and with certain incoming...
phagocytic vacuoles. Vacuoles containing *L. amazonensis* or *C. burnetii* appear to function as lysosomal traps, in both cases, host cells are depleted of secondary lysosomes. Vacuoles containing *L. amazonensis*, because they stain for the GTP-binding proteins Rab7 and Rab9, are thought to be prelysosomes. Recently, however, Rab7 was found in a subpopulation of lysosomes in normal cells. It is likely that *L. amazonensis*-containing vacuoles enlarge by fusion with endocytic vesicles without balanced membrane retrieval. Fusion between *L. amazonensis*-containing vacuoles has been recorded, albeit uncommonly, in time-lapse movie sequences of infected macrophage cultures (M. Pouchelet, pers. commun.).

Far less is known about *C. burnetii*-containing vacuoles. The membranes of these vacuoles stain prominently for CD63, a lysosomal marker, but not for transferrin receptors, found in early endosomes (S. Paul, pers. commun.). In contrast to *L. amazonensis*-containing vacuoles, the large vacuoles containing *C. burnetii* appear to arise by the fusion of smaller vacuoles.

**Games vacuoles play with inert particles**

Our cohabitation experiments came as a sequel to studies of the kinetics and selectivity of transfer of inert particles to vacuoles occupied by *L. amazonensis* or *C. burnetii*. Fusion was inferred by observing the colocalization of particles and parasites. We found that *L. amazonensis*-containing vacuoles in macrophages fuse with phagosomes containing yeast-derived particles, but, rather surprisingly, not with immunoglobulin-G-coated erythocytes or latex beads. Fusion is probably dictated by the nature of the receptors that recognize the particles, the signals being encoded in the receptor's cytosolic domains; the persistence of the signals may depend on the rate of degradation of ligands and receptors in the vacuolar environment. Vacuoles containing *L. amazonensis* in Chinese hamster ovary (CHO) cells also only fuse with some types of incoming vacuoles. In contrast, *C. burnetii*-containing vacuoles in CHO cells are promiscuous and fuse efficiently with the phagosomes tested.

**From inert particles to living parasites**

As *C. burnetii*-containing vacuoles appeared to be particularly prone to fusion, we chose them as 'recipient vacuoles'. CHO or other cells were

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**Box 1. Dual infection, co-infection and super-infection**

*Summary of role of dual infection.* Applied to cells, "dual infection" and "co-infection" denote infection with two different pathogens, genus, strains or stages of the same organism with no implication on timing. Super-infection implies two successive rounds of infection with the same or a different organism. Cells in culture have been often co-infected with a virus (HIV, or cytomegalovirus) and a non-viral pathogen (e.g., *Mycobacterium avium*, *Leishmania species*, *Toxoplasma gondii*; see, for example, Ref. 2). However, we only encountered two reports of cells doubly infected with bacterial and/or protozoan pathogens, which included *T. gondii* and *T. cruzi*, and *T. gondii* and *M. avium*, respectively. Doubly infected vacuoles were not, or only rarely, found in the doubly infected cells.

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**Fig. 1. Ultrastructure of Chinese hamster ovary cells (a) infected for 5 days with *Coxiella burnetii* alone and (b) infected for 3 days with *C. burnetii* superinfected with *Leishmania amazonensis*, and fixed 2 days later. The stars mark the lumen of the *Coxiella*-containing vacuoles. Individual *Coxiella* bacteria are marked by arrows and an arrow indicates an oomastigote of *Leishmania*.**

Electron micrographs are courtesy of C. Daguett (Pasteur Institute, Paris, France). Scale bar = 2 μm in (a) and 1 μm in (b). Reproduced from Ref. 14 with permission from the American Society for Microbiology.
infected with the bacteria, and 2 or 3 d later, when more than 90% sported large *C. burnetii*-rich vacuoles, cells were superinfected with *L. amazonensis* amastigotes, and fixed at different times for microscopic observation. Large vacuoles that contained both *C. burnetii* and *L. amazonensis* were observed 6 h after superinfection, and the numbers of amastigotes increased with time. Isolated *L. amazonensis* amastigotes transform into fully flagellated motile promastigotes when shifted to lower temperatures. When doubly infected cells were placed at 25°C for 24–48 h, amastigotes in the mixed vacuoles transformed into promastigotes, which, by whipping their flagella, swirled *C. burnetii* within the shared vacuole. Furthermore, essentially all the amastigotes were killed by L-leucyl-O-methyl ester, to which amastigotes, but not host cells or promastigotes, are vulnerable. The evidence indicates that *L. amazonensis* can remain alive for several days within the mixed vacuoles.

It is clearly important to distinguish morphological colocalization of two organisms—the outcome of vacuolar fusion—from their cohabitation, which requires proof of at least temporary survival and, even better, multiplication of the two partners. Whereas the former has been achieved, the latter is still incomplete, as precise counts of viable *C. burnetii* are not easily obtained.

**C. burnetii or *L. amazonensis* vacuoles as hosts for other organisms**

Infected forms of *T. cruzi* normally enter cells via newly assembled lysonosomal compartiments from which parasites exit to the cytosol and multiply as amastigotes. When *C. burnetii*-infected cells were superinfected with *T. cruzi* tissue-culture trypomastigotes, flagellates reminiscent of the Loch Ness monster circled incessantly within the adoptive *C. burnetii*-infected vacuoles, as documented in video sequences. The vacuole also contained numerous amastigote-like forms. The implication is that, in the superinfected cells, the flagellates do not enter the cytosol, but transform into amastigote-like forms within vacuoles occupied by *C. burnetii* [Rabinovich, M. et al. (1995) Memorias Instituto Oswaldo Cruz 90 (Suppl. 1), 5–8]. This hypothesis is supported by the finding that low medium pH can trigger the transformation of isolated *T. cruzi* trypanastigotes into amastigote-like forms.

Paradigms of restricted vacuole fusion are provided by *Myobacterium tuberculosis* and *Mycobacterium avium*, which lodge in maturation-blocked unacidified vacuoles that may fuse with endosomes, but not with secondary lysosomes or phagolysosomes. The proposal that ammonia is the signal that specifies the fusion-restricted phenotype gained support from the finding that *M. tuberculosis* secretes glutamin synthetase into the vacuolar lumen.

Ultrastructural studies performed in association with Chantal de Chastellet also demonstrate that *M. avium* and *C. burnetii* can efficiently colocalize in vacuoles of doubly infected macrophages; furthermore, both organisms are structurally well preserved in the doubly infected vacuoles. In contrast, colocalization of *M. avium* and *L. amazonensis* was less frequently observed. Although *C. burnetii*-containing vacuoles have features of phagolysosomes they have not yet been adequately characterized. Endosome-like vacuoles containing activated ferritin eagerly fuse with *Mycobacterium microti*-containing vacuoles formed in macrophages in the presence of ammonium chloride. Consequently, it may be fruitful to compare the compositional and functional features of these two types of vacuoles.

**The 100 vacuoles question**

Parasite colocalization and eventual cohabitation requires movement, docking and fusion of vacuoles enclosing different parasites; fusing vacuoles may or may not be phenotypically similar. The biochemical and molecular mechanisms that underlie these events are unknown. A basic question that needs to be answered, case by case, is: does fusion of parasite vacuoles and its regulation by parasite-derived signals involve 'normal' fusion pathways as defined for vacuoles that contain nonviable organisms or particles?

The answers should come from the convergence of independent areas of research. (1) Work from different laboratories has defined highly conserved mechanisms for budding and fusion of small vesicles involved in intracellular protein transport, exocytosis and endocytosis in different eukaryotic cells. For example, the ATPase NSF (N-ethylmaleimide-sensitive factor), SNAPS (soluble NSF attachment proteins), vSNAREs and rSNAREs (SNAP receptors), Rab proteins (guanosine-triphosphate binding proteins), ARFs (adenosine diphosphate-ribosylation factors) and many other proteins involved in vesicle recognition, docking and fusion have been characterized, their genes cloned, and in some cases expressed and mutated. The mechanisms that involve these proteins provide a detailed backdrop against which fusion of normal and pathogen-containing vacuoles can be reset. (2) Compositional changes of membrane and contents of normal phagosomes have been elucidated in situ and in phagosome-enriched cell fractions. (3) Fusion of endosomes with phagosomes containing killed bacteria has been reconstituted in vitro and shown to display requirements common...
to those known for the endocytic and secretory pathways; a Ca²⁺- and annexin-dependent mechanism for phagosome–endosome fusion has also been revealed.

2) Vacuoles enclosing Mycobacteria or Leishmania species have been isolated from infected macrophages and their ultrastructural, antigenic and biochemical characterization initiated. Advances can also be expected from less invasive in situ methodology, involving reversible cell permeabilization, microinjection and genetically modified host cells that hyperexpress or do not express proteins involved in normal vesicle fusion. Collaboration between cell biologists and parasitologists is needed so as to apply the impressive advances in research on membrane trafficking to parasite-infected cells. There is a need for better microscopic methodology to measure fusion between phagocytic vacuoles. Fusion between endosomes and phagosomes has been studied by measuring the formation of complexes formed when vacuole populations containing, for instance, a biotinylated particle, are coincubated with another set of vacuoles containing an avidin-derivated molecular ligand. This strategy does not work when both vacuole populations contain particles, as contact of the particles within fused vacuoles does not necessarily take place.

Why doubly infected vacuoles?
Although vacuoles containing two different parasites may not exist in nature, artificially constructed doubly infected vacuoles may have some use.

1) Doubly infected vacuoles can test for compatibility of two different pathogens within a common intracellular microenvironment. Did exclusionary mechanisms evolve to reduce pathogen competition for growth factors or to prevent undesirable genetic exchanges? Can there be a need for better in vivo microscopy methodology to measure fusion between phagocytic vacuoles? Alternatively, can growth enhancement occur by some sort of complementation between pathogens?

2) Compositional and functional features of doubly infected vacuoles can provide information regarding dominance of parasite signals that specify different vacuolar phenotypes.

3) Colocalization in vacuoles can entrap pathogens in unusual locations and, therefore, can implicitly test for pathogen survival in potentially stressing micro-environments.

Acknowledgements
Most of the studies summarized here were performed at the Institut Pasteur, Paris, and URA 361 of the CNRS, France and supported by the Institut Pasteur and CNRS. Current work by M.R. is supported by NIH grant AI 26616 to Dr. G. Kaplan. We thank D.G. Russell and B.L. Clemens for constructive comments. M.R. dedicates this paper to the memory of Robert Fauré (1930–1993).

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