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# Poly(I):poly(C)-Enhanced Alveolar and Peritoneal Macrophage Phagocytosis: Quantification by a New Method Utilizing Fluorescent Beads[1,2] (42501)

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*Abstract.* Phagocytosis is an important immune function to quantify. This immune response may be modulated by exposure to biological response modifiers or by exposure to pollutants. A new technique for quantifying nonspecific phagocytosis of alveolar and peritoneal macrophages in the same animal has been developed that utilizes fluorescent polystyrene beads. When incorporated into inhalation studies, this technique can be used to determine whether the toxic effect of an inhaled pollutant is local (effect on alveolar macrophages), systemic (effect on peritoneal macrophages), or both local and systemic. This method results in a determination of both the level of phagocytosis (the percentage of phagocytic macrophages) and the macrophage specific activity (the number of beads phagocytized per macrophage). This method also allows a determination of adherence by quantifying the number of particles in contact with, but not phagocytized by, the macrophage. Macrophage preparations were incubated with fluorescent beads for 2 hr and cyto-centrifuged onto a glass slide. Fluorescent beads present on the slide or cell-associated but not ingested by phagocytosis were removed by immersing the slide containing the macrophage preparation in methylene chloride for 15–30 sec. Fluorescent beads ingested by phagocytosis were then easily quantified with a fluorescence microscope. This technique was used to assess (1) the baseline levels of phagocytosis for rat alevolar and peritoneal macrophages from the same animal and (2) the kinetics and level of enhanced phagocytosis for alevolar and peritoneal macrophages after injection with the interferon inducer polyinosinate-polycytidylate (poly(I):poly(C)). The kinetics of enhanced alveolar and peritoneal macrophage phagocytosis by poly(I):poly(C) were similar; however, stimulated phagocytic levels of peritoneal macrophages never reached the phagocytic activity observed for the resident, highly phagocytic alveolar macrophages. This elevated phagocytic activity is most likely due to interferon stimulated by particulate matter in the large volume of air processed by the lungs and is important for host defense against a number of different inhaled microorganisms. 

To determine the level of nonspecific phagocytosis for macrophages, many studies utilize clear latex beads. Using this technique, it is extremely difficult to determine whether a latex bead is cell-associated or phagocytized. Gardner *et al.* (1) reported an improved technique that removed cell-associated clear latex beads by a 3-hr incubation with xylene. The technique described in this study utilized fluorescent polystyrene beads to assess the percentage phagocytosis as well as the degree of phagocytic activity per cell. Uningested beads were removed by virtue of their solubility in methylene chloride, thus facilitating the determination of the level of phagocytosis (percentage of phagocytic macrophages) and the macrophage specific activity (number of beads phagocytized per macrophage). This technique was used to assess (1) the baseline levels of percentage phagocytosis for rat alveolar and peritoneal macrophages and (2) the kinetics and level of phagocytosis for alveolar and peritoneal macrophages following injection with the interferon inducer poly(I):poly(C). Field *et al.* (2) first reported that natural and synthetic double-stranded RNAs were good interferon inducers in mice and that the best

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[2] The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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inducer was poly(I):poly(C). Interferon has been reported to stimulate phagocytosis levels (3, 4, 5). While Davis *et al.* (6) reported that poly(I):poly(C) also induced interferon in rats, there have been no previous studies on the effect of interferon or interferon inducers on rat macrophages. This is also the first study of the kinetics of enhanced alveolar and peritoneal macrophage phagocytosis after administration of an interferon inducer.

**Materials and Methods.** Male Fischer 344 rats (CDF), 49–60 days old and 175–195 g, that were used in all studies were purchased from Charles River Laboratory. Animals were purchased VAF (virus antibody-free). After receipt, animals were kept in designated rooms as part of a sentinel rat program. They were screened for numerous pathogens, and serum samples were tested for viral antibody to reovirus type 3, pneumonia virus of mice, encephalomyelitis virus, Sendai virus, mouse adenovirus, mouse hepatitis virus, Toolan H-1 virus, Kilham rat virus, rat coronavirus, sialodacryoadenitis virus, and lymphocytic choriomeningitis virus. Results of all these tests were negative.

Alveolar macrophages were obtained by bronchoalveolar lavage. Animals were anesthetized with sodium pentobarbital and bled by cardiac puncture, and serum was collected for assay of interferon. Rats were euthanized by exsanguination. A 45° incision was made in the surgically exposed trachea. A 16-gauge blunt needle catheter was inserted into the trachea and secured by suture with OO-black braided silk (Ethicon). The lungs of each rat were lavaged six times with a 5-ml syringe, 5 ml per wash, with Hanks' balanced salt solution without calcium and magnesium (HBSS), prewarmed to 37°C. The first lavage wash fluid was allowed to remain in the lungs for 5 min before slow aspiration. The lavage procedure was repeated an additional five times in rapid succession. The six lavage washes from each animal were pooled and centrifuged at 300*g* for 10 min. A total cell and viability count was performed by trypan blue exclusion. The number of macrophages was determined by centrifuging with a Shandon Cytospin (Shandon Southern Instruments, Inc.), followed by fixation and stain with the Diff-Quik hematological stain (Harleco) for enumeration of cell types. Typical cell yields from bronchoalveolar lavage were $1.9 \times 10^6$ total cells with greater than 90% viability by trypan blue exclusion. Mean values for cell types recovered were as follows: 96% macrophages (by differential and by nonspecific esterase stain), 1.9% lymphocytes, and 1.5% neutrophils. Peritoneal macrophages were obtained from the same animal by injecting 10 ml of HBSS into the abdominal cavity, moving the animal from side to side for 1 min, and then making a 4-cm sagittal slit in the abdominal wall to recover the wash fluid with a 10-$cm^3$ syringe (without a needle). Peritoneal macrophages were enumerated as described above for alveolar macrophages. Cell suspensions were adjusted to contain $2.0 \times 10^5$ macrophages/ml in Dulbecco's modified Eagle medium (GIBCO) containing 10% fetal bovine serum (GIBCO), penicillin (100 units/ml), and streptomycin (100 $\mu$g/ml) (D-MEM-FBS-10).

Fluorescent polystyrene beads (1.05 $\mu$m in diameter) obtained from Duke Scientific (Palo Alto, CA) were used throughout this study. Covaspheres, fluorescent polystyrene spheres from Covalent Technology Corp. (Ann Arbor, MI), could also be used for the phagocytosis assay described in this study. One bottle of fluorescent beads ($1 \times 10^8$ beads/5 ml) and 10 ml of D-MEM-FBS-10 were centrifuged at 16,000*g* for 10 min in a sterile 15-ml Corex centrifuge tube. The pellet of fluorescent beads was resuspended in 1 ml of D-MEM-FBS-10 ($1 \times 10^7$ beads/100 $\mu$l). One milliliter of cells ($2.0 \times 10^5$ cells/ml) was then incubated with 100 $\mu$l of beads (ratio of fluorescent beads to cells, 50:1) in siliconized (Sigmacote, Sigma) 16 × 125-mm screw cap tubes placed on a rotating rack in a 37°C incubator for 2 hr. Samples were diluted and centrifuged at 300*g* for 5 min. The pellet was resuspended in 1 ml of D-MEM-FBS-10 and centrifuged in Shandon Cytospin chambers ($1.0 \times 10^4$ cells/chamber) onto a glass slide. The slides were fixed and stained with the Diff-Quik hematological stain, dried, and then immersed for 15–30 sec in a Coplin jar containing 100% methylene chloride to remove beads not phagocytized by macrophages. Gloves, mask, safety glasses, and laboratory coat were worn while working with methylene chloride in a chemical safety hood. Slides were read at a 1000× magnification with a Nikon Optiphot fluorescence microscope with halogen illu-

mination to determine the cell type and with mercury illumination to view the fluorescent beads. An 80A blue filter was used for halogen illumination and a 546-nm green interference filter was used for mercury illumination. Macrophages (200) were examined, and the number of beads per macrophage was counted and recorded. Those macrophages containing one or more fluorescent beads were scored positive for phagocytosis.

Polyinosinate-polycytidylate (poly(I):poly(C)), a synthetic polyribonucleotide, was obtained as a gift from Dr. John J. Gavin, Miles Research Laboratories, Inc., Research Products Division (Elkhart, IN). Poly(I):poly(C) was injected intraperitoneally at a dose of 400 $\mu$g per rat. Rabbit antiserum to rat interferon-$\alpha/\beta$ was purchased from Lee Biomolecular Research, Inc. (San Diego, CA). Rabbit antiserum to rat interferon-$\alpha/\beta$ (500 neutralizing units per rat), or normal rabbit serum for control animals, was administered iv 8 hr prior to alveolar macrophage phagocytosis assay.

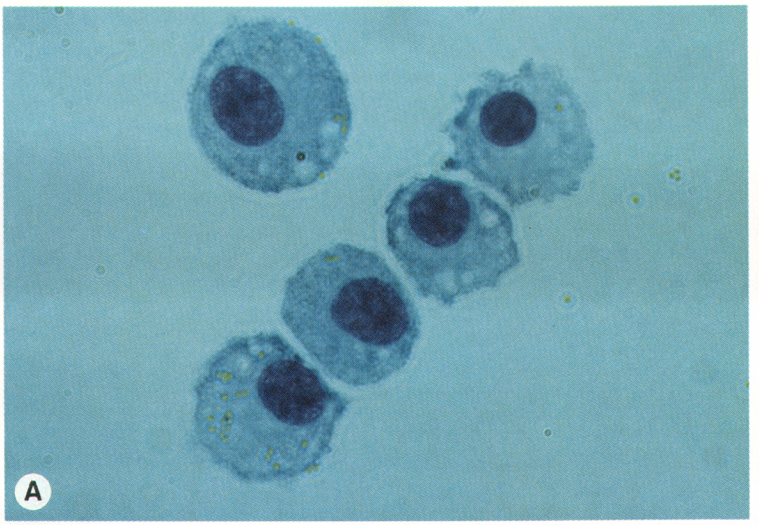
Interferon was quantified by a cytopathic effect (CPE) reduction bioassay that was modified by Wiedbrauk and Burleson (7) from the technique of Burleson and Herzog (8). Briefly, rat LMS-C2 cells were plated in 96-well plates and incubated for 24 hr. Samples to be assayed for interferon activity were diluted in medium, and twofold dilution series were added to confluent cell monolayers and incubated for 24 hr. The LMS-C2 cells were then challenged with vesicular stomatitis virus and incubated for 16 hr. The cell monolayers were stained with 1% crystal violet in 70% methanol for 30 min at room temperature. The interferon titer was defined as the inverse of the dilution resulting in 50% protection of the cell monolayer as compared with virus and cell controls. Since there was no international rat interferon standard, titers were reported in laboratory units. A laboratory interferon standard was used in every bioassay.

Statistical analysis for the phagocytosis and interferon experiments was performed utilizing one-way univariate (ANOVA) and multivariate (MANOVA) analyses of variance. The data were first tested for homogeneity of variance and normality. Transformations were used as needed. Duncan's multiple range procedure was used to test for significant differences among the time points when a significant ANOVA effect was found ($P < 0.05$). Each data point for phagocytosis or interferon titer resulted from individual animals of two replicate experiments of five rats each (one group had a total of nine rats). The data for interferon levels, peritoneal macrophage phagocytosis, and alveolar macrophage phagocytosis were all obtained from the same animals. Each data point for the effect of interferon antiserum on poly(I):poly(C)-stimulated phagocytosis resulted from individual animals of two replicate experiments of four rats each. Data from the antiserum to rat interferon experiment were analyzed using two-way ANOVA. Significant effects were subtested using $t$ tests with a Bonferroni correction for multiple comparisons.

**Results.** Photomicrographs (Fig. 1) showed the results of the phagocytosis assay. Figure 1A shows macrophages using halogen illumination to determine the cell type. Figure 1B shows macrophages before methylene chloride treatment to remove beads not ingested by phagocytosis, and Fig. 1C shows the same macrophages containing only phagocytized fluorescent beads after treatment with methylene chloride. Note cell A (Figs. 1B and 1C) is a macrophage that would have been incorrectly scored positive without methylene chloride treatment, and the number of beads in cell B would also have been incorrectly scored.

Fischer 344 rats were injected ip with the interferon inducer poly(I):poly(C) at a dose of 400 $\mu$g/rat. Individual rats were bled by cardiac puncture and serum was collected for assay of interferon. Interferon was present in the sera at 2 hr, and a peak interferon response was

FIG. 1. Photomicrograph of a cytospin preparation of alveolar macrophages after incubation with fluorescent polystyrene beads. (A) Photomicrograph of Diff-Quick stained alveolar macrophages using halogen illumination. (B) Photomicrograph using both halogen and mercury illumination of alveolar macrophages before methylene chloride treatment. (C) Photomicrograph of alveolar macrophages after methylene chloride treatment. Magnification ×1170.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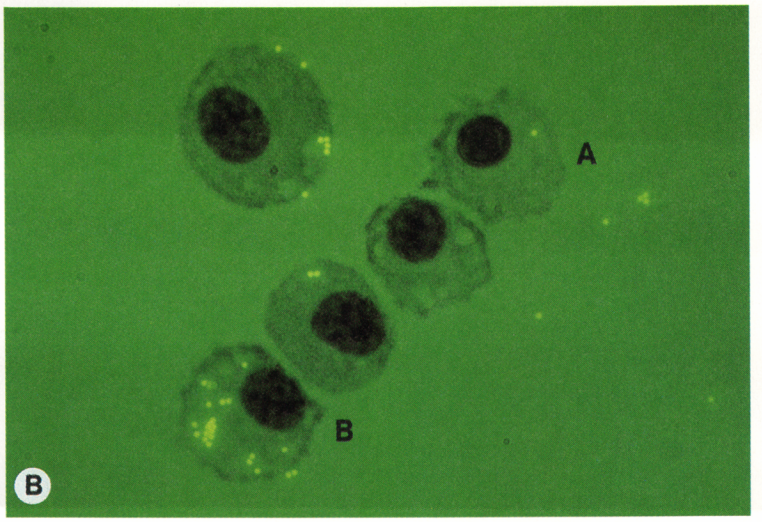
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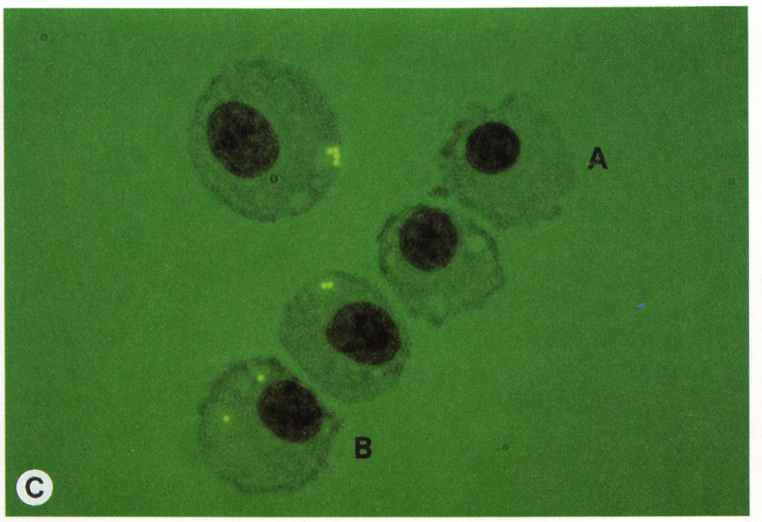
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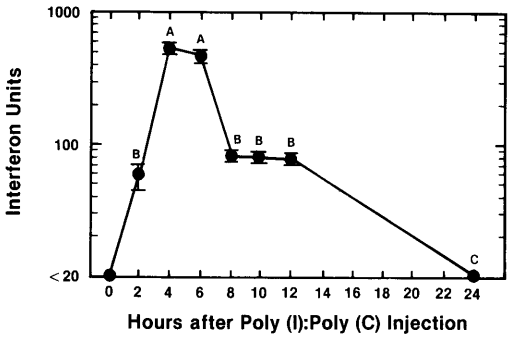


FIG. 2. Kinetics of interferon production following ip injection with poly(I):poly(C). Mean values ± SE are indicated. Subtesting was performed when a significant ANOVA effect ($P < 0.05$) was detected. Duncan's multiple range procedure was used. Any points identified by the same letter are not significantly different from one another.

obtained 4–6 hr after induction with poly(I):poly(C) (Fig. 2). Circulating interferon was not detected in the sera 24 hr after poly(I):poly(C) injection.

The kinetics of enhanced percentages of phagocytic alveolar macrophages was determined following injection with poly(I):poly(C) (Fig. 3A). Fifty-one percent of the resident alveolar macrophages from nonstimulated animals were phagocytic. There was a statistically significant increase in percentage phagocytosis at 2 hr (58.5% positive) and an increase to 73.2% at 4 hr after poly(I):poly(C) administration. At all other times tested, there was an increase ($P < 0.05$) in the percentage of phagocytic cells after poly(I):poly(C) injection when compared to nonstimulated resident alveolar macrophages. A biphasic response with peaks at 4 and 10 hr was also observed. The responses at 6 and 8 hr were significantly less ($P < 0.05$) than the response at 4 or 10 hr. The number of beads ingested per macrophage was also determined (Fig. 3B). Nonstimulated resident alveolar macrophages phagocytized an average of 2.3 beads. The mean number of beads phagocytized increased to 3.3 at 4 hr after administration of poly(I):poly(C). The mean number of beads per cell was increased ($P < 0.05$) at 4, 6, 8, 10, 12, and 24 hr after poly(I):poly(C) when compared to the mean number of beads for nonstimulated resident alveolar macrophages. Again, a biphasic response with peaks at 4 and 10 hr was observed. The responses at 6 and 8 hr were less ($P < 0.05$) than the response at 10 hr. Although the response at 4 hr was qualitatively higher, it was not significantly different from the 6- and 8-hr responses.

The kinetics of peritoneal macrophage phagocytosis was also determined after injection of poly(I):poly(C) (Fig. 4A). The percentage of phagocytic macrophages increased from 11.6% for nonstimulated resident peritoneal macrophages to 14.5% at 2 hr and to 30.1% 4 hr after injection of poly(I):poly(C). The percentage of phagocytic macrophages was increased ($P < 0.05$) at 4, 6, 8, 10, 12, and 24 hr after poly(I):poly(C) when compared to the nonstimulated resident peritoneal macrophages. Resident peritoneal macrophages phagocytized a mean number of 1.4 beads. This number increased to 1.6–1.9 beads/macrophage 4–10 hr after poly(I):poly(C) injection. The mean number of beads per cell was increased ($P < 0.05$) at 4, 6, 8, 10, and 24 hr

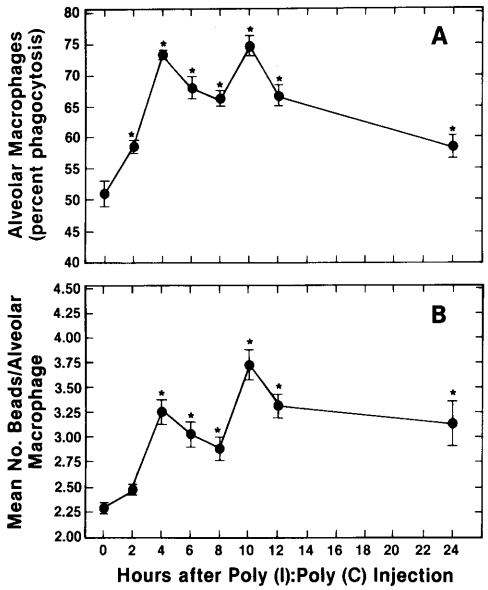


FIG. 3. Kinetics of phagocytosis for alveolar macrophages following ip injection with poly(I):poly(C). (A) Level of phagocytosis: percentage of phagocytic macrophages. (B) Degree of macrophage phagocytic activity: the number of fluorescent beads phagocytized per macrophage. Mean values ± SE are indicated. Subtesting was performed when a significant ANOVA effect ($P < 0.05$) was detected. Duncan's multiple range procedure was used. Results are reported for all points different from nonstimulated resident macrophage controls. Significance ($P < 0.05$) is indicated by an asterisk(*).

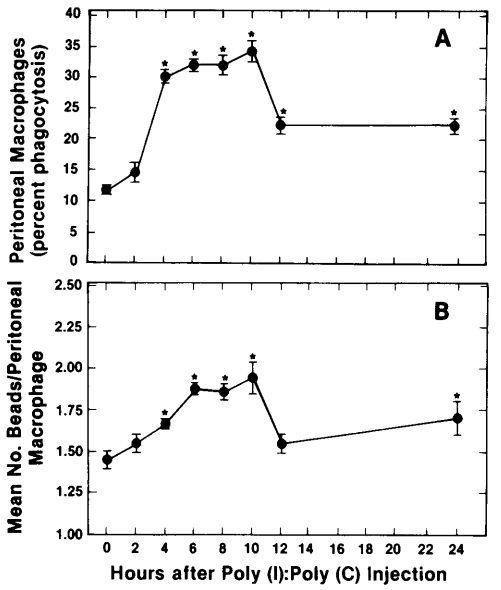


FIG. 4. Kinetics of phagocytosis for peritoneal macrophages following ip injection with poly(I):poly(C). (A) Level of phagocytosis: percentage of phagocytic macrophages. (B) Degree of macrophage phagocytic activity: the number of fluorescent beads phagocytized per macrophage. Mean values ± SE are indicated. Subtesting was performed when a significant ANOVA effect ($P < 0.05$) was detected. Duncan's multiple range procedure was used. Results are reported for all points different from nonstimulated resident macrophage controls. Significance ($P < 0.05$) is indicated by an asterisk(*).

after poly(I):poly(C) administration when compared to the mean number of beads for nonstimulated resident peritoneal macrophages (Fig. 4B).

Alveolar and peritoneal macrophages exhibited similar kinetics upon stimulation by poly(I):poly(C) (Figs. 3 and 4), although the phagocytosis level for nonstimulated resident alveolar macrophages (Fig. 3A) was higher ($P < 0.05$) than for peritoneal macrophages (Fig. 4A) at any time after stimulation by poly(I):poly(C). The macrophage specific activity, as determined by the mean number of beads phagocytized per macrophage, was also greater ($P < 0.05$) for nonstimulated resident alveolar macrophages (Fig. 3B) than for poly(I):poly(C)-stimulated peritoneal macrophages (Fig. 4B).

Rabbit antiserum to rat interferon-$\alpha/\beta$ was used to determine whether the poly(I):poly(C)-enhanced level of phagocytosis was due to interferon (Fig. 5). Alveolar macrophages were used for this study. Alveolar macrophages obtained from animals injected with poly(I):poly(C) and normal rabbit serum had a higher ($P < 0.05$) level of phagocytosis than macrophages from animals receiving either phosphate-buffered saline (PBS) and normal rabbit serum, PBS, and antiserum to rat interferon-$\alpha/\beta$ or poly(I):poly(C) and antiserum rat interferon-$\alpha/\beta$ (Fig. 5). Animals injected with PBS and antiserum to rat interferon-$\alpha/\beta$ had a significantly lower ($P < 0.05$) level of phagocytosis when compared to animals injected with PBS and normal rabbit serum. The total cell number and viability, and the percentage of macrophages, lymphocytes, and neutrophils were similar in the bronchoalveolar lavage fluid for all groups.

**Discussion.** In our efforts to assess local pulmonary immunity, we have described a method to quantify nonspecific (non-receptor-mediated) phagocytosis, not confounded by cell-associated beads, for alveolar and peritoneal macrophages obtained from the same an-

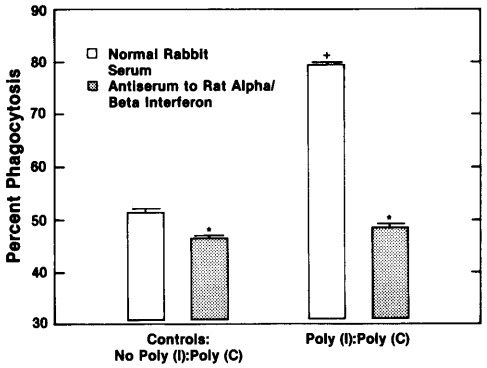


FIG. 5. Effect of antiserum to rat interferon-$\alpha/\beta$ on poly(I):poly(C)-enhanced phagocytosis of alveolar macrophages. Normal rabbit serum or rabbit antiserum to rat $\alpha/\beta$ interferon (500 neutralizing units per rat) was injected iv. Phosphate-buffered saline (PBS) or poly(I):poly(C) was injected ip. All injections were made 8 hr prior to alveolar macrophage phagocytosis assay. Mean values ± SE are indicated. When a significant ANOVA effect was seen ($P < 0.05$), subtesting was performed using $t$ tests with a Bonferroni correction. The (*) indicates a significant difference ($P < 0.05$) between normal rabbit serum and rabbit antiserum to rat interferon-$\alpha/\beta$. The (+) indicates a significant difference between controls (no poly(I):poly(C) and poly(I):poly(C)-injected animals.

imal. This method could be used to indicate whether the toxic effect of inhaled pollutants is local (effect on alveolar macrophages), systemic (effect on peritoneal macrophages), or both local and systemic. This technique also allows an assessment of toxicants on the innate or stimulated macrophage immune function parameters. It will also allow an evaluation of inhaled pollutants on several macrophage functions: (i) the level of macrophage phagocytosis, (ii) the macrophage specific activity for an individual macrophage as measured by the number of fluorescent beads phagocytized per macrophage, (iii) adherence of beads to macrophages, and (iv) the ability of the macrophages to be modulated by the interferon-inducer poly(I):poly(C). Other methods for determining nonimmune phagocytosis have been described elsewhere (1, 9). Clear latex particles have been used to assess phagocytosis; however, it is extremely difficult to determine whether a clear latex particle is actually ingested or merely residing beside the macrophage. Gardner *et al.* (1) resolved this problem with clear latex beads by incubating the slide preparation of macrophages for 3 hr in xylene to remove nonphagocytized beads. However, even then microscopic quantitation was very difficult if the animal had been exposed to a particulate pollutant present in the macrophage preparation. Visualization of particles has been vastly improved by the use of fluorescent polystyrene beads for phagocytosis studies as described in this report. Methylene chloride removal of unphagocytized beads obviates the uncertainty of whether a particle is actually phagocytized or merely residing on or near the cell surface. This method is also useful for phagocytosis studies utilizing adherent macrophages. Another advantage of this technique is that fluorescence is permanent; slides have been viewed after 1 year with no decrease in fluorescence intensity.

These studies revealed that the kinetics of stimulated phagocytosis for alveolar and peritoneal macrophages were similar and that both roughly paralleled the kinetics of interferon production. Antiserum to rat interferon-$\alpha/\beta$ prevented the enhanced phagocytosis for alveolar macrophages obtained from poly(I):poly(C)-injected animals, thereby indicating interferon to be responsible for modulating the enhanced phagocytic levels. Also, control animals injected with antiserum to rat interferon-$\alpha/\beta$ (500 neutralizing units per rat) resulted in a significant decrease in the level of phagocytosis compared to animals receiving normal rabbit serum. Burleson *et al.* have shown that an even greater decrease was observed for baseline alveolar macrophage phagocytosis levels after injection with 5000 neutralizing units of antiserum to rat interferon-$\alpha/\beta$ compared to animals receiving normal rabbit serum (manuscript submitted). Thus, basal levels of endogenous interferon play an important immunomodulatory role in maintaining elevated alveolar macrophage phagocytosis. The stimulation of macrophage specific activity followed the same kinetics as the percentage phagocytosis. Imanishi (10) reported that when human monocytes were stimulated with human interferon *in vitro,* the percentage phagocytosis paralleled the increased number of beads per macrophage. The poly(I):poly(C)-stimulated level of phagocytosis or specific activity of peritoneal macrophages never attained the innate level or specific activity of resident, nonstimulated alveolar macrophage phagocytosis in the present study. This elevated phagocytic activity of resident alveolar macrophages may be due to the constant stimulation by particulate matter, perhaps by interferon induction, in the large volume of air processed by the lungs every day. Basal levels of interferon, not detectable by interferon bioassays, are important for homeostatic control of immune functions. Endogenous interferon has been reported to be responsible for baseline natural killer cell activity (11, 12) and for maintaining the natural resistance of peritoneal macrophages to vesicular stomatitis and encephalomyocarditis viruses (13, 14). Any dysfunction of the resident or stimulated phagocytic levels may result in a host more susceptible to infection by a number of different pathogenic microorganisms.

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