Interferon Suppresses Pinocytosis but Stimulates Phagocytosis in Mouse Peritoneal Macrophages: Related Changes in Cytoskeletal Organization

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ABSTRACT Treatment of thioglycolate-elicited macrophages with mouse β-interferon markedly reduces pinocytosis of horseradish peroxidase and fluorescein isothiocyanate (FITC)-dextran but stimulates phagocytosis of IgG-coated sheep erythrocytes. Experiments with FITC-dextran have revealed that the overall decrease in pinocytosis is due to a nearly complete inhibition of pinocytosis in a large fraction of interferon-treated macrophages. In the remaining cells pinocytosis continues at a rate similar to that in untreated control cells. A considerable reduction in the number of cells pinocytosing FITC-dextran was observed within 12 h from the beginning of interferon treatment. Measurement of the overall level of pinocytic activity with horseradish peroxidase showed a progressive decline through 72 h of treatment. In the interferon-sensitive subpopulation, there were marked changes in cytoskeletal organization. Microtubules and 10-nm filaments were aggregated in the perinuclear region while most of the peripheral cytoplasm became devoid of these cytoskeletal structures as observed by fluorescence and electron microscopy. In addition, interferon treatment of macrophages appeared to disrupt the close topological association between bundles of 10-nm filaments and organelles such as mitochondria, lysosomes, and elements of the Golgi apparatus and endoplasmic reticulum. Such alterations in the distribution of microtubules and 10-nm filaments were not seen in the interferon-insensitive subpopulation.

We have investigated the mechanism of the interferon-induced enhancement of phagocytic activity by binding IgG-coated sheep erythrocytes to mouse peritoneal macrophages at 4°C and then initiating a synchronous round of ingestion by warming the cells to 37°C. Thioglycolate-elicited macrophages that had been treated with mouse β-interferon ingested IgG-coated erythrocytes faster and to a higher level than control cells in a single round of phagocytosis. In interferon-treated cultures, phagocytic cups became evident within 30 s of the shift of cultures from 4° to 37°C, whereas in control cultures, they appeared in 2 min. Cytochalasin D, an inhibitor of actin assembly and polymerization, abolished phagocytic activity in both control and β-interferon-treated macrophages. However, to inhibit phagocytosis completely in thioglycolate-elicited interferon-treated macrophages, twice as much cytochalasin D was required in the treated as in control cultures. Accelerated association of actin filaments with the plasma membrane during engulfment of the erythrocytes appears to be a major factor contributing to the interferon-induced increase in phagocytic rate. Using monoclonal antibody (2,4G2) to the trypsin-resistant FcRII receptors, no difference was detected between control and interferon-treated macrophages in the abundance of cell surface receptors for IgG.

In conclusion, the stimulation of phagocytosis by interferon treatment of macrophages

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appears to reflect increased efficiency of the phagocytic process and may involve alterations of the plasma membrane and associated actin filaments; the suppression of pinocytosis may be due to alterations in these structures as well as to disruption of the extended network of microtubules and 10-nm filaments.

Interferons are a group of inducible proteins that inhibit the replication of many different viruses as well as the proliferation of a variety of normal and tumor cells both in the whole organism and in culture (13, 26, 28, 47). In general, the action of interferons is analogous to that of hormones. Interferons bind to specific cell surface receptors and subsequently activate and amplify cellular responses that regulate cell physiology (3, 6, 9, 10, 27). We have shown that the decrease in the proliferation of human β-interferon-treated human fibroblasts and tumor (HeLa) cells is associated with increased assembly and organization of actin-containing microfilaments (30, 55). Interferons inhibit cell locomotion across a solid substrate, saltatory movements of subcellular organelles, and lateral movement of cell surface receptors—activities that involve the function of microfilaments (30, 32). We have suggested that the increased incidence of abortive mitoses in interferon-treated cell populations (30, 43) is due to the altered organization of microfilaments that function in the formation of the cleavage furrow during cytokinesis (31, 43). These results have led to the conclusion that interferon treatment affects in a major way the organization of the cytoskeleton and the motile activities of cells that depend on the integrity of cytoskeleton (31, 42, 43).

In mouse peritoneal macrophages, interferon treatment increases, rather than inhibits, the phagocytosis of tumor cells, carbon and latex particles, and erythrocytes (8, 12, 14, 16). However, we will report that interferon treatment inhibits the pinocytosis of fluorescein isothiocyanate (FITC)[1]-dextran and horseradish peroxidase by thioglycolate-elicited mouse peritoneal macrophages, and define the quantitative parameters of the inhibition. It will be shown that in macrophages in which pinocytic activity has been suppressed by interferon treatment, microtubules and 10-nm filaments are no longer distributed in the form of an extended network, but have aggregated in the perinuclear region.

We also report that β-interferon treatment of thioglycolate-elicited mouse peritoneal macrophages increases the rate of phagocytosis of IgG-coated sheep erythrocytes in a single round of phagocytosis. Interferon treatment facilitates the association of actin filaments with the plasma membrane in the regions of attached erythrocytes, as a result of which phagocytic cups form with increased speed. Evidence will be presented showing that the cytochalasin D sensitivity of the process is decreased in interferon-treated cells.

Preliminary reports of a part of this work have appeared previously (43, 53, 54).

MATERIALS AND METHODS

Macrophages: Resident peritoneal cells (24–30% macrophages) and thioglycolate broth-elicited peritoneal cells (72–86% macrophages) were obtained by peritoneal lavage from the Nelson-Collins strain of mice as described (23). The macrophages were allowed to adhere to the surface of the culture dishes for 2 h at 37°C in Eagle's minimum essential medium (MEM); nonadherent cells were removed by repeated washing with Ca^{2+}- and Mg^{2+}-free PBS, pH 7.2 (PBS-def). The macrophages were maintained in MEM supplemented with 10% heat-inactivated (56°C, 45 min) fetal bovine serum and washed again before use in experiments.

Interferon: Interferon concentrations are expressed in terms of antiviral activity units (U) per milliliter. The antiviral activity of interferon preparations was assayed with vesicular stomatitis virus in mouse L cells, using the National Institutes of Health mouse interferon preparation No. G-002-904-511 as the standard. The activity was assayed by reduction of the viral cytopathic effect.

Two preparations of mouse β-interferon (Lee Biomolecular Research Laboratories, Inc., San Diego, CA; cat. No. 20171; containing <0.1% α-interferon and no detectable γ-interferon) were used in most experiments. Batch 81008 had a specific activity of $>1 \times 10^7$ U/mg protein, and batch 82014, 2×10^8 U/mg protein. A chemically homogeneous preparation of mouse β-interferon, kindly provided by Dr. Peter Lengyel of Yale University, was used in some experiments.

Immunofluorescence Microscopy: Cells cultured on No. 1 glass coverslips were fixed with 3.7% formaldehyde in PBS-def (pH 7.2) at room temperature for 20 min. After being rinsed with PBS-def several times, the samples were extracted with acetone at 20°C for 2 min. After air drying, we incubated coverslip cultures either with nitrobenzooxadiazole (NBD)-phallacidin (Molecular Probes, Inc., Plano, TX) for staining polymerized actin (1), or with rabbit antibodies to tubulin or vimentin in a moist chamber for 30 min at room temperature. The specificities of these antibodies for staining microtubules and 10-nm filaments have been established in a previous report (51). The coverslips were then washed extensively with PBS-def containing 1 M glycine, incubated with fluorescein-conjugated goat anti-rabbit IgG (0.5 mg/ml) for 30 min at room temperature, washed with PBS-def containing 1 M glycine, and mounted onto glass slides in a solution consisting of PBS-def and glycerol in a 1:1 ratio. Immunofluorescent staining was visualized using UV light (470–530 nm) and stained cells were photographed on Tri-X Kodak film at ASA 800 with a Zeiss photomicroscope III equipped with epifluorescence illumination and a 63× phase objective.

For labeling of the cytoskeletal structures in cells that had pinocytosed horseradish peroxidase, the coverslip cultures were first processed for cytochemical localization of peroxidase as described below and then reacted with antibody for visualization of the cytoplasmic fibers by immunofluorescence.

Electron Microscopy: For cytoskeletal studies, macrophages cultured in Petri dishes were fixed for 30 min at room temperature in 1% glutaraldehyde in PBS-def. After a brief rinse with PBS-def, the cells were postfixed for 30 min with a 1% solution of osmium tetroxide in PBS-def. Cells were then rinsed twice with PBS-def, dehydrated rapidly through a graded series of alcohol concentrations, and flat-embedded in Epon 812 (19). Liquid nitrogen was used to remove the polymerized Epon from Petri dishes. The embedded cultures were examined in a Zeiss inverted microscope and cells selected for sectioning were marked with a needle controlled by a Leitz micromanipulator. These cells were cut out of the main block and remounted cell-side up for thin sectioning parallel to the substrate side of the cell. Sections were cut with a diamond knife on a Sorvall Porter-Blum MT-2 microtome (E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT) and supported on 300-mesh copper grids with either parlodion or Formvar coating. These sections were then stained with hot uranyl acetate (17) and lead citrate (36). Ultrastructural observations were made with a Philips 300 electron microscope at 80 kV.

Decoration of Actin-containing Microfilaments with Heavy Meromyosin and Electron Microscopy: Heavy meromyosin (HMM) used for decoration of actin filaments was prepared from the rabbit back muscle according to the procedure described initially by Szent-Györgyi (41) and modified by Pollard et al. (35). Glycerination was carried out using the method of Chang and Goldman (5). Cells were sequentially extracted at room temperature with 50, 25, 12.5, and 5% glycerol in PBS-def for 30 min each. The samples were then incubated with HMM (2 mg/ml) in PBS-def containing 5% glycerol for 30 min at 22°C, rinsed thoroughly with PBS-def, and fixed with 1% glutaraldehyde in PBS-def containing tannic acid for 30 min (2). After being rinsed with PBS-def, the samples were fixed with 1% osmium tetroxide in PBS-def, rinsed again with PBS-def, dehydrated through a graded series of alcohol solutions, and flat-embedded in Epon 812 and sectioned for ultrastructural examination (17).

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[1] *Abbreviations used in this paper:* E(IgG), IgG-coated erythrocytes; FITC, fluorescein isothiocyanate; HMM, heavy meromyosin; NBD, nitrobenzooxadiazole; MEM, Eagle's minimum essential medium; PBS-def, Ca^{2+}- and Mg^{2+}-free PBS.

Determination of Pinocytic Activity: Pinocytic activity was evaluated qualitatively by the uptake of FITC-dextran (67,000 dalton; Sigma Chemical Co., St. Louis, MO) (49). Macrophage cultures were incubated for 30 min at 37°C with medium containing FITC-dextran (5 mg/ml), rinsed several times with warm PBS containing Ca^{2+} and Mg^{2+}, pH 7.2, fixed with 3.7% formaldehyde in PBS, and washed again with PBS. The samples were mounted in PBS containing 50% glycerol and examined in a Zeiss epifluorescence microscope with 63× objective, and cells containing fluorescent vesicles were scored. Cells were photographed with Kodak Tri-X film at 800 ASA. The pinocytic rate of all macrophages in the culture was quantitated by measuring the uptake of horseradish peroxidase from the culture medium (40). Briefly, 4×10^5 resident peritoneal cells or 2.5×10^5 thioglycolate-elicited peritoneal cells were seeded into the 16-mm wells of a 24-well Costar plate (Costar Data Packaging Co., Cambridge, MA). After overnight incubation at 37°C, the cultures were washed twice with warm MEM to remove the nonadherent cells and 0.5 ml of MEM or interferon in MEM was added to each well. Incubation at 37°C was continued for varying periods, after which the medium was removed, the cells were washed with warm PBS, and the macrophages were overlaid with 0.5 ml MEM containing fetal bovine serum (10%) and horseradish peroxidase (1 mg/ml). After incubation at 37°C for periods indicated below (usually 30 min), triplicate cultures were chilled on ice. The culture medium was removed and the macrophages were washed vigorously and rapidly six times with ice-cold MEM and once with ice-cold PBS. The cells were treated with 0.05% (vol/vol) Triton X-100 in double-distilled water, 0.4 ml/well, for 15 min at 37°C, and processed for determination of bound peroxidase using H_2O_2 and *o*-dianisidine as substrates as described (15, 25). The results are expressed in nanograms peroxidase ingested per microgram of cell protein. They are corrected for nonspecific adsorption of peroxidase to cell-free control wells (0.0034 μg). Cell protein was quantitated by the method of Lowry et al. (18). The amount of cell protein per well was equivalent to 20–40 μg.

Cytochemical Localization of Horseradish Peroxidase: For cytochemical localization of horseradish peroxidase by light microscopy, 1×10^6 cells were plated onto No. 1 glass coverslips placed in 35-mm plastic tissue culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA). For electron microscopy, the cells were plated on 35-mm culture dishes without coverslips. Control and interferon-treated cultures were incubated for 30 min at 37°C in medium containing peroxidase (2 mg/ml). After being washed four times with medium without peroxidase, the samples were fixed for 20 min at room temperature in 2.5% glutaraldehyde in PBS and incubated for 30 min at room temperature in 0.1 M cacodylate buffer containing diaminobenzidine and H_2O_2 (11). Coverslip cultures were mounted on glass slides for examination by light microscopy. For electron microscopy, the cells were fixed at 4°C with 1% osmium tetroxide in 0.1 M cacodylate buffer and then dehydrated and embedded as described above. The presence of peroxidase in organelle was evaluated in sections that had not been stained with either uranyl acetate or lead citrate.

Preparation of IgG-coated Erythrocytes: Sheep erythrocytes (Laboratory Animal Research Center, The Rockefeller University) were coated with rabbit anti-E IgG (Cordis Laboratories, Inc., Miami, FL) as described (22). The coated particles, designated E(IgG), were adjusted to a final concentration of 1% vol/vol in PBS with Ca^{2+} and Mg^{2+}. Mouse monoclonal IgG2a and IgG3 anti-sheep erythrocyte antibodies were a generous gift from Dr. Betty Diamond of the Albert Einstein College of Medicine.

Phagocytosis Assay: 4×10^5 resident peritoneal cells or 1.5×10^5 thioglycolate-elicited cells were seeded into each well of Costar plates and processed as described above. Each well contained a 12-mm glass coverslip (Rochester Scientific, Rochester, NY) and 0.5 ml MEM. At the time points indicated in Results, duplicate coverslips were removed, dipped five times into warm MEM, and placed into a fresh Costar plate containing 0.5 ml of warm MEM in each well. Rabbit IgG-coated sheep erythrocytes in a volume of 0.1 ml were added to each well, and the incubation was continued for 45 min at 37°C. The coverslips were then removed from the wells, treated for 30 s to 1 min with hypotonic NH_4Cl buffer to lyse E(IgG) that were attached but not ingested, and fixed in 1.25% glutaraldehyde in PBS. The number of erythrocytes ingested was determined by phase-contrast microscopy using a 100× phase-three oil immersion objective. At least 100 macrophages in random fields were counted. The percentages of macrophages that ingested erythrocytes multiplied by the average number of erythrocytes ingested per macrophage is expressed as the phagocytic or ingestion index (22). The ingestion index is equivalent to the number of particles ingested per 100 macrophages in the culture. For electron microscopy, 4×10^6 thioglycolate-elicited macrophages were seeded onto 35-mm plastic Petri dishes, and processed as described above.

Quantitation of Macrophage Fc Receptors with ^{125}I-labeled Monoclonal Rat Anti-mouse Macrophage Fc Receptor Antibody 2.4G2: Macrophage Fc receptors were quantitated using ^{125}I-labeled 2.4G2 Fab as described previously (24, 46). All measurements were done in duplicate, and the average of two determinations is expressed as nanograms ^{125}I-Fab bound per coverslip. All results have been corrected for nonspecific absorption of the antibody fragment to cell-free coverslips that were prepared and processed in parallel with the experimental samples.

RESULTS

Inhibition of Pinocytosis by Interferon

Treatment with interferon decreases the pinocytic activity in cultures of thioglycolate-elicited mouse peritoneal macrophages. In a fraction of the treated cell population, pinocytosis is markedly depressed, while in the remaining cells pinocytosis continues at a rate similar to that in untreated control cells. Control macrophages (Fig. 1, *a* and *b*) endocytose FITC-dextran in numerous pinosomes that appear as bright spots under UV illumination, as demonstrated previously by Walter et al. (49), and accumulate (FITC)-dextran in secondary lysosomes. In the interferon-treated (batch 81008, 5,000 U/ml) cultures (Fig. 1, *c* and *d*), the majority of macrophages contain less than 10 fluorescent vesicles per cell even after an extended period (1 h) of incubation with (FITC)-dextran.

Examination of populations of approximately 500 cells in control or interferon-treated cultures showed that after treatment of thioglycolate-elicited macrophages with mouse interferon at 5,000 U/ml for 12 h, ~80% of the interferon-treated cells still take up some dextran as compared with ~95% of the control cells (Fig. 2*A*). After interferon treatment at 10,000 U/ml, only ~40% of the cell population showed uptake of (FITC)-dextran at levels similar to those in 95% of the control cells. In the remaining 60% of the treated cells, the pinocytic activity was much reduced, and remained so during the entire 72-h period of interferon treatment. Thus, in terms of the number of actively pinocytosing cells, interferon batch 81008 caused 15% inhibition at 5,000 U/ml and 58% inhibition at 10,000 U/ml. Full recovery of pinocytic activity to the control level after incubation of the cultures with interferon-free medium required a minimum of 2–4 d (Fig. 2*B*).

In contrast to the results obtained with thioglycolate-elicited macrophages, interferon treatment (5,000 or 10,000 U/ml for up to 72 h) did not suppress pinocytic activity of resident peritoneal macrophages, as indicated by the percentage of such macrophages taking up FITC-dextran.

To quantitate the reduction in pinocytosis caused by interferon treatment of thioglycolate-elicited macrophages, horseradish peroxidase was used as a pinocytic marker. As shown in Fig. 3, interferon treatment for 72 h reduced the pinocytic activity from a control value of 2.5 ng of peroxidase taken up per microgram of cell protein to the values of 1.8 (72% of control) and 1.2 (48% of control) at 5,000 and 10,000 U/ml, respectively.

The results in Fig. 3 indicate that pinocytic activity declines progressively upon treatment of thioglycolate-elicited macrophages with interferon at 5,000 or 10,000 U/ml. However, even after 72 h, interferon-treated macrophage cultures were still capable of considerable pinocytosis of horseradish peroxidase, which is in agreement with results obtained with FITC-dextran (Fig. 2*A*).

Effects of Interferon on the Organization of Microtubules and 10-nm Filaments

Previous studies have indicated that cytoskeletal structures such as microtubules and 10-nm filaments play a role in the pinocytic process (33, 34). Alterations of the organization of

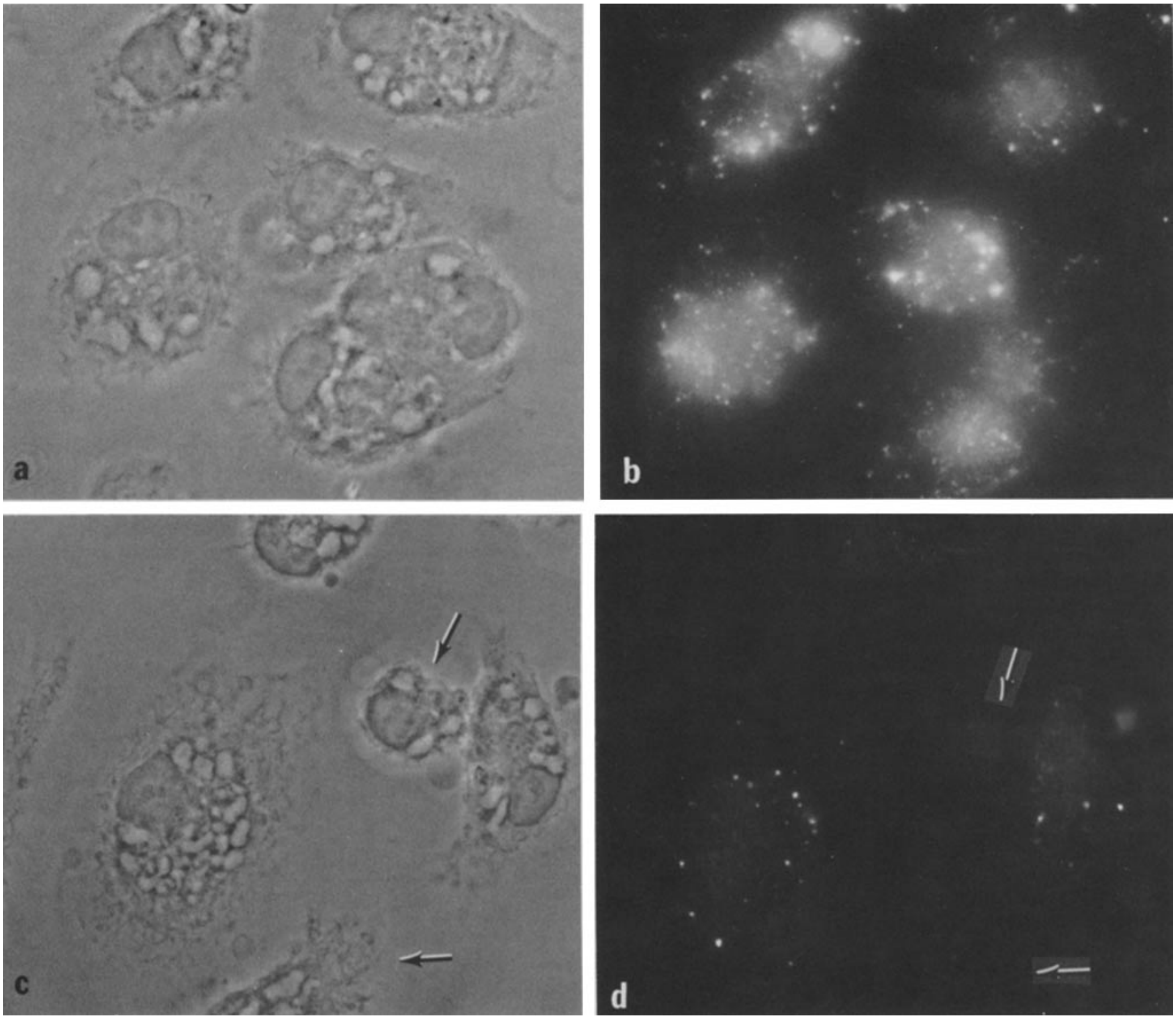

FIGURE 1 Uptake of fluorescent dextran in pinocytic vesicles by control (*a* and *b*) and β-interferon-treated (*c* and *d*) thioglycolate-elicited mouse peritoneal macrophages. Cultures of macrophages were incubated at 37°C with or without mouse fibroblast interferon (batch 81008; 5,000 U/ml) for 72 h and then with fluorescent dextran (5 mg/ml) for 1 h. The samples were then washed extensively with PBS and fixed with 1% glutaraldehyde in PBS-def before examination. (*a*) Phase-contrast micrograph of control macrophages; (*b*) the same cells, viewed with fluorescence optics, show pinocytosis as evidenced by the small, bright granules in the cytoplasm; (*c*) phase-contrast micrograph of interferon-treated cells; (*d*) the same cells, viewed with fluorescence optics, show the absence of pinocytosis in two of the cells (arrows) and a marked reduction of this activity in the other two cells. × 1,000.

such cytoskeletal structures may thus be expected to affect the pinocytic process.

We have found that interferon treatment caused a major reorganization of microtubules in macrophages. As shown in Fig. 4*a*, the microtubules in control cells form a radial pattern extending from the cell center to the periphery. In contrast, as illustrated in Fig. 4*b*, many cells in interferon-treated (batch 81008, 10,000 U/ml, 72 h) cultures, display a lack of microtubules in the periphery of the cells. Most of the microtubules are aggregated in the perinuclear region.

Similarly, we have found that interferon treatment causes the disappearance of organized 10-nm filaments from the periphery of the cytoplasm. As shown in Fig. 4*c*, 10-nm filaments are distributed throughout the cytoplasm of control cells, whereas in the interferon-treated (batch 81008; 10,000 U/ml, 72 h) cultures (Fig. 4*d*), 10-nm filaments aggregate in the perinuclear region in many cells.

Results of electron microscopic examination confirm our findings of cytoskeletal reorganization as a result of interferon treatment of macrophages. In control macrophages, there are oriented arrays of microtubules and 10-nm filaments that extend into the long processes and the cortical region of the plasma membrane. In contrast, in interferon-treated macrophages (batch 81008, 10,000 U/ml for 72 h), microtubules are found only in the immediate vicinity of the nucleus and 10-nm filaments are present as aggregated bundles in the centrosomal region.

Interferon caused similar reductions in the number of cells engaged in pinocytic activity (as measured by the uptake of FITC-dextran) and in the number of cells that possess a

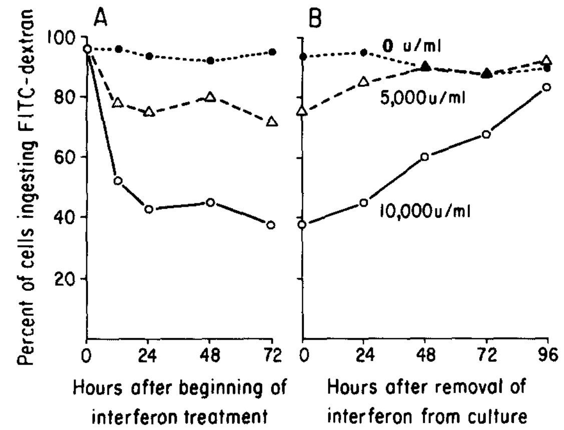

FIGURE 2 (*A*) Relationship between interferon concentration and the reduction in the number of thioglycolate-elicited macrophages engaged in pinocytosis. After incubation of control and interferon-treated (batch 81008; 5,000 or 10,000 U/ml) macrophages at 37°C for various periods, they were further incubated with fluorescent dextran (5 mg/ml) for 30 min. After extensive washing with PBS, the cultures were fixed with 1% glutaraldehyde and mounted on slides. Percent of cells ingesting fluorescent-dextran was determined by direct counting of cells containing the bright granules illustrated in Fig. 1*b*. Approximately 500 cells were evaluated per variable. Maximal dose-dependent reduction in pinocytic activity was observed 12 h after addition of interferon to the cultures. (*B*) Recovery of pinocytic activity after removal of interferon from the culture medium. Cultures were treated with interferon for 72 h after which they were washed with interferon-free medium. The cultures were assayed for pinocytic activity immediately after washing or after incubation in interferon-free medium for 24, 48, 72, or 96 h. Approximately 200 cells were examined per variable in duplicate cultures. ●, control; △, interferon, 5,000 U/ml; ○, interferon, 10,000 U/ml.

normal distribution of 10-nm filaments (Fig. 5). As shown in Fig. 5*A*, in the control cultures, 90% of the cells took up FITC-dextran. In contrast, in the interferon-treated cultures, the percentage was reduced to 80 and 45, respectively, after treatment at 5,000 and 10,000 U/ml (batch 81008) for 72 h. Fig. 5*B* shows that 95% of the cells in the control cultures displayed a fully extended distribution of 10-nm filaments, whereas after treatment with interferon at 5,000 or 10,000 U/ml, approximately 80% and 30%, respectively, of the cells had 10-nm filaments extending to the periphery.

To ascertain whether in the interferon-treated cultures those cells that do not pinocytose are cells that exhibit an abnormal distribution of 10-nm filaments, macrophage cultures were treated with interferon at 10,000 U/ml (batch 81008) for 72 h, and then incubated with peroxidase-containing medium for 30 min in the continued presence of interferon. Fig. 6*a* illustrates an interferon-treated cell that displays a normal distribution of 10-nm filaments extending from the perinuclear region to the cell periphery. In this cell almost all of the large vacuoles and vesicles are filled with peroxidase (Fig. 6*b*). Fig. 6*c* demonstrates that in another interferon-treated cell, 10-nm filaments are aggregated in the perinuclear region. In this cell, peroxidase is absent from large vacuoles (Fig. 6*d*).

Lack of Effect of Interferon on Cell Size

We have determined the surface area of control and interferon-treated cells as described by Pfeffer et al. (30) and Phaire-Washington et al. (34). Based on three experiments, the area of substrate covered by control thioglycolate-elicited macrophages was 494 ± 75 μm^2 whereas that for interferon-treated (batch 81008; 10,000 U/ml, 72 h) macrophages was 442 ± 80 μm^2. It appears, therefore, that the reduction in pinocytosis in the interferon-sensitive macrophages cannot be explained simply by a decrease in surface area of the plasma membrane available for pinocytosis.

Enhancement of Phagocytosis by Interferon

The time course of the development of enhanced phagocytic ability for E(IgG) was determined for both thioglycolate-elicited and resident mouse peritoneal macrophages treated with interferon at 5,000 U/ml. The phagocytosis assay was performed on control and interferon-treated cells after periods of incubation which ranged from 0 to 72 h. Fig. 7 shows that thioglycolate-elicited macrophages exhibit a higher level of phagocytic activity than resident macrophages. The activity in control cells increased rapidly during the first 12 h of incubation and then leveled off. An interferon-induced increase in phagocytosis was observed as early as 4 h after the beginning of treatment of either resident or thioglycolate-elicited macrophages, which was also observed by Hamburg et al. (14). In resident macrophages treated with interferon, the enhanced phagocytic activity increased through the first 24 h of treatment and then leveled off at approximately 2.3 times the control level for resident macrophages. In contrast, in the thioglycolate-elicited macrophages, the increase continued until 48 h after the addition of interferon, and reached a level approximately 2.7 times above the control level for the thioglycolate-elicited cells. After 48-h treatment, the thioglycolate-stimulated macrophages ingested >30 erythrocytes per cell.

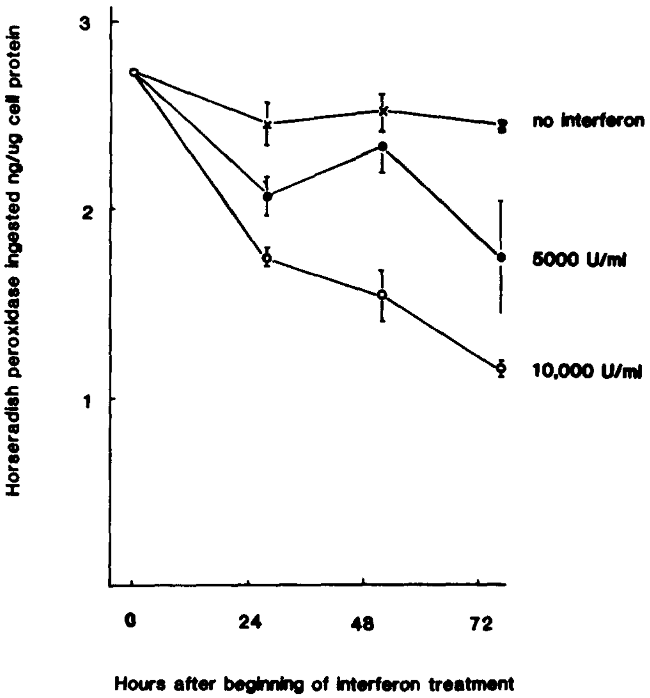

FIGURE 3 Relationship between interferon concentration and the reduction in the uptake of horseradish peroxidase in thioglycolate-elicited macrophages. Cultures of control and interferon-treated (batch 81008; 5,000 or 10,000 U/ml) macrophages were incubated in horseradish peroxidase-containing medium for 60 min at 37°C and processed for the quantitation of cell-associated horseradish peroxidase as described in Materials and Methods.

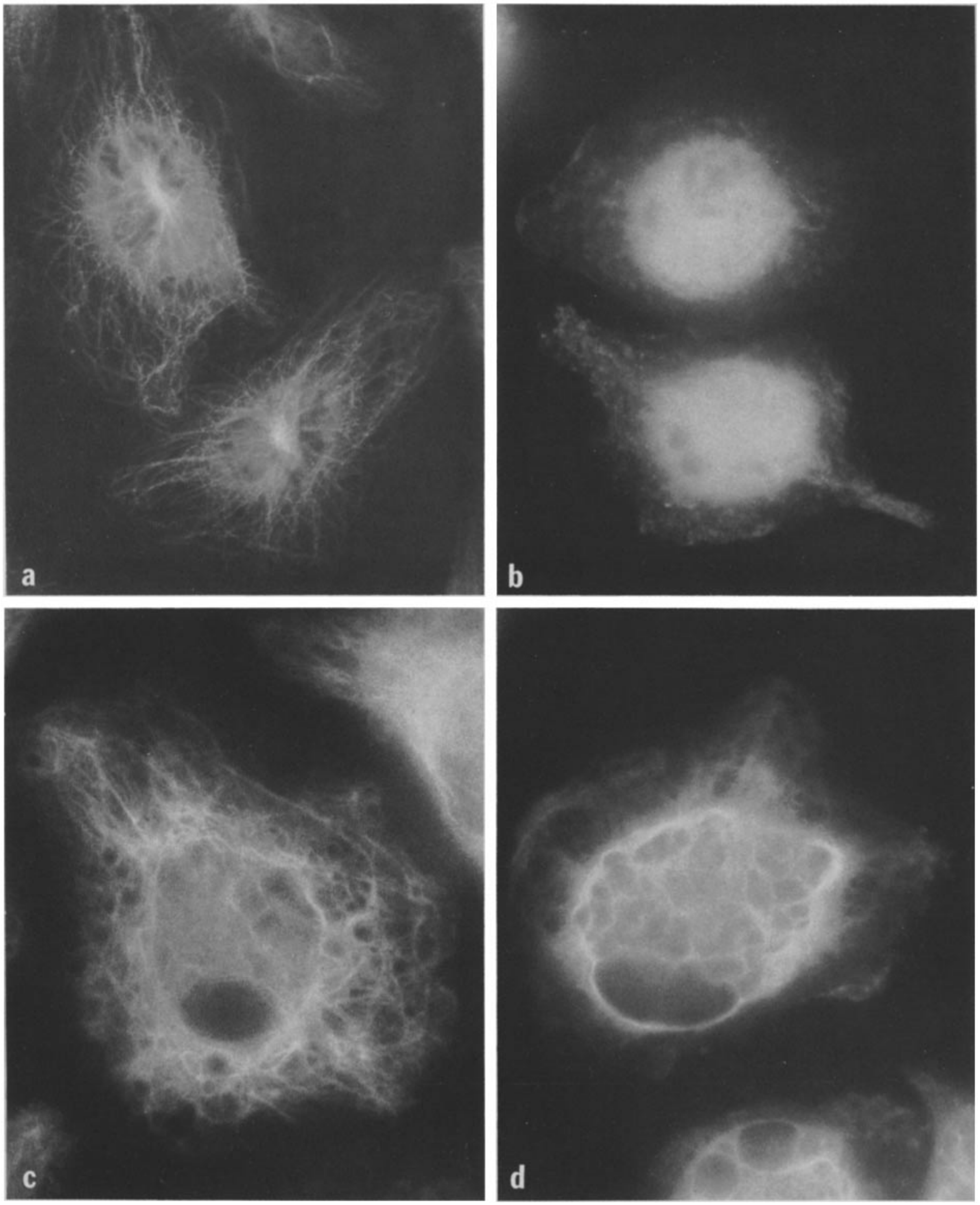

FIGURE 4 Alterations in the cytoplasmic organization of microtubules and 10-nm filaments in thioglycolate-elicited peritoneal macrophages treated with mouse interferon (batch 81008; 10,000 U/ml) for 72 h. Control and interferon-treated cells were processed for indirect immunofluorescence microscopy as described in Materials and Methods. (*a*) Typical radial pattern of microtubules in the control cells; (*b*) lack of a distinct network of microtubules observed in the majority of interferon-treated cells, accompanied by bright fluorescent staining by tubulin antibody in the nuclear region; (*c*) pattern of 10-nm filament distribution in control cells illustrates the extension of filaments from the perinuclear region to the cell periphery; (*d*) shows the perinuclear aggregation of these filaments in most of the interferon-treated cells; few filaments are found in the peripheral cytoplasm. (*a* and *b*) × 1,300; (*c* and *d*) × 1,700.

Abundance of Fc Receptors

Hamburg et al. (14) showed that treatment of resident macrophages with interferon for 8 h did not increase the number of trypsin-resistant Fc receptors (FcRII) expressed by these cells. To determine whether interferon treatment alters FcRII expression in thioglycolate-elicited macrophages, we quantitated the amount of FcRII on these cells using radio-

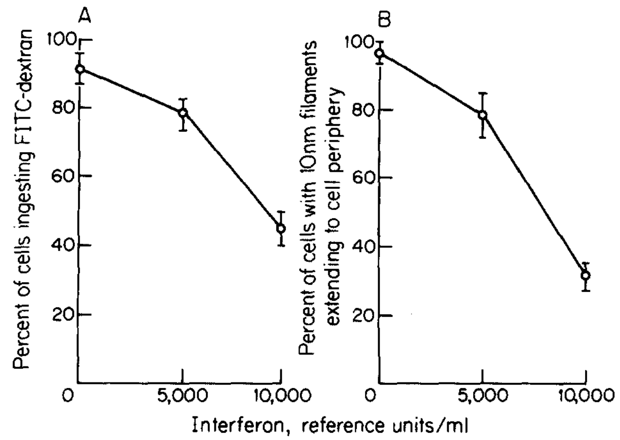

FIGURE 5 Correlation between reduced pinocytic activity (*A*) and altered organization of 10-nm filaments (*B*) in interferon-treated thioglycolate-elicited macrophages (batch 81008; 5,000 or 10,000 U/ml, 72 h). Approximately 500 cells from each sample were scored both for reduced uptake of fluorescent dextran and for redistribution of 10-nm filaments into perinuclear aggregates. Note the similarity in the percentages of cells displaying reduced pinocytosis or altered organization of 10-nm filaments. Data represent mean results of three experiments.

iodinated Fab fragments of monoclonal antibody 2.4G2 (anti-FcRII) (45). We have found that the number of FcRII increases in parallel in control and interferon-treated thioglycolate-elicited macrophages over the course of 72 h. Thus, increased FcRII expression does not account for the interferon-induced increase in phagocytosis of E(IgG) by thioglycolate-elicited macrophages.

Enhancement in a Single Round of Ingestion

To determine whether interferon treatment increased the rate or the extent of phagocytosis in thioglycolate-elicited and resident macrophages, or whether it affected both parameters, we conducted experiments under conditions that provide synchronization of the process of phagocytosis, which makes dissection of the process possible. E(IgG) were added to macrophages at 4°C, and the incubation was continued for 1 h at that temperature. At 4°C binding of E(IgG) to the plasma membrane of the macrophages takes place efficiently, but ingestion does not occur. It is important to note that there appeared to be no significant difference in the number of E(IgG) that initially attached to thioglycolate-elicited or resident macrophages irrespective of whether they had or had not been treated with interferon (Fig. 8). After removal of unbound E(IgG) by washing with cold PBS-def, the macrophages were immediately incubated in medium at 37°C allowing the ingestion of the bound E(IgG) to proceed. Phagocytosis of E(IgG) was examined at 0.5, 2, 4, 6 and 8 min after the temperature shift. Interferon-treated thioglycolate-elicited macrophages showed markedly enhanced phagocytic activity within the first 2 min after the temperature was raised from 4° to 37°C (Fig. 8*A*). Both in the treated and control thioglycolate-elicited macrophages, phagocytosis reached a plateau value by 4 min from the temperature shift, which provides an estimate of the duration of a round of phagocytosis. The plateau value for interferon-treated thioglycolate-elicited macrophages was ~2.3-fold higher than that for control cells. There was no further increase in the ingestion index when the control and interferon-treated cells were incubated for an additional 7 min. It therefore appears that when ~33 E(IgG) attach per cell, not all of the adsorbed erythrocytes could be ingested by the macrophage in a single round of phagocytosis. In resident macrophages no significant phagocytic activity was observed within 2 min from the temperature shift, and at 4 min the activity was approximately two-thirds of that in thioglycolate-elicited macrophages (Fig. 8*B*). No interferon-induced enhancement was observed during a single round of phagocytosis in resident macrophages. This is somewhat surprising in view of the results obtained under conditions of unsynchronized phagocytosis over a 45-min period, where interferon treatment has a considerable enhancing effect on phagocytosis (Fig. 7). Under both sets of conditions, resident control cells show lower phagocytic activity than thioglycolate-elicited control cells. The above results obtained with thioglycolate-elicited peritoneal macrophages show that interferon increases both the rate of the phagocytic process and the total number of particles ingested per thioglycolate-elicited macrophage per round of ingestion, without affecting the number of particles initially bound per cell. Availability of actin filaments in the zone adjacent to the plasma membrane, achieved through a redistribution of cytoskeletal elements, may play an important role in phagocytosis. We have therefore investigated the microfilament distribution in control and interferon-treated thioglycolate-elicited macrophages, first in cells not engaged in phagocytosis, and then in phagocytizing cells under conditions that provide synchronization of the process.

Actin Filaments in Nonphagocytosing Cells

The distribution of actin filaments in control and interferon-treated cultures of macrophages was investigated by staining the formaldehyde-fixed and acetone-extracted samples with NBD-phallacidin. Interferon treatment did not induce any apparent differences in actin distribution in the noningesting macrophages, as observed by fluorescence microscopy. In both control and interferon-treated cultures of thioglycolate-elicited macrophages, fluorescent staining was found in a diffuse pattern in the cytoplasm, as well as in localized regions associated with membrane ruffling, with a dotted pattern in the vicinity of the ruffling cell edge.

Actin Filaments in Phagocytosing Cells

To investigate the involvement of actin filaments in receptor-mediated phagocytosis, we used a temperature shift experiment as described above. E(IgG) were bound to the surface of thioglycolate-elicited macrophages at 4°C, after which the temperature was raised to 37°C. At various intervals after the temperature shift, the cells were fixed and processed for phase-contrast and fluorescence microscopy or for electron microscopy. Fig. 9*a* is a phase-contrast micrograph that shows erythrocytes being ingested by an interferon-treated macrophage within 30 s after the temperature shift. In Fig. 9*b*, actin filaments that have been stained with NBD-phallacidin are seen in the cytoplasm underlying the nascent phagocytic cups. It should be pointed out that F-actin filaments are very heavily concentrated in the areas of phagocytosis, with little if any fluorescence in regions of the plasma membrane distinct from sites of ingestion. In contrast, control macrophages (Fig. 9, *c* and *d*) had not yet begun forming phagocytic cups 1 min after the temperature shift. Fluorescence staining of actin filaments was still largely diffuse in the cytoplasm, except for

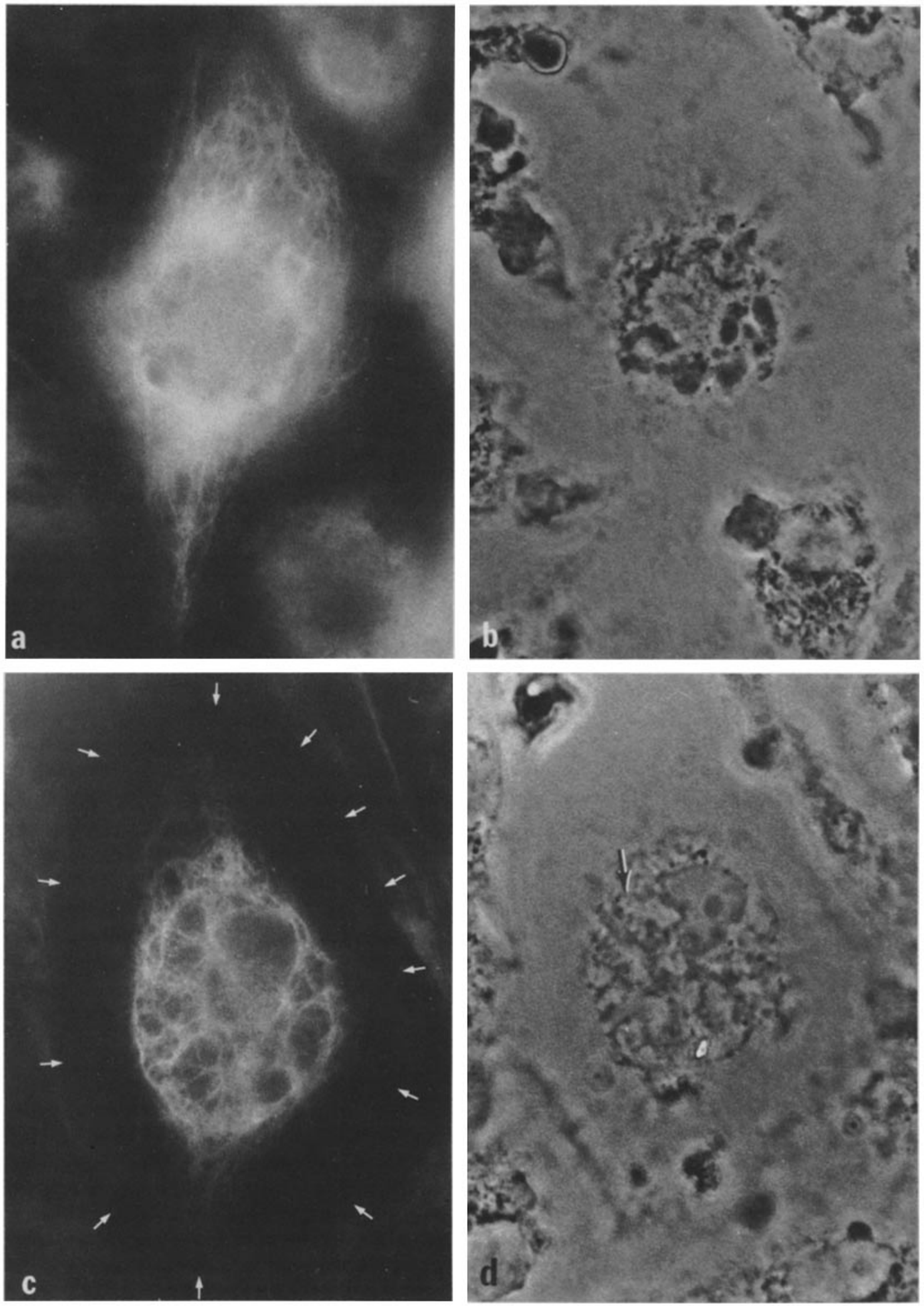

FIGURE 6 Simultaneous localization of 10-nm filaments and the endocytosed horseradish peroxidase in interferon-treated thioglycolate-elicited mouse peritoneal macrophages. Cultures of macrophages were treated with interferon (batch 81008; 10,000 U/ml, 72 h) and then incubated with peroxidase (5 mg/ml) in medium for 30 min at 37°C. The samples were fixed with 3.7% formaldehyde in PBS, processed initially for the visualization of peroxidase, and then stained for 10-nm filaments. *a* Illustrates the extension of 10-nm filaments from the perinuclear region to the cell periphery in an interferon-treated cell, and *b* shows the abundant presence of endocytosed peroxidase in large vacuoles as well as small vesicles in this cell. *c* shows perinuclear aggregation of filaments and absence of filaments from the cell periphery (arrows) in another interferon-treated cell, and *d* illustrates the absence of peroxidase from large vacuoles (arrow) in this cell. (*a* and *b*) × 900; (*c* and *d*) × 1,400.

some concentration of actin filaments in a few locations of plasma membrane where E(IgG) had attached.

By 2 min after the temperature shift, the interferon-treated macrophages display erythrocytes partially enclosed in phagocytic cups, and by 3–4 min, they show internalized particles in phagosomes. These phagosomes do not have associated actin filaments demonstrable by staining with NBD-phallacidin. In control macrophages, phagocytic cups have begun to

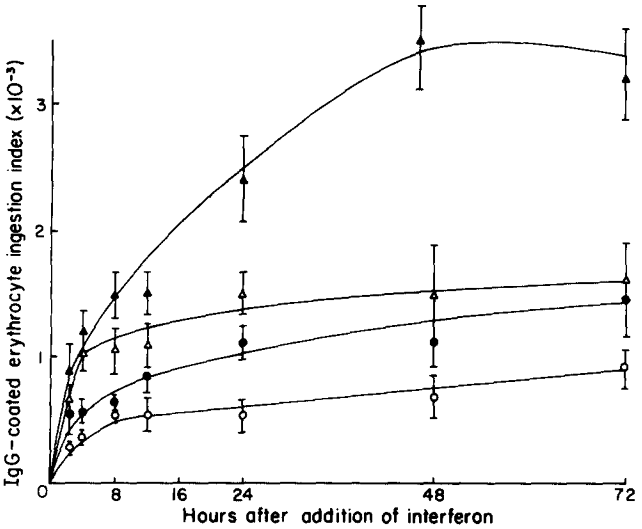

FIGURE 7 Time course of development of enhanced ability for phagocytosis of E(IgG) by interferon-treated thioglycolate-elicited and resident macrophages. Mouse peritoneal macrophages were treated with interferon (5,000 U/ml) for various periods and incubated with E(IgG) for 45 min at 37°C. Values are the means of triplicate determinations and bars indicate the standard deviations. (△) Thioglycolate-elicited macrophages; (▲) interferon-treated thioglycolate-elicited; (○) resident; (●) interferon-treated resident macrophages. The assay for phagocytosis was done as described in Materials and Methods, and the activity of uptake was expressed as the ingestion index, i.e., the number of erythrocytes ingested per 100 macrophages.

form by 2 min and phagocytic cup formation is well developed by 3–4 min. Complete internalization of particles in phagosomes requires ~6 min in control cells.

The relationship between actin organization and formation of the phagocytic cup was further characterized by means of ultrastructural identification of HMM-decorated microfilaments in glycerinated cells. Cultures of macrophages with attached E(IgG) were shifted from 4° to 37°C to initiate the process of ingestion. Approximately 30 s after the temperature shift, the cultures were glycerinated and incubated with HMM. Glycerination disrupts the plasma membrane as a result of which the cytoplasm becomes depleted of soluble materials. The glycerinated specimen still contains most of the plasma membrane, and the insoluble cytoskeletal matrix as well as the nucleus. Fig. 10*a* shows an ultrastructural profile representative of control macrophages with E(IgG) bound on the cell surface. The E(IgG) appear as membrane-bounded ghosts attached to the macrophage, which itself appears as a membrane-bounded structure with a few actin filaments sparsely distributed in the cytoplasm. In contrast, in interferon-treated cells (Fig. 10*b*) a dense filamentous network was found in the vicinity of the plasma membrane 30 s after the temperature shift, and distinct phagocytic cups surrounding each E(IgG) had already begun to be formed. Each phagocytic cup was composed of the plasma membrane and an associated matrix of actin filaments (Fig. 11).

Effects of Cytochalasin D

Cytochalasin D, a drug that inhibits the formation of polymerized F-actin, was used to examine the contractile function of microfilaments in regulating the formation of the phagocytic cup. In thioglycolate-elicited control macrophages, exposure for 45 min to cytochalasin D at a concentration of 1 μg/ml was sufficient to inhibit phagocytosis completely, but in interferon-treated (5,000 U/ml, 72 h) cultures, 31% of the cells were still able to ingest some E(IgG) despite the application of cytochalasin D. The ingestion index was 210, or 6.7% of that in interferon-treated cultures to which cytochalasin D had not been added. Increasing the concentration of cytochalasin D to 2 μg/ml completely abolished phagocytosis by interferon-treated thioglycolate-elicited macrophages. In cultures of resident macrophages, phagocytosis of E(IgG) was essentially completely inhibited by the application of cytochalasin D, 1 μg/ml, to either control or interferon-treated control.

Comparison of Interferon Preparations

The pinocytosis-inhibiting activity of three mouse β-interferon preparations (Lee Biomolecular batches 81008 and 82014, and homogeneous interferon from Dr. P. Lengyel) was compared relative to the antiviral activity of the three preparations. Thioglycolate-elicited macrophages in monolayer cultures were treated for 24 h with interferon at various concentrations, after which either the pinocytic activity of the cells was evaluated by uptake of FITC-dextran (5 mg/ml; 45 min) or the phagocytic activity was assayed by ingestion of E(IgG) (1 h at 4°C, then 4 min at 37°C). The numbers of FITC-dextran–positive cells or the numbers of E(IgG) ingested per cell in treated cultures were related to the value in controls and the percent values plotted against $\log_{10}$ interferon concentrations. The relationships between dose and the effects appeared to be exponential. When the preparations were compared in terms of the concentrations in antiviral units per milliliter at which a 40% reduction in the number of FITC-dextran–positive cells occurred, the pinocytosis-inhibiting ac-

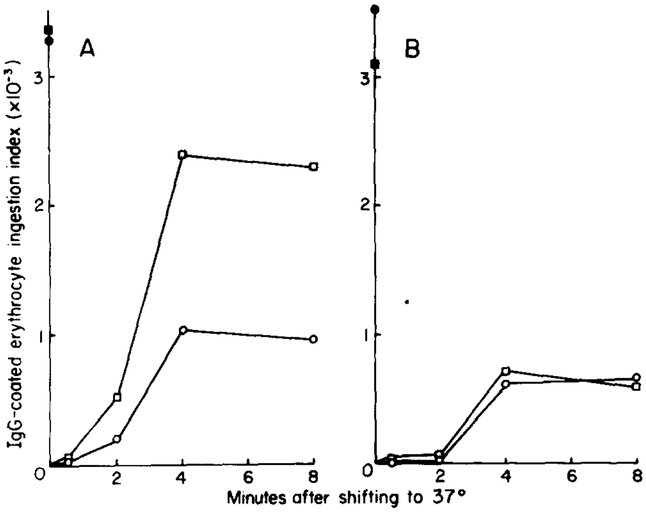

FIGURE 8 Effects of interferon treatment on the rate and extent of ingestion of E(IgG) in a single round of phagocytosis by thioglycolate-elicited (*A*) and resident (*B*) mouse peritoneal macrophages. Macrophage cultures were treated with interferon, 5,000 U/ml, for 72 h at 37°C and then shifted to 4°C for 1 h. IgG-coated sheep erythrocytes were added to the cultures and, after 1 h at 4°C, the cultures were shifted to 37°C. At various times after the temperature shift, the cultures were fixed with 1% glutaraldehyde in PBS-def. Phagocytic activity is represented by the number of erythrocytes ingested per 100 macrophages: (○) Control; (□) interferon-treated. Number of adherent erythrocytes per 100 macrophages at the time of temperature shift: (●) control; (■) interferon-treated.

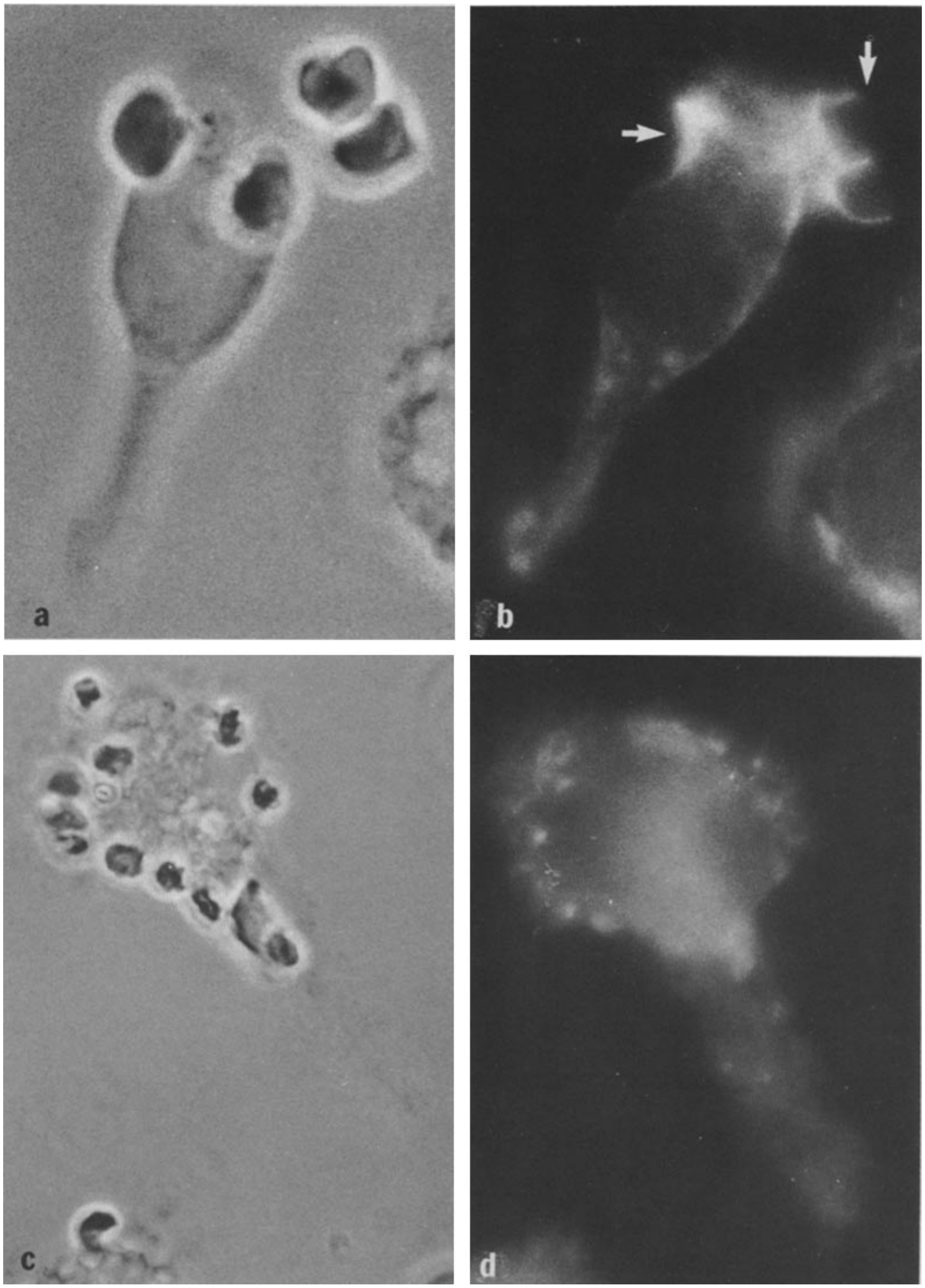

FIGURE 9 Phase-contrast (a) and fluorescence (*b*) microscopic views of cells with actin-containing phagocytic cups. The thioglycolate-elicited macrophages were treated with interferon, 5,000 U/ml, for 72 h. IgG-coated sheep erythrocytes were then added to the cultures at 4°C. After incubation for 1 h, the cultures were shifted to 37°C. At 30 s after the temperature shift, the cultures were fixed and processed for actin staining with NBD-phallacidin. Note the bright fluorescent cup found beneath the erythrocytes undergoing ingestion (arrows). Phase-contrast (c) and fluorescence (*d*) microscopic views of control thioglycolate-elicited macrophages showing a diffuse pattern of actin staining. Note the lack of distinct phagocytic cups and of an increase in the amount of actin in the regions of erythrocytes. (*a* and *b*) × 2,600; (*c* and *d*) × 1,300.

tivities of the three preparations, 81008/82014/homogeneous interferon, normalized with respect to the activity of 81008, were 1:2:10. These results are based on one comparison of 8100 and 82014, and one of 82014 and homogeneous interferon. It can be estimated that the marked pinocytosis-inhibiting effect of batch 81008 at 10,000 U/ml would be obtained with the preparation of homogeneous interferon at $\sim$1,000 U/ml. In terms of the concentration required to enhance phagocytic activity twofold, the results were as follows: 81008: 900 U/ml (two experiments); 82014: 60 U/ml (three experiments); homogeneous interferon: 120 U/ml (one experiment). These estimates indicate that relative to antiviral activity,

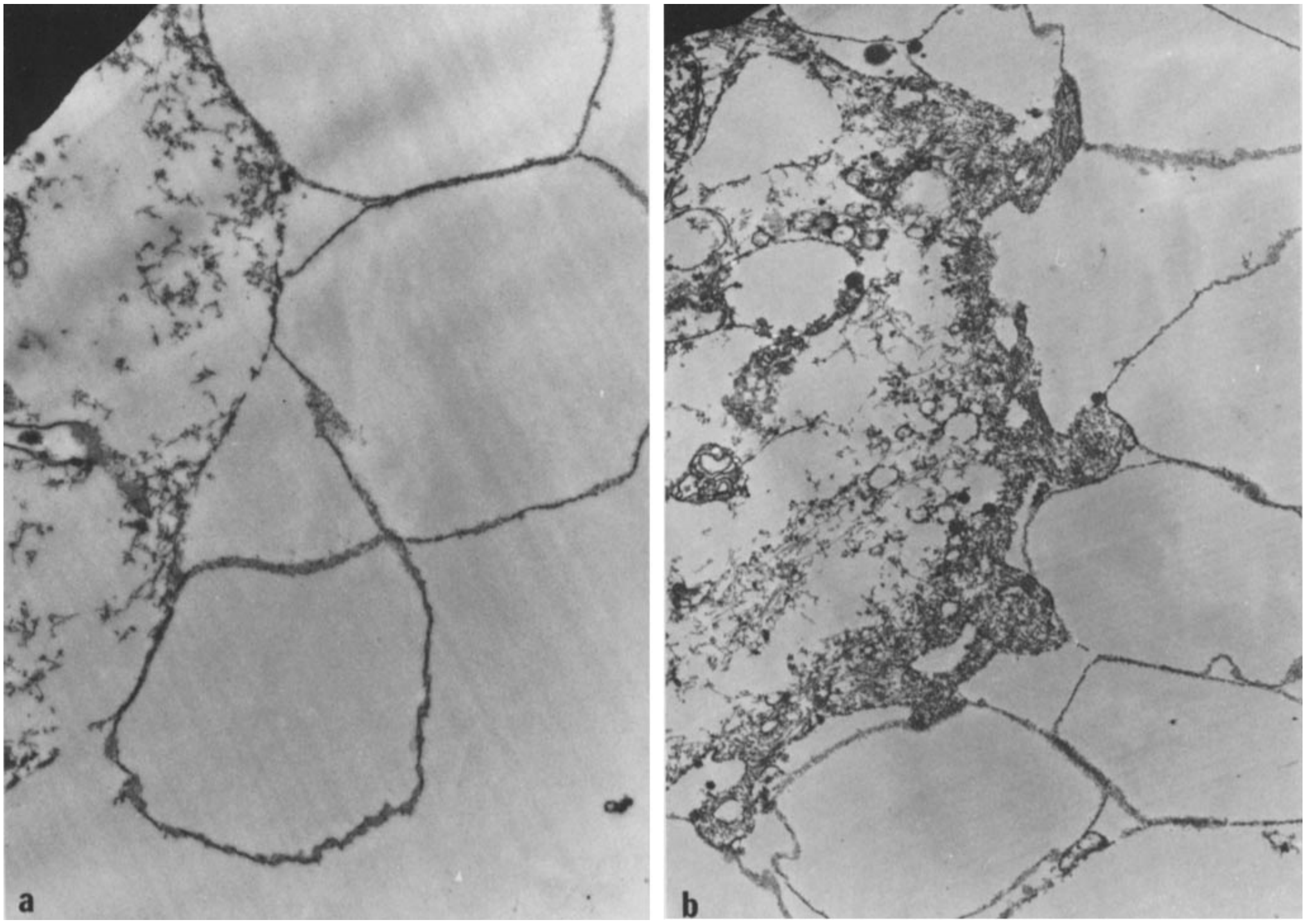

FIGURE 10 Accelerated phagocytic cup formation in interferon-treated thioglycolate-elicited macrophages. Control (*a*) and interferon-treated (5,000 U/ml, 72 h) (*b*) cultures were incubated with E(IgG) for 1 h at 4°C. At approximately 30 s after shifting to 37°C, the cultures were glycerinated and incubated with HMM before processing for thin-section electron microscopy as described in Materials and Methods. The erythrocytes are represented by the membrane-bounded ghosts. Nascent cups in the interferon-treated cells (*b*) can be recognized by the contour of the cortical membrane region containing an abundance of HMM-decorated actin filaments. Control cells (*a*) lack nascent phagocytic cups. (a) × 40,000; (b) × 30,240.

batch 81008 was considerably less active than batch 82014 or homogeneous interferon in enhancing the phagocytic activity of macrophages.

The basis for the differences among interferon preparations in their cell-modulating activities relative to antiviral activity has not been determined. It is possible that the ratios for antiviral to cell-modulating activities of β-interferon subtypes vary, and that different preparations or batches of β-interferon may differ in their subtype compositions.

DISCUSSION

We have demonstrated that β-interferon treatment of thioglycolate-elicited mouse peritoneal macrophages in culture inhibits pinocytosis in a dose-dependent manner, although it stimulates phagocytosis in such cells. The processes of pinocytosis and phagocytosis are affected in macrophages within 12 h after the beginning of interferon treatment. Interferon-treated populations of thioglycolate-elicited macrophages are heterogeneous with respect to pinocytic activity in that at a high interferon concentration, pinocytosis is suppressed >90% in most of the cells and is inhibited only slightly, if at all, in the rest of the cells. Interferon causes disorganization of the network of microtubules and 10-nm filaments in those thioglycolate-elicited macrophages in which it inhibits pinocytic activity. Interferon has no effect on the organization of microtubules and 10-nm filaments or on pinocytic rate in the remaining cells. We have not determined whether those thioglycolate-elicited macrophages that are unresponsive to the inhibitory action of interferon on pinocytosis also do not exhibit enhanced phagocytosis due to interferon.

The biological difference between resident and thioglycolate-elicited macrophages is illustrated by the lack of effect of interferon on the pinocytic activity of the resident macrophages, which are also less responsive to the phagocytosis-enhancing activity of interferon. The basis for these differences between resident and thioglycolate-stimulated macrophages is not clear at present. Interferon-induced suppression of pinocytosis and enhancement of phagocytosis has also been observed with macrophages stimulated with protease peptone and other agents (unpublished observations; 8, 14). The heterogeneity in the response of macrophages to interferon action resembles that which has been observed in many other cell systems (e.g., see references 28–30, 55).

Effects on macrophages have been demonstrated with a variety of interferon preparations. Although there are quantitative differences among interferon preparations with respect to the concentrations (antiviral units per milliliter) required

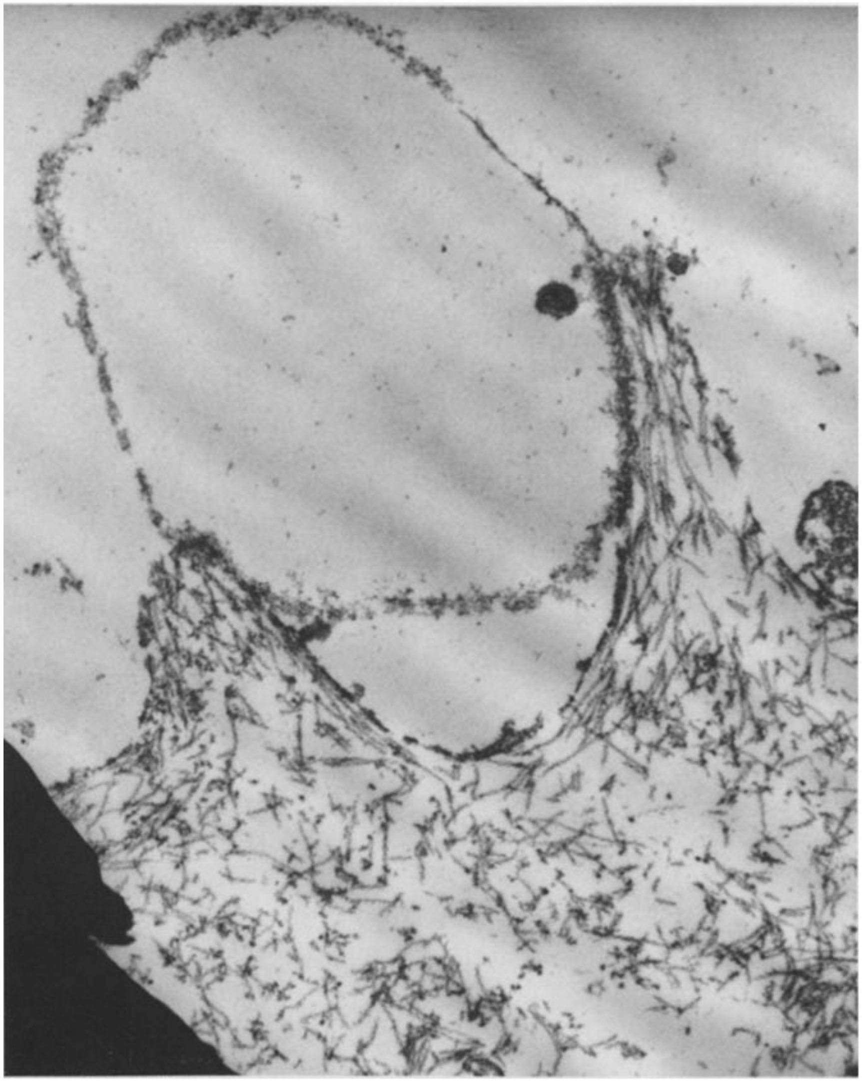

FIGURE 11 The actin-containing microfilament meshwork in the forming phagocytic cup of an interferon-treated macrophage. The sample was prepared as described for Fig. 10*b*. × 86,000.

to inhibit pinocytosis or enhance phagocytosis, there is strong evidence that it is the interferon in the preparations that is causing the effects; i.e., homogeneous mouse β-interferon affects these processes in a similar manner, and the effects of the partially purified preparations can be neutralized by anti-β-interferon antibody (data not shown).

The reorganization of cytoskeletal structures caused by interferon treatment of macrophages differs sharply from the reorganization resulting from colchicine treatment. Colchicine depolymerizes microtubules, whereas interferon treatment results in the displacement of microtubules from the peripheral cytoplasm and causes microtubules to coil around the nucleus, without evidence of depolymerization. Similarly, 10-nm filaments form a pattern of loosely coiled bundles in the perinuclear region of interferon-treated cells, whereas colchicine induces the formation of a perinuclear cap composed of tightly entangled 10-nm filaments. Interferon treatment causes extensive changes in the plasma membrane and its associated structures in a variety of cells (39, 42). In the HeLa line of human carcinoma cells these alterations include increased rigidity of the lipid bilayer (27) and an increase in the submembranous meshwork of microfilaments (55). If interferon perturbs the plasma membrane in macrophages, this could lead to the dissociation of microtubules and 10-nm filaments from the plasma membrane region, and their retention and aggregation in the centrosomal region. Microtubules and 10-nm filaments form tracks guiding the movement and transport of particles and organelles in the cytoplasm (50–52). When this system is highly organized, as in phorbol myristate acetate-treated macrophages, phagocytic activity takes place at an accelerated rate. However, the importance of the radial network for microtubules and 10-nm filaments in maintaining a basal level of pinocytosis is unclear.

We have found that β-interferon treatment of thioglycolate-elicited mouse peritoneal macrophages results in the ingestion of attached E(IgG) at an increased speed and to a higher level in a single round of phagocytosis. These effects are mediated through enhanced assembly of microfilaments into structures that surround phagocytic cups and appear to provide the motile force for ingestion of particles.

To date, three types of Fc-receptors, FcRI, FcRII, and FcRIII, have been identified on the surface of mouse macrophages (7, 33). The trypsin-sensitive FcRI and FcRIII bind IgG2a or IgG3, respectively. The trypsin-insensitive FcRII bind only aggregated mouse IgG and IgG2b as well as aggregated rabbit IgG. Our results as well as those of Hamburg et al. (14) show that the number of FcRII receptors, as estimated by binding of the iodinated monoclonal antibody 2.4G2, does not increase as a result of the interferon treatment. However, Vogel et al. (48) have reported that interferon treatment increases the abundance of FcRI assayed by binding of ^{51}Cr-labeled IgG2a. We have obtained evidence that interferon treatment also enhances the ingestion of erythrocytes opsonized with monoclonal IgG2a and IgG3 (data not shown). Two conclusions can be drawn from all of these findings: (*1*) Interferon-induced enhancement of phagocytic activity is a general phenomenon mediated by all three types of Fc receptors; and (*2*) some but not all types of receptors increase in abundance in response to interferon treatment. Nevertheless, in phagocytosis mediated via receptors belonging to any of the three types, actin-containing microfilaments, as judged by NBD-phallacidin staining, are markedly enriched in the cytoplasm immediately surrounding the phagocytic cups. Therefore, the accelerated reorganization of actin filaments during ingestion of E(IgG) by interferon-treated macrophages may explain the enhanced phagocytic activity via all three types of receptors, and may extend to the ingestion of other particles, e.g., latex beads whose uptake is also stimulated in interferon-treated macrophages (data not shown).

In cell systems so far examined, interferon has been found to alter the organization of microfilaments and affect cell functions dependent on microfilaments. In the macrophage system, there is a remarkable dichotomy in the actions of interferon; pinocytosis is suppressed, while phagocytosis is stimulated. At the structural level, the enhancement in actin filaments surrounding the forming phagosomes in interferon-treated macrophages is consistent with the observations of increased organization of microfilaments into fibers in fibroblasts (30) and into a thickened submembranous meshwork in HeLa cells growing in suspension (55). However, at the functional level, the enhancement of phagocytosis by interferon in macrophages stands in striking contrast to the inhibitory action of interferon on locomotion of fibroblasts (30) and capping of concanavalin A receptors in HeLa cells (32). It seems possible that interferon may inhibit pinocytosis in macrophages by altering the organization of submembranous microfilaments in a manner inconsistent with the performance of their role in pinocytic vesicle formation and internalization, although serving to enhance functional competence in phagocytosis. It is also possible that the aggregated state of the microtubules and 10-nm filaments in the interferon-treated macrophages contributes to the failure of the pinocytic process. Finally, the inhibition of pinocytosis in interferon-treated macrophages may be mediated through an increase in rigidity of the plasma membrane lipid bilayer. This suggestion is based on the following observations: (*a*) increasing the ratio of the saturated to unsaturated fatty acids in the phospholipid bilayer of macrophages is associated with an increase in the rigidity of the plasma membrane and decreased fluid phase endocytic activity (20, 21); (*b*) interferon treatment of HeLa cells and fibroblasts increases the rigidity of the plasma membrane lipid bilayer (27); and (*c*) interferon treatment of mouse sarcoma S-180 cells causes a reduction in unsaturated fatty acids in membrane lipids, which would be expected to result in a decrease in membrane fluidity (4). Whether interferon treatment increases the rigidity of the plasma membrane lipid bilayer in mouse macrophages remains to be determined.

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