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The (α2→8)-Linked Polysialic Acid Capsule of Group B *Neisseria meningitidis* Modifies Multiple Steps during Interaction with Human Macrophages

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Group B *Neisseria meningitidis* causes systemic disease, including meningitis, after initial colonization and subsequent penetration of nasopharyngeal mucosa, a tissue which is richly populated by macrophages. In an initial effort to characterize the interaction of *N. meningitidis* and mature human macrophages, the influence of the (α2→8)-linked polysialic acid capsule on the interaction of *N. meningitidis* with human monocyte-derived macrophages was investigated with a capsulate case isolate and an isogenic Tn*916*-derived noncapsulate transformant. The capsulate strain was fourfold less adherent to the macrophage surface after cold incubation, although adherence of both strains was significantly increased after opsonization with nonimmune C5-depleted serum. When opsonized inocula were adjusted so that they adhered to macrophages in equal numbers, the two strains were internalized at equivalent rates and both entered membrane-bound compartments (phagosomes). Colocalization of bacteria with the late endosomal and lysosomal marker lysosome-associated membrane protein revealed that fusion of lysosomes with phagosomes containing the capsulate organism was significantly reduced 10 and 30 min after entry, but by 1 h, no difference between the strains was observed. Once internalized, meningococci were effectively killed, although more rapid killing of the capsulate strain was observed over the first 3 h. These results indicate that the (α2→8)-linked polysialic acid capsule modifies the interaction of meningococci with human macrophages at multiple steps, including adherence to the macrophage surface and phagosome-lysosome fusion. Moreover, the discordance between the kinetics of phagosome-lysosome fusion and bacterial killing suggests that a nonlysosomal mechanism may be responsible for a significant fraction of macrophage killing of *N. meningitidis*.

Group B *Neisseria meningitidis* colonizes the upper respiratory tracts of 1 to 5% of a given population (1) but in a small proportion of individuals may invade this mucosal surface, enter the bloodstream, and cause systemic disease, including meningitis. It is intuitive that in order to cause systemic disease, the organism must overcome the host defenses of the upper respiratory tract. These include the mucociliary apparatus, the epithelial layer, the subepithelial extracellular matrix, and the underlying phagocytic and lymphoid elements. Mononuclear phagocytes, including macrophages, constitute a large fraction of the cellular population of the upper respiratory tract (19). Meningococci penetrating to the subepithelial mucosa should be subject to phagocytosis and killing by macrophages; entry into the bloodstream therefore implies either failure or subversion of this facet of host defence. Supporting evidence for the general role of macrophages in surveillance and control of colonizing bacteria within the human nasopharynx and for their potential role as a reservoir of infection has been provided by the recent observation of nontypeable *Haemophilus influenzae* within mononuclear phagocytes of nasopharyngeal mucosa of colonized humans (5). Group B *N. meningitidis* cells have been observed within mononuclear phagocytes during experimental infection of human rhinopharyngeal mucosa (20).

Evidence that implicates the group B (α2→8)-linked polysialic acid capsule as a major virulence determinant includes the exclusive recovery of capsulate organisms from the blood and cerebrospinal fluid of patients, while both capsulate and noncapsulate strains are isolated from the nasopharynx of the same patients and from epidemiologically related carriers (2). Invasive group B disease is generally seen in individuals who lack protective antibody and are therefore dependent upon nonimmune mechanisms, including alternative complement pathway-mediated clearance. (α2→8)-Linked polysialic acid has been shown to restrict the deposition of C3b on the meningococcal surface during incubation of the organisms in nonimmune serum, suggesting that sialic acid can prevent alternative pathway-mediated stabilization of C3b (11). In the nonimmune host, this is likely to have two consequences: (i) it will reduce complement-mediated bactericidal activity, and (ii) it will reduce complement receptor mediated-recognition of meningococci by phagocytes, including macrophages.

This study tested the hypothesis that (α2→8)-linked polysialic acid capsule reduces adherence of group B *N. meningitidis* to human monocyte-derived macrophages in vitro. The influ-

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ence of the polysialic acid capsule on internalization and postinternalization events was also investigated. The process of phagocytic killing occurs in an ordered sequence of events, which includes adherence to the surface of macrophages, internalization, delivery of phagosomes to lysosomes, and killing; the influence of capsule at each of these steps was examined. An isogenic pair of meningococcal strains was studied, one of which expresses no capsule as a consequence of Tn*916* insertional mutagenesis of the first gene in the sialic acid biosynthesis pathway (27, 28). The results suggest that the ($\alpha 2 \rightarrow 8$)-linked polysialic acid capsule modifies meningococcus-macrophage interaction at more than one step, including adherence to the macrophage surface and delivery to lysosomal compartments.

MATERIALS AND METHODS

Bacteria. Strain NMB (Cap^+) is a serogroup B strain of *N. meningitidis* originally isolated from the cerebrospinal fluid of a patient with meningitis. The spontaneous phase variation of NMB to Cap^- is $\leq 10^{-4}$. Strain M7 (Cap^-) is a stable mutant of this strain, identified from a library of Tn*916*-containing transformants. Strain M7 expresses no capsular polysaccharide by colony immunoblots, agar diffusion assays, rocket electrophoresis, or enzyme-linked immunosorbent assay (27, 28) and does not express sialylated lipo-oligosaccharide on routine culture (27a). The M7 mutant has a single Tn*916* insertion in the chromosome (27). The insertion is in *synX*, the first gene of the capsule biosynthesis operon (28). The group B encapsulated phenotype can be restored to the M7 mutant by transformation with parental DNA containing the intact *synX* (26). Prior to each experiment, bacteria were removed from storage at −70°C and grown overnight on enriched chocolate agar (PML Microbiologicals, Tualatin, Oreg.). Two colonies were then transferred to 10 ml of fresh Mueller-Hinton broth (Becton Dickinson, Cockeysville, Md.) and incubated for 4 h at 37°C in 5% CO_2.

Opsonization of bacteria. To avoid complement-mediated lysis of bacteria, complement component C5-depleted human serum (Sigma Chemical Co., St. Louis, Mo.) was used to opsonize bacteria. Prior to use, immunoglobulin G (IgG) was removed from serum with immobilized protein A (Pierce, Rockford, Ill.). To prevent complement activation during this process, serum was pretreated with 2 mM EDTA, and serum was reconstituted with calcium and magnesium after collection from the column. After broth culture, bacteria were washed three times in 10 ml of phosphate-buffered saline (PBS) and resuspended in 1.5 ml of RPMI 1640-glutamine containing 10% PBS (control) or 10% IgG-free C5-depleted serum and were then incubated on a rotating stage at 37°C for 30 min. After this treatment, both strains contained bound C3 fragments, as indicated by immunofluorescence microscopy with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C3 antibody (Accurate Chemicals, Westbury, N.Y.). In contrast, neither strain bound detectable IgG by immunofluorescence microscopy with FITC-conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, West Grove, Pa.); bacteria opsonized with untreated C5-depleted serum reacted positively with both anti-C3 and anti-IgG antibodies, demonstrating that Ig was successfully removed from the serum by protein A.

Isolation and culture of human macrophages. Peripheral blood mononuclear cells were isolated from whole buffy coats received together with the corresponding plasma from the San Francisco Irving Memorial Blood Transfusion Center. Mononuclear cells were first isolated by separation in Ficoll and subsequently purified on Percoll density gradients to separate monocytes from lymphocytes (6). Autologous serum was recovered by inducing the clotting of plasma by addition of calcium chloride (10 mM). Macrophages were cultured at a cell density of 10^6 per well in 24-well culture plates (Corning, Corning, N.Y.) in 1 ml of RPMI 1640 supplemented with 2 mM L-glutamine and 10% autologous serum at 37°C in 5% CO_2 in humidified air. Macrophages destined for microscopy were inoculated into wells containing 12-mm-diameter glass coverslips (Fisher Scientific, Pittsburgh, Pa.) by the method described by Zimmerli et al. (33). After 24 h, the cells were washed to remove the nonadherent population, and then they were reincubated with 2 ml of serum-containing medium. Culture medium was exchanged at 3-day intervals until the cells were used after 12 days in culture. Two hours prior to the experiments, medium was replaced by serum-containing medium including 2% bovine serum albumin to limit bacterial adherence to plastic surfaces. The cells were then washed once with 1 ml of serum-free medium just prior to the experiments.

Adherence studies. To study the effect of the group B capsule on initial adherence of *N. meningitidis*, bacteria were incubated with macrophages at 4°C. At this temperature, binding occurs but phagocytosis and killing are inhibited.

After opsonization, bacteria were declumped by vortexing for 30 s in the presence of 3-mm-diameter glass beads (Fisher Scientific), viable bacteria were counted by a standard dilution technique, and 250 μl of inoculum from the final suspension was instilled into macrophage-containing wells (to give an inoculum size of approximately 10^7 CFU, a multiplicity of infection of approximately 10:1). Wells without cells were inoculated in parallel to monitor the adherence of bacteria to the plastic surfaces of wells. Meningococci and macrophages were

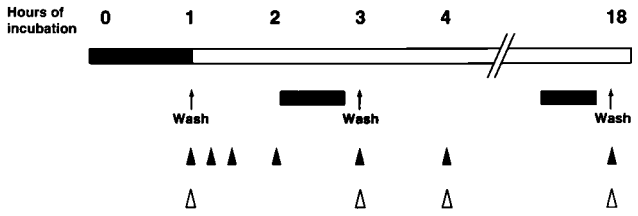

FIG. 1. Schematic diagram of experimental procedure for measurement of internalization and survival of *N. meningitidis* after inoculation into cultures of human monocyte-derived macrophages. Hours of incubation after inoculation are shown. Incubation was at 37°C except for the slashed area (4°C). ▲, fixation of macrophages for immunofluorescence and confocal microscopy; △, treatment of macrophages with 1% saponin for viable counting of cell-associated bacteria. Solid bar, treatment of wells with gentamicin (200 μg/ml) to kill extracellular bacteria.

then incubated for 1 h at 4°C, the infected supernatant was aspirated gently, and the wells were carefully washed three times with 500 μl of cold PBS with a micropipette. For microscopy, cells were then fixed with 4% paraformaldehyde in PBS at pH 7.4. For viable counting of associated viable organisms, 1 ml of 1% saponin was added to the wells, followed by gentle rotation for 10 min. The saponin was aspirated and expelled 10 times with a micropipette to thoroughly lyse the cells, and then the saponin was transferred to Eppendorf tubes and vortexed through glass beads to break up the cells and adherent bacteria. Viable counts were then taken from the saponin lysate. Control experiments with freshly grown bacteria demonstrated that this procedure did not alter the viability of either strain. To adjust for adherence to plastic surfaces of wells, viable counts recovered from wells containing no macrophages were subtracted from viable counts in test wells (adjusted viable count) within each treatment. From each saponin lysate, macrophage nuclei were counted in a Neubauer chamber to record the number of macrophages present in each well. To take account of minor variations in the size of the inocula and the number of macrophages present in wells, an adherence index (AI) was calculated for each well as follows: AI = (adjusted viable count/macrophage count)/inoculum.

Internalization and survival. The experimental method for study of internalization and survival of *N. meningitidis* in macrophages is summarized in Fig. 1. To study events subsequent to macrophage binding, inocula were adjusted so that the two strains became bound to macrophages in equal numbers. To quantitate initial binding, one set of wells were lysed with saponin and adherent bacteria, and macrophages were counted as described above. After 1 h of incubation, cells on glass coverslips were washed twice and fixed for immunofluorescence and laser confocal microscopy studies to examine intracellular events (described below). After this period, extracellular bacteria were killed by replacement of the supernatant with 2 ml of RPMI 1640 containing 200 μg of gentamicin per ml and incubation at 37°C for 30 min. (The MIC of gentamicin is 2 μg/ml for each of the two strains.) Cells were then washed twice and incubated for 30 min with warm RPMI 1640 and then lysed with saponin to count associated bacteria or fixed for microscopy. The remaining cells were bathed with RPMI 1640-glutamine containing 10% autologous serum and incubated for a further 1 h at 37°C in 5% CO_2 in humidified air. Cells were then washed and again lysed with saponin or fixed as described above. To study survival over a prolonged period, the remaining cells were left untouched overnight for a further 13.5 h, whereupon they were retreated with RPMI 1640-gentamicin, washed, and saponin lysed.

Immunofluorescence. For immunofluorescence, cells were fixed with 4% paraformaldehyde as noted above. Cells were then washed three times with 1 ml of PBS. To distinguish adherent, extracellular bacteria from those that were intracellular, cells were incubated with FITC-conjugated rabbit anti-human C3b antibody diluted 1:20 in PBS for 15 min. Cells were washed three times with PBS and refixed with 4% paraformaldehyde for 15 min and washed three times with PBS. Incorporation of meningococci into fused phagolysosomes was assessed by colocalization of bacteria with the late endosomal and lysosomal marker lysosome-associated membrane protein (LAMP-1) (12, 25, 33). The use of the LAMP-1 stain as a sensitive marker of phagosome-lysosome fusion has been validated (33). To visualize LAMP-1, 0.4 ml of H4A3 hybridoma supernatant (Developmental Studies Hybridoma Bank, Iowa City, Iowa) diluted 1:10 in RPMI 1640 with 0.1% saponin (to permeabilize cells) was added to wells. After 15 min, excess primary antibody was washed off three times with PBS, and the monolayer was incubated with 0.4 ml of CY3-conjugated purified goat anti-mouse IgG (37.5 μg/ml, final concentration) (Zymed, San Francisco, Calif.) in PBS containing 5% goat serum, 0.1% Triton X-100, 0.02% sodium dodecyl sulfate and 0.5 μg of 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, Eugene, Oreg.) (a nucleic acid stain which counterstains nuclei and bacteria) for 15 min. Coverslips were then washed three times with PBS and twice with distilled water, air dried, removed from the wells, and mounted on microscope slides with 5 μl of Slowfade (Molecular Probes). Specimens were then evaluated blind with a Leitz DMRB microscope equipped with fluorescence filters (Chroma, Brattleboro, Vt.) and a Wild photo system. For

optimal selectivity between probe signals when a specimen was stained with multiple dyes, all filter cubes were equipped with band-pass excitation (ex) and emission (em) filters with the following characteristics: blue, ex, 360 (±20) nm; em, >420 nm; green, ex, 480 (±20) nm; em, >510 nm; red, ex, 540 (±10) nm; em, >576 nm. A triple band filter set was used to simultaneously view DAPI-, fluorescein-, and CY3-labelled specimens. For each treatment in each experiment, 100 consecutive cells were evaluated. Bacteria, labelled with FITC-conjugated anti-C3 antibody and/or with DAPI, were denoted as macrophage associated when they overlay the body of a macrophage. Internalized bacteria were estimated by subtracting the number of anti-C3-positive bacteria from the number of DAPI-positive macrophage-associated bacteria (because cells were treated with anti-C3 prior to permeabilization, thus denying access of the antibody to internalized bacteria). Bacteria were considered to be within phagolysosomes when they were surrounded by a thin, smooth and brightly fluorescent ring of LAMP as revealed by CY3 fluorescence (33).

Laser confocal microscopy. To confirm that bacteria entered cells and that intracellular bacteria were contained in vesicles composed of membrane derived from the plasma membrane, macrophages were labelled with the lipid soluble fluoroprobe 1,1′-dioctadecyl-*d,d,d′,d′*-tetramethylindocarbocyanine (DiI) (Molecular Probes) by the method of Honig and Hume, prior to inoculation of meningococci (9). Prior to opsonization, bacteria were labelled by incubation with 250 μg of FITC per ml in PBS at 37°C for 30 min, followed by three washings in PBS. The internalization assay was then conducted as described above, and cells were washed and fixed with 2.5% gluteraldehyde 30 min after the warming step. Glutaraldehyde was washed off twice with PBS–0.1% bovine serum albumin. Glass coverslips were removed and mounted with 5 μl of Slowfade. Specimens were examined blind with an MRC-600 confocal imaging system (Bio-Rad, Hercules, Calif.) with a double labelling filter set combination. At a given focal plane, bacteria viewed through the FITC channel (ex, 480 nm; em, 535 nm), were examined for colocalization with diI examined through a rhodamine filter (ex, 540 nm; em, 580 nm). Control slides containing DiI only or FITC only were used to adjust settings to exclude spectral overlap. To compare internalization into membrane compartments between the two strains, the number of DiI-positive meningococci per 50 consecutive meningococci was counted.

Statistical analysis. Means of adherence indices, macrophage association, percentage internalization, and incorporation into phagolysosomes of each of the two strains were calculated and compared by Wilcoxon's signed-rank analysis.

RESULTS

Group B capsule inhibits meningococcal binding to macrophages. As shown in Fig. 2, there was a 4.5-fold difference between Cap^+ and Cap^- *N. meningitidis* in the adherence of unopsonized bacteria to the surface of macrophages. The Cap^- strain was consistently more adherent. There was no difference in the adherence to the plastic surfaces of control wells of the two strains or in the number of recovered macrophage nuclei in the saponin lysates (range, 1.8×10^5 to 8.0×10^5). Opsonization of bacteria with C5-depleted serum significantly increased the adherence of both strains two- to threefold (see Fig. 2). Opsonization did not increase adherence to plastic. When infected macrophages were fixed and stained with DAPI and examined by fluorescent microscopy, the percentage of macrophages with associated organisms and the number of organisms per 100 macrophages, respectively, were as follows: unopsonized Cap^+, 4% and 12; unopsonized Cap^-, 20% and 92; opsonized Cap^+, 13% and 41; and opsonized Cap^-, 89% and 910.

Capsulate and capsule-deficient meningococci are internalized at an equivalent rate by macrophages. When inocula were adjusted so that the numbers of Cap^+ and Cap^- strains associated with macrophages were approximately equal after 1 h of cold incubation, there was no significant difference in the overall numbers of meningococci associated with macrophages during the subsequent period of warm incubation (Fig. 3A and B). After the initial 1-h period of cold incubation, 100% of DAPI-positive bacteria (Cap^+ and Cap^-) were also FITC positive, indicating they were extracellular, although Cap^- organisms exhibited brighter fluorescence. After 10 min of incubation at 37°C, the majority of meningococci of each strain were intracellular, as indicated by their lack of access to FITC-conjugated anti-C3 antibody (Fig. 3C); by 60 min, approximately 75% of the associated organisms of each strain were internalized (Fig. 4).

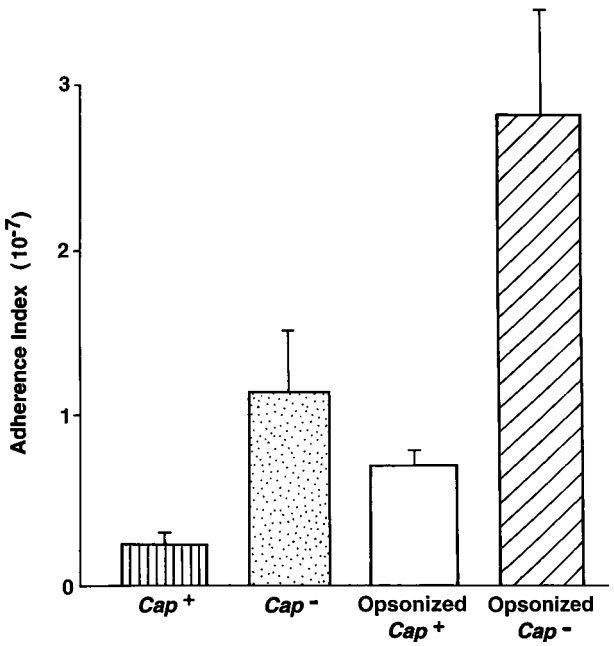

FIG. 2. Adherence of unopsonized and opsonized capsulate (Cap^+) and noncapsulate (Cap^-) *N. meningitidis* cells to monocyte-derived macrophages after incubation at 4°C for 1 h. The inocula of Cap^+ and Cap^- cells were $(4.7 \pm 2.7) \times 10^7$ and $(4.8 \pm 2.6) \times 10^7$ CFU, respectively. The inocula of opsonized Cap^+ and Cap^- cells were $(3.9 \pm 1.8) \times 10^7$ and $6.8 \pm 4.7 \times 10^7$ CFU, respectively. Results are means ± standard error for duplicates from three experiments with heterologous macrophages. In a typical experiment, when an inoculum of Cap^+ cells of 2.5×10^7 was incubated with macrophages for 1 h, the saponin lysate yielded 7.0×10^5 CFU of Cap^+ cells and 5.0×10^5 macrophage nuclei, while the plastic (control) well yielded 2.1×10^5 CFU, producing an adherence index of 0.4×10^{-7}: an inoculum of Cap^- cells of 2.2×10^7 CFU yielded 2.1×10^6 CFU and 4.8×10^5 macrophage nuclei in the saponin lysate, with 2.0×10^5 CFU being recovered from the control well, producing an adherence index of 1.98×10^{-7}. Cap^+ versus Cap^-, $P = 0.014$; unopsonized Cap^+ versus opsonized Cap^+, $P = 0.014$; unopsonized Cap^- versus opsonized Cap^-, $P = 0.023$ (Wilcoxon's signed-rank test).

There was no difference in the rate of internalization of Cap^+ and Cap^- strains. In a separate experiment, in which meningococci were labelled with FITC and inoculated onto macrophages pretreated with the phospholipid membrane marker DiI and fixed for confocal microscopy 30 min after the warming step, there was also no difference between Cap^+ and Cap^- strains; 83.3 and 82.5% of Cap^+ and Cap^- strains, respectively, colocalized with DiI in an intracellular location (Fig. 5), demonstrating that both organisms were taken up into intracellular compartments. Therefore, the group B capsule does not inhibit phagocytosis (internalization) once bacteria are bound to the cell surface.

Group B capsule delays incorporation of the late endosome-lysosome marker LAMP-1 into phagosomes. After 10 min of warming of infected macrophages, 19% of macrophage-associated Cap^- organisms were observed to colocalize with LAMP-1 and, by inference, to be within terminal phagolysosomes (Fig. 3D and 4B). This increased to 40% by 60 min of warming. In contrast, incorporation of Cap^+ organisms into phagolysosomes was significantly lower at 10 min and 30 min after warming, with only 3% of the Cap^+ organisms colocalizing with LAMP-1 by 30 min. By 60 min, there was no significant difference between the two strains (Fig. 3D). Therefore, while the (α2→8)-linked polysialic acid capsule does not block

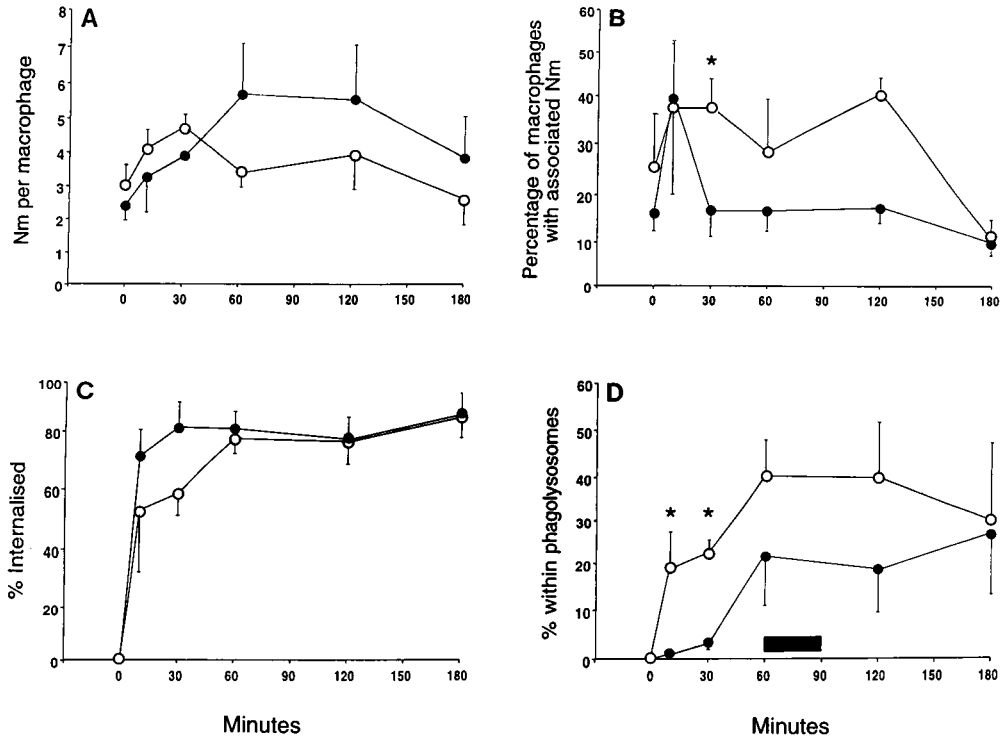

FIG. 3. Binding, phagocytosis, and intracellular trafficking of *N. meningitidis*. Bacteria were incubated with macrophages at 4°C for 1 h to permit binding. The inocula of Cap^+ (●) and Cap^- (○) cells were adjusted so that equal numbers of Cap^+ and Cap^- cells were bound to macrophages at the end of this period. Unbound bacteria were then removed by washing, and warm buffer was added to initiate phagocytosis (time zero in this figure). (A) Number of *N. meningitidis* (Nm) organisms per macrophage. (B) Number of macrophages with associated meningococci. (C) Percentage of organisms shown to be internalized by protection from anti-C3 antibody. (D) Percentage of organisms colocalizing with LAMP and therefore within phagolysosomal compartments. Data are shown as means ± standard errors of five experiments with macrophages from different human sources. Bar, 30-min period of gentamicin treatment. Significance is shown by the asterisks ($P < 0.05$; Wilcoxon's signed-rank test).

fusion of lysosomes with phagosomes, it does decrease the rate at which phagosomes acquire a lysosomal marker.

Efficient killing of intracellular *N. meningitidis*. Despite differences in incorporation of the two strains into terminal phagolysosomes, there was efficient, time-dependent killing of both strains (Table 1). Although both strains were killed over the period of warm incubation, there was significantly more killing of the Cap^+ strain after 3 h of warm incubation (Table 1). Mean challenge inocula for opsonized capsulate (Cap^+) and noncapsulate (Cap^-) *N. meningitidis* cells were $(7.25 \pm 1.83) \times 10^7$ and $(2.40 \pm 0.4) \times 10^7$, respectively, per 10^6 macrophages. In a typical experiment, 7×10^7 CFU of Cap^- cells and 2.5×10^7 CFU of Cap^+ cells were inoculated onto macrophages. After incubation at 4°C for 1 h and washing of cells, 1.25×10^6 and 0.75×10^6 CFU of Cap^- and Cap^+ cells were recovered from the saponin lysate, which contained 5×10^5 and 4.5×10^5 macrophage nuclei, respectively. After further warm incubation at 37°C for 1 h, followed by gentamicin treatment, washing of cells, and further incubation for 30 min, 2.4×10^4 CFU/5.8×10^5 nuclei of Cap^- cells and 3.5×10^3 CFU/6×10^5 nuclei of Cap^+ cells were recovered. After a further 60 min of warm incubation, 1.5×10^3 CFU/2.5×10^5 nuclei of Cap^- cells and 1.6×10^2 CFU/3.7×10^5 nuclei of Cap^+ cells were recovered. No meningococci were recovered from control wells that did not contain macrophages after gentamicin treatment, demonstrating that meningococci recovered from macrophage-containing wells were protected from gentamicin killing, which is assumed to be due to their intracellular location.

DISCUSSION

This study has demonstrated that the possession of (α2→8)-linked polysialic acid capsule by group B *N. meningitidis* results in decreased bacterial binding to the surface of human macrophages. Although capsule does not affect the rate of internalization of meningococci into membrane-bound vesicles once they are bound to the surface of macrophages, it does appear to delay the incorporation of LAMP-1 into the phagosome (i.e., phagosome-lysosome fusion is retarded).

Reduced adherence to the macrophage surface was demonstrable both by traditional washing and viable counting techniques and by direct fluorescent microscopy. Opsonization with nonimmune C5-depleted serum increased macrophage association of both capsulate and noncapsulate organisms. Our results suggest (i) that there are noncomplement and non-Fc receptors on human macrophages that will bind meningococci in the absence of serum, albeit poorly; (ii) that coating of meningococci with C3b and/or other serum proteins in the absence of Ig increases binding to macrophages, presumably via complement receptors; and (iii) that the presence of the (α2→8)-linked polysialic acid capsule decreases the affinity of the macrophage for the meningococcus in both cases.

Negative charge, steric hinderance, and masking of adhesin ligands on meningococci by polysialic acid may partly explain this observation. Increased C3b binding to noncapsulate meningococci (11) (up to 150% more bound C3b compared with capsulate meningococci) is also a likely explanation for the enhanced adherence of the opsonized Cap^- strain. Since mac-

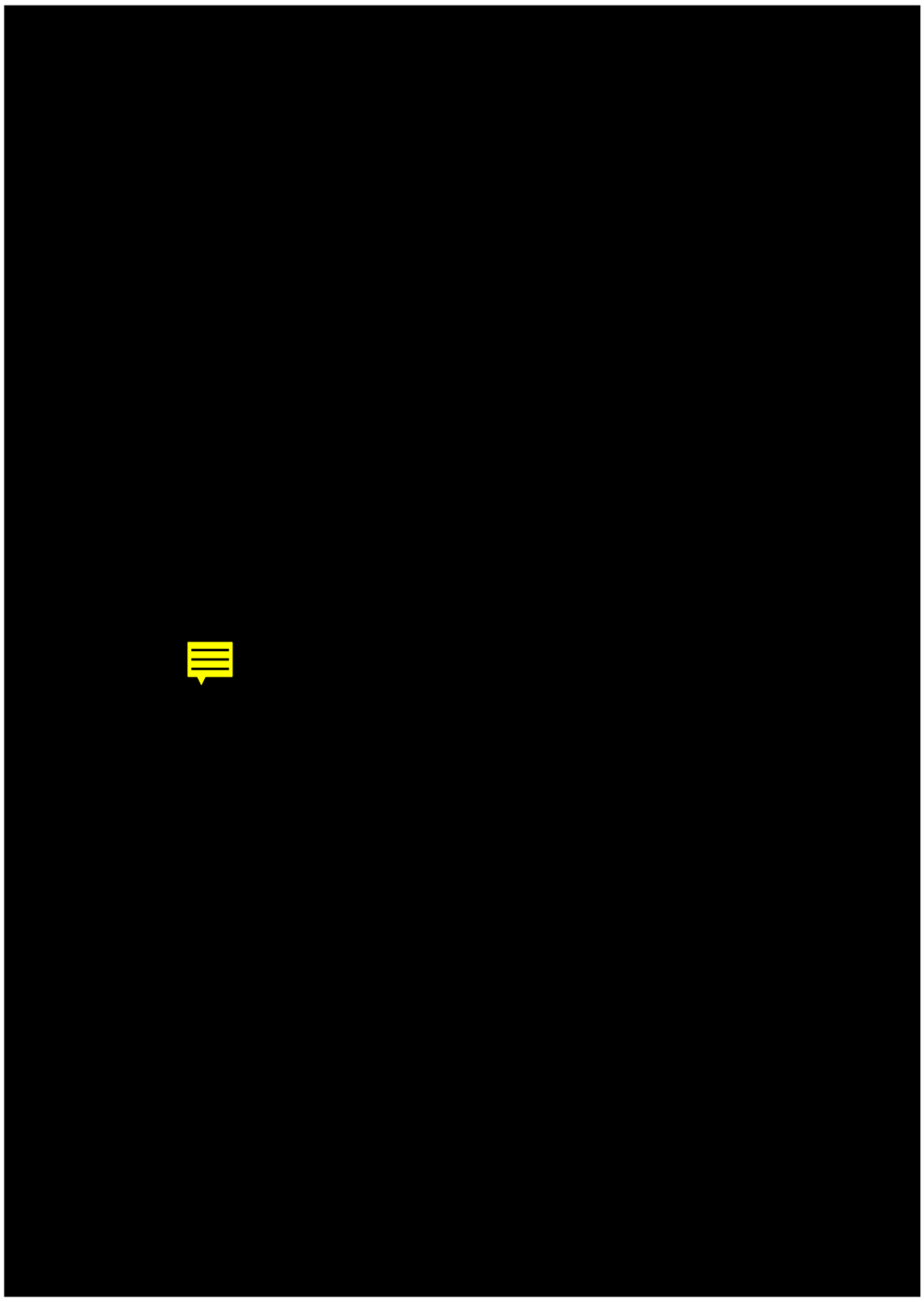

FIG. 4. Fluorescence microscopy of binding and internalization of *N. meningitidis* by macrophages. (A) Macrophages infected with Cap^- cells after 30 min of warm incubation and stained with DAPI, which stains both intra- and extracellular meningococci. (B) Same field with more extensive treatment. After fixation, cells were incubated with FITC-conjugated anti-C3b antibody (green) and then refixed and permeabilized. The cells were then incubated with anti-LAMP primary and secondary antibody (CY3 conjugated [red]) and DAPI. External organisms (but not internalized) take up green anti-C3b antibody, and meningococci within phagolysosomes are surrounded by a red ring of lysosome-associated LAMP antibody (arrow). (Bar, 10 μm).

rophages appear to be able to kill meningococci, the ability of the capsule to diminish binding to macrophages is an important virulence mechanism.

When the inoculum was adjusted so that equivalent numbers of meningococci adhered to the surface of the macrophages during cold incubation, internalization of organisms as detected by anti-C3b antibody and fluorescent-labelled plasma membrane occurred rapidly on warming of macrophages. With both techniques, capsulate and noncapsulate meningococci were observed to be internalized at approximately the same rate, and 75 to 85% of bound organisms were subsequently internalized by 30 min of warm incubation. This suggests that once bound, meningococci activate a receptor that transduces internalization, and this process is not affected by the presence

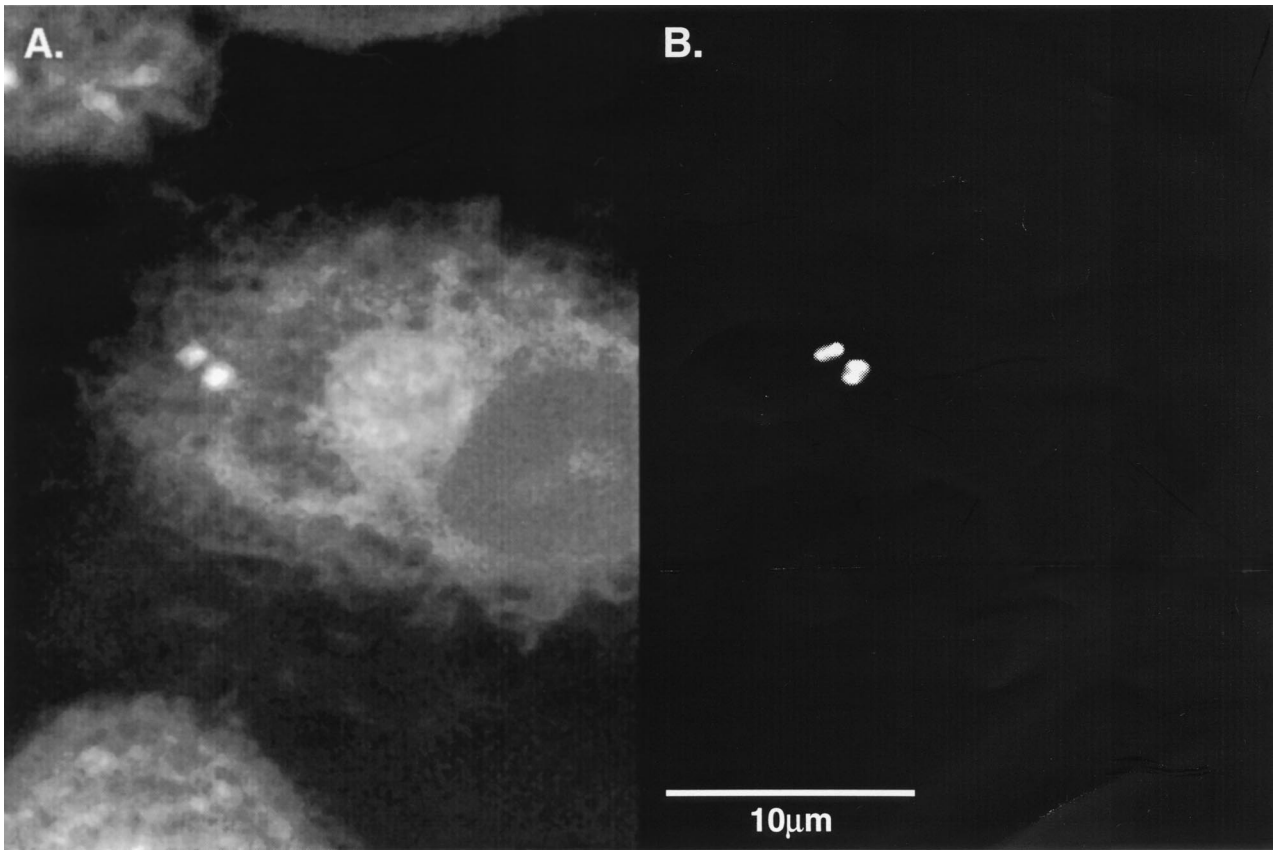

FIG. 5. Confocal microscopy of macrophages infected for 30 min with Cap$^+$ *N. meningitidis*. (A) Viewed through the red channel, a strong signal from the rhodamine-labelled phospholipid membrane marker DiI is seen. (B) Same field viewed through the green channel (FITC), showing organisms colocalizing with the DiI signal and establishing that they are intracellular and are surrounded by a phagosome membrane.

of (α2→8)-linked polysialic acid capsule. Therefore, with reference to macrophages, the polysialic acid capsule is not antiphagocytic once meningococci have been successfully bound. When meningococci were incubated with macrophages in the absence of serum, internalization still occurred (data not shown); therefore, the receptors that bind nonopsonized group B *N. meningitidis* are also capable of mediating phagocytosis.

The precise mechanisms of general microbial killing by macrophages have not been comprehensively determined. It is known that macrophages can kill by production of reactive oxygen metabolites, but mononuclear phagocytes, particularly resident macrophages, produce markedly less toxic oxygen products cell-for-cell than do polymorphonuclear neutrophils (21). Another major mechanism for dealing with phagocytosed particles is delivery of phagosomes to compartments that evolve characteristics of lysosomes in that they incorporate LAMP (12). Destruction of organisms within terminal phagolysosomes occurs by means of hydrolytic enzymes and other peptides (8). In this study, noncapsulate organisms colocalized with LAMP very rapidly. In contrast, capsulate organisms did not colocalize with LAMP to approximately the same extent until 60 min of warm incubation had elapsed. This can be interpreted as demonstrating that the presence of (α2→8)-linked polysialic acid capsule interferes with the maturation of phagosomes containing *N. meningitidis*. Despite the delay in incorporation of LAMP into phagosomes containing capsulate bacteria, there was no effect on killing; indeed, intracellular capsulate bacteria were killed more rapidly than capsule-deficient bacteria. This is a surprising result and suggests that meningococci can be rapidly killed within phagosomes prior to fusion with lysosomes. It has been shown that capsulate group B meningococci can be phagocytosed by and activate the respiratory burst of monocytes over very short periods of incubation (7.5 min) in vitro (22) and that this is enhanced in the presence of immune serum.

TABLE 1. Recovery of *N. meningitidis* from macrophages

Strain	CFU recovered/100 macrophages after:			
	Incubation at 4°C for 1 h	Incubation at 37°C for[a]:		
		2 h	3 h	18 h
Cap$^+$	194.40 ± 39.00	0.98 ± 0.60	0.10 ± 0.10	0
Cap$^-$	150.90 ± 25.90	3.10 ± 1.00[b]	0.79 ± 0.20[c]	0

[a] After 1 h and 17.5 h at 37°C, extracellular bacteria were killed by gentamicin (200 μg/ml) for 30 min. Results are means ± standard errors from five experiments with macrophages from separate donors.
[b] Not significant.
[c] $P = 0.014$.

Gentamicin penetrates phagocytes relatively poorly (18); this was the basis of our assay of intracellular viable meningococci. There is a theoretical possibility that the killing of Cap$^+$ organisms, which occurred despite phagosome-lysosome fusion, as reflected by delayed acquisition of LAMP-1 by phagosomes, was due to accumulation of effective concentrations of gentamicin within this compartment, which was not achieved in

the LAMP-1-positive phagosomes containing Cap^- organisms. Phagosomes become rapidly acidified to pH 5.5 to 6.0. At this pH, the activity of gentamicin is greatly reduced. Differential gentamicin-mediated killing of Cap^+ and Cap^- organisms might therefore have occurred if Cap^+ organisms selectively blocked acidification of their phagosomes, but we did not directly examine this possibility.

Pathogens have evolved a number of mechanisms to modify interactions with macrophages. First, they may avoid coating by complement and/or Ig and therefore may be resistant to opsonophagocytosis. The classic example of this is the neutrophil antiphagocytic activity of the pneumococcus capsule, but the reduced adherence of capsulate meningococci that we have observed may also be due to the reduced complement binding that has been described by Jarvis and Vedros (11). Second, microorganisms might avoid killing by selecting a receptor which transduces a series of events which result in a benign intracellular course (e.g., *Leishmania* spp. [17]). Third, microorganisms may escape from the phagosome before it has fused with the lysosome (e.g., *Listeria monocytogenes* [29]). Fourth, as we have observed with *N. meningitidis*, some organisms appear to be capable of interfering with phagosome-lysosome maturation. *Mycobacterium tuberculosis* can also retard the maturation of the phagosome in which it sits (3, 7). Finally, microorganisms may be resistant to the toxic contents of phagolysosomes (e.g., *Leishmania* spp. [30]). The examples given above are all classic intracellular pathogens; however, some invasive pathogens have been shown to be capable of avoiding killing by macrophages. These include *Salmonella* spp. which initially colonize gut mucosa; bacteremia presumably ensues when mucosal surveillance fails. Mutants of *Salmonella typhimurium* that are incapable of survival within macrophages are avirulent in a mouse model (4). *S. typhimurium* can respond to environmental stress (e.g., oxygen stress) to produce resistance to macrophage defensins (16).

There have been a number of studies that have investigated the interaction of meningococci with fresh peripheral blood monocytes, although it is noteworthy that in culture, monocyte-derived macrophages require several days to fully differentiate and express certain crucial receptors, for example, FcR III (31) and the mannose receptor (24). In a series of experiments to examine antibody-dependent anti-meningococcal activity of monocytes and of purified lymphocytes, group-specific antibacterial activity was removed when immune serum from donors immune to group A and group C organisms was preincubated with purified group A and group C polysaccharide, respectively (13, 14, 23). In the present study, which used mature macrophages and different assay conditions, killing proceded without the presence of Ig.

McNeil et al. (15) investigated the influence of capsule, pili, and opacity proteins on meningococcal interaction with cultured peripheral blood monocytes in a human serum-free system over 4 h. Capsulate group A meningococci associated in much lower numbers than capsule-deficient organisms. Pili did not affect bacterial association with monocytes. Capsule-deficient bacteria derived from a group A clone that failed to express the outer membrane proteins Opc and Opa did not associate with monocytes. In contrast, those that expressed the Opc or the Opa B protein did associate in greater numbers with monocytes, but monocytes appeared to kill these variants very efficiently in a phagocytic (tumbling tube) killing assay. Over a 4-h chase, McNeil et al. found a gradual decrease in both capsulate and capsule-deficient group A bacteria, showing that the monocytes were able to gradually kill those organisms that were internalized. However, bacteria were not completely eradicated over the 4-h period.

One enigma of meningococcal disease is the reason why presumably small colonies of organisms within the nasopharynx are capable of disseminating via the bloodstream. Although capsulate meningococci are relatively serum resistant, they are likely to be exposed to the activity of macrophages as soon as they penetrate the epithelial membrane of the nasopharynx, and it is difficult to envisage how small numbers of organisms penetrating the submucosa can avoid killing by whole blood even in the nonimmune host. This study has shown that the ($\alpha 2 \rightarrow 8$)-linked polysialic acid capsule of meningococci reduces recognition by macrophages. Within the relatively complement-deficient environment of the submucosa, this would presumably permit the formation of microcolonies better able to withstand the bactericidal activity of whole blood once the bloodstream is penetrated. This study also suggests that in the case of those meningococci that are successfully phagocytosed by macrophages, the presence of the ($\alpha 2 \rightarrow 8$)-linked polysialic acid capsule modifies their intracellular fate. This might be of significance if one or more of the varied microbicidal mechanisms of macrophages is impaired in any particular host, in which case failure of maturation of phagolysosomes could result in intracellular multiplication of meningococci. It has been suggested that mononuclear phagocytes might be capable of trafficking not only into but out of nasopharyngeal mucosa and that in such circumstances, the macrophage may act as a Trojan horse for the dissemination of capsulate organisms from the nasopharynx (15).

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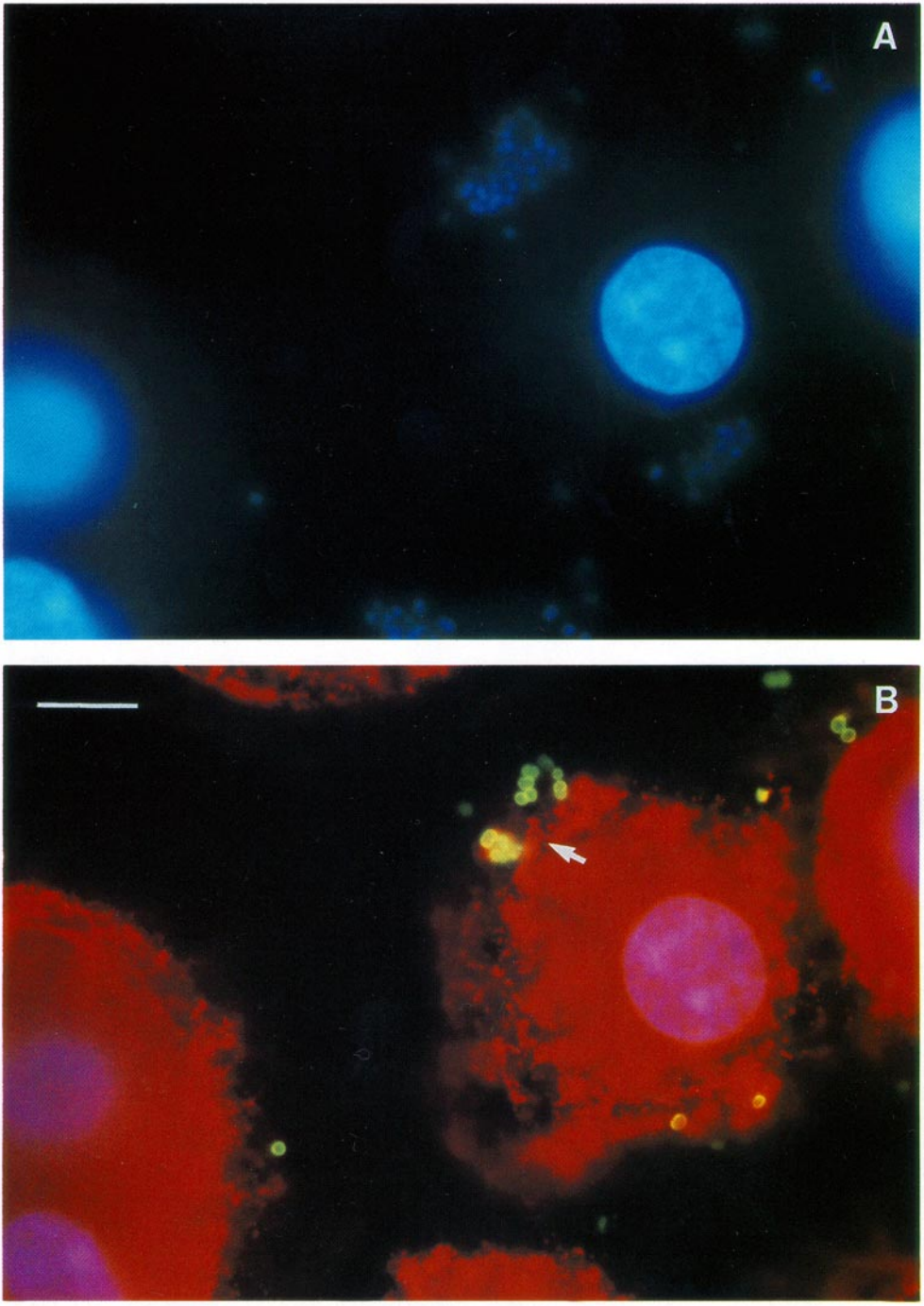
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