The Role of Macrophages in Particle Translocation from Lungs to Lymph Nodes

Abstract. *Red fluorescent and green fluorescent microspheres were instilled into separate but adjacent areas of dog lung lobes. After 7 days, the tracheobronchial lymph nodes that drained both of the instilled areas contained many macrophages with all red or all green microspheres but rarely both. This indicates that the particles did not translocate passively and that lung macrophages phagocytized the microspheres in the lung and carried them to the tracheobronchial lymph nodes. In addition, two populations of pulmonary alveolar macrophages (PAM's), one that had phagocytized red microspheres in vivo and one that had phagocytized green microspheres, were lavaged from the lungs of dogs, mixed into one population, and instilled back into a previously unexposed lung lobe of the same dogs. As in the first experiment, the tracheobronchial lymph nodes that drained the instilled area contained numerous macrophages with either all red or all green microspheres. This suggested that the instilled PAM's had migrated to the tracheobronchial lymph nodes. Thus, lung macrophages, including PAM's, probably play a critical role in the induction of lung immunity and in protection from disease by determining particle translocation.*

ALLEN G. HARMSEN
BRUCE A. MUGGENBURG
M. BURTON SNIPES
DAVID E. BICE
Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, Albuquerque, New Mexico 87185

Inhalation of a sufficient number of insoluble particles can result in the development of such lung diseases as pneumoconiosis, in the case of mineral dusts, hypersensitivity pneumonitis, for certain organic dusts, and pneumonia, when microorganisms are inhaled. The principal defense mechanisms that prevent such diseases are the clearance of particles by mucociliary processes in conducting airways and the transport of particles from the lung to the regional tracheobronchial lymph nodes (TBLN's) (*1*). Lung macrophages probably play key roles in both clearance processes by engulfing particles and, if the particles are indigestible, retaining them in intracellular vesicles. In the case of mucociliary clearance, many particles are transported up the airways within pulmonary alveolar macrophages (PAM's) and are eventually swallowed (*2*). The role of lung macrophages in clearance of particles to the TBLN's (*3*) is not so clear.

The TBLN is the site of immune cell proliferation after antigen is deposited in the lung (*4*). Also, for induction of an immune response in the lung to occur, antigen must first be translocated to the draining TBLN's (*5*). Therefore, the induction of pulmonary immunity, as well as sensitization to inhaled allergens, may be controlled by this clearance process.

Translocation of cytotoxic particles from the lungs to the TBLN's can alter normal immunological responses in the TBLN's (*6*). Clearance of infectious microorganisms to the TBLN's has also been suggested as a possible means of dissemination of pathogens deposited in the lungs (*7*).

Particles translocated to the TBLN's are generally found within macrophages of the TBLN's. However, it was not known whether the particles are transported from the lungs to the TBLN's within lung macrophages or whether they are transported in a free state and phagocytized within the TBLN's by macrophages (*8*).

We developed a method to determine if particles instilled into the lung reach TBLN's as free particles or associated with lung macrophages. Fluorescent microspheres were instilled into the lungs of anesthetized dogs with a fiber-optic bronchoscope. Red fluorescent microspheres 1.3 μm in diameter were instilled into an airway of the right intermediate lung lobe, and green fluorescent microspheres (also 1.3 μm in diameter) were instilled into an adjacent airway of the same lung lobe. Seven days after instillation two dogs were anesthetized and the

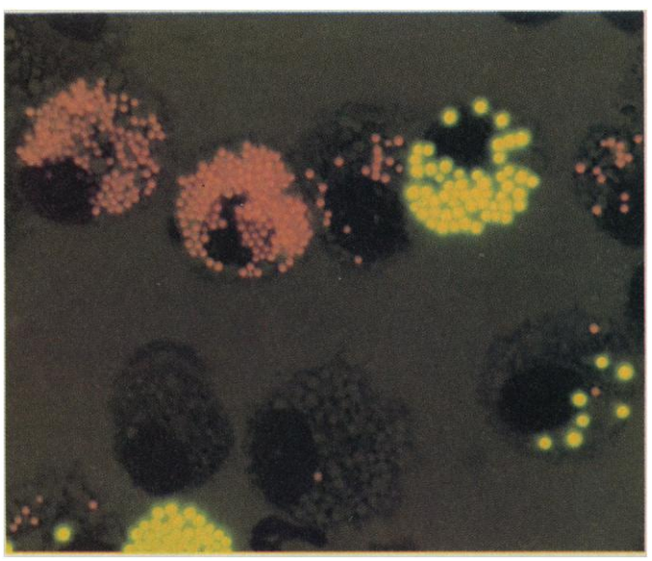
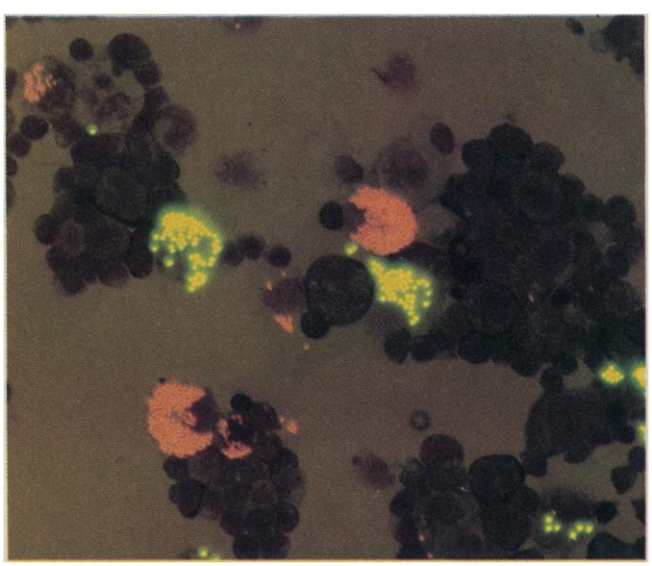


Fig. 1 (top). Cytocentrifuge preparation of cells recovered by pulmonary lavage 7 days after instillation of microspheres. The PAM's containing red or green microspheres can be seen (magnification × 1000). Fig. 2. (bottom). Cytocentrifuge preparation of cell suspension from the TBLN's 7 days after instillation of microspheres. Macrophages containing red or green microspheres can be seen around the lymph node cells (magnification × 400).



instilled areas of their lungs were lavaged (*9*). While anesthetized, the animals were killed by exsanguination. The TBLN that received drainage from the instilled lung lobe (*10*) was removed, and the lavage fluids and TBLN were examined for the presence of macrophages containing microspheres (Figs. 1 and 2). Twenty-two days after instillation, this procedure was repeated with two more dogs.

The particle-laden PAM's recovered in lung lavage fluids contained an average of 20 microspheres at 7 days and 28 microspheres at 22 days. Most of the microspheres were either all red or all green (Fig. 1). Less than 5 percent of the PAM's contained a combination of the two colored microspheres (Fig. 3A). A similar pattern of particle-laden macrophages was observed in suspensions of cells from the TBLN that drained the area of the lung that was instilled (Fig. 2). In the TBLN, the particle-laden macrophages contained an average of 14 microspheres at 7 days and 17 microspheres at 22 days. Eighty percent of these macrophages contained either all red microspheres or all green microspheres (Fig. 3B). This suggested that most of the particles arrived at the TBLN associated with macrophages.

Approximately equal numbers of red and green particles were translocated to the same TBLN (Table 1). If the microspheres arrived at the TBLN as free particles, microspheres of both colors would have been phagocytized by lymph node macrophages, and most of them would have contained a combination of the red and green microspheres. Evaluation of frozen sections of the TBLN confirmed that most microspheres were macrophage-associated and that macrophages containing red or green microspheres were evenly distributed throughout the lymph node.

Table 1. Number of microspheres translocated to TBLN after instillation of 5×10^{10} red and 5×10^{10} green microspheres into lung lobes. Counts were determined from the 1-ml portions of TBLN digest cell suspensions described in Fig. 3. The solutions were diluted in distilled water and filtered through 0.22-μm pore size polycarbonate filters (Nuclepore). The number of colored microspheres on the filter was determined by fluorescence microscopy, and the total number in the TBLN was calculated. Values are the mean ± standard deviation of the results for two dogs at each time.

Color	Number ($\times 10^8$)	Percentage of total instilled
	7 days	
Red	1.0 ± 0.4	0.20 ± 0.08
Green	0.7 ± 0.2	0.14 ± 0.04
	22 days	
Red	6.7 ± 2.0	1.3 ± 0.4
Green	4.9 ± 1.5	1.0 ± 0.3

Different rates of translocation for the colored microspheres could have caused the observed macrophage pattern in the TBLN. However, the number of red and green microspheres in the TBLN was approximately equal at 7 days and again at 22 days after instillation, suggesting that the rates of accumulation of the colored microspheres were equal (Table 1).

As a control, a mixture of red and green microspheres (approximately 2.5×10^{10} each) were instilled into the left cardiac lung lobes of two dogs. After 7 days, PAM's from the left cardiac lung lobes and macrophages from the left TBLN's were analyzed for the distribution of red and green microspheres. Most of the PAM's contained a mixture of red and green fluorescent microspheres (Fig. 4A). The distribution of colored microspheres was nearly the same in the macrophages from the TBLN's (Fig. 4B). Figure 4 shows that for both the PAM's and macrophages from the TBLN's, the distribution was slightly toward the right of 50 percent. Although we instilled about 2.5×10^{10} of each color of micro-

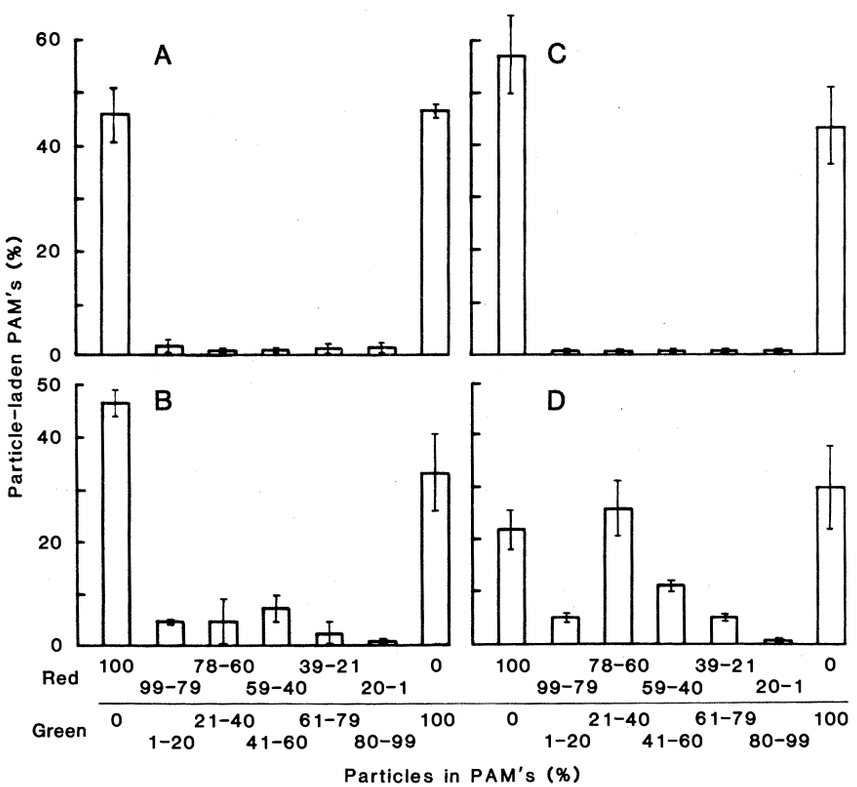


Fig. 3. Percentages of particle-laden macrophages from lung and lymph nodes that contained various ratios of red and green fluorescent microspheres. Four beagles were instilled (*8*) with 5.0×10^{10} red microspheres in one airway of the right intermediate (RI) lung lobe and with 5.0×10^{10} green microspheres in an adjacent airway of the same lung lobe. The procedure was repeated in the left cardiac (LC) lung lobe. After 7 days, two dogs were anesthetized with halothane, their RI and LC lung lobes were lavaged (*9*), and the dogs were exsanguinated. After 22 days, the procedure was repeated with the remaining two dogs. The right middle TBLN, which drains the RI lung lobe, and the left TBLN, which drains the LC lung lobe, were removed (*10*) from each dog. Half of each TBLN was placed in Karnovsky's fixative for frozen sections, and the other half was placed in RPMI 1640 medium (Gibco) containing collagenase (2 mg/ml), protease (72.5 mg/ml), deoxyribonuclease I (Sigma, 125 μg/ml), and fetal bovine serum (Gibco, 5 percent). The TBLN's were minced and incubated with the enzyme solution for 1 hour. A 1-ml portion of each cell suspension was saved for total microsphere counts. The remainder of each cell suspension was washed, and cytocentrifuge preparations were made of it and of each of the lavage fluids. The cell preparations were stained and examined by fluorescent microscopy. The number of red and green particles in 50 to 100 individual macrophages were determined, and the percentage of the particles in each macrophage calculated. (A) PAM's lavaged from the RI lobe 7 days after instillation. (B) Macrophages from the right middle TBLN 7 days after instillation. (C) PAM's lavaged from the RI lobe 22 days after instillation. (D) Macrophages from the right middle TBLN 22 days after instillation. Bars represent the mean ± standard error of results for two dogs. Results for the LC lung lobe and the left TBLN were similar.

sphere, the results suggest that more green microspheres were actually instilled than red microspheres. Regardless, these results indicate that if the red and green microspheres are mixed together in the lung, PAM's and macrophages from the TBLN subsequently contain a mixture of colored microspheres.

The distribution of colored microspheres within individual PAM's in the lavage fluids at 22 days was essentially identical to that observed at 7 days (Fig. 3C). This suggests that, during the 22 days after instillation, the colored microspheres remained in separate areas of the lung lobe. Similar to the results at 7 days, many macrophages from the TBLN contained microspheres of a single color at 22 days (Fig. 3D). However, at 22 days there appeared to be more macrophages from the TBLN with microspheres of both colors than at 7 days [45 ± 5 percent (mean ± standard error) compared with 20 ± 5 percent, respectively]. An increase in mixing of colored microspheres within individual macrophages from the TBLN could have been due to the release of microspheres from these macrophages and the subsequent phagocytosis of both colors by other macrophages in the TBLN. Alternatively, after 7 days, more free particles could have arrived at the TBLN than during the 7-day period.

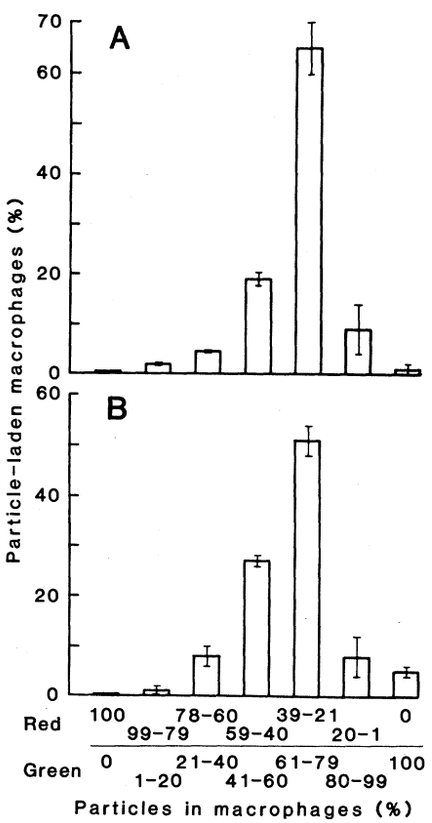


Fig. 4. Percentages of particle-laden macrophages from lungs and from TBLN's that contained various ratios of red and green microspheres. Two beagles were instilled with a mixture of 2.5×10^{10} red and 2.5×10^{10} green microspheres in the left cardiac lung lobe. After 7 days, PAM's collected from the left cardiac lung lobe and macrophages from the left TBLN were analyzed for particle color distribution. (A) PAM's 7 days after instillation. (B) Macrophages from the left TBLN 7 days after instillation. Bars represent the mean ± standard error of the results for two dogs.

These results suggest that most particles translocated from lungs to TBLN's are carried there by either interstitial lung macrophages or PAM. In lung sections taken 7 days after instillation, microspheres were seen within macrophages in both locations. It is possible that the microspheres were phagocytized in the alveoli, and the particle-laden PAM then penetrated the epithelium and migrated through the interstitium into the lymphatics. Alternatively, the particles might have entered the interstitium either independently or within migratory PAM that subsequently released the particles in the interstitium. Interstitial macrophages could then have phagocytized the microspheres, entered the lymphatics, and carried the particles to the TBLN. All these mechanisms were suggested previously (*11*).

To determine whether PAM's that contain particles can migrate from the alveolus of the lung to the TBLN's, red microspheres were instilled into one area of lung and green microspheres into another in six dogs. After 4 or 14 days the PAM's were collected by lavage, pooled, and instilled into a third area of lung. This resulted in the deposition of autologous PAM's, half of which were labeled with red microspheres and half with green, within the same alveoli. After 3 days, the distribution of microspheres in PAM's from the instilled lung lobe and in macrophages from the TBLN was determined.

The distribution of the relative number of red and green microspheres is shown in Fig. 5. As in the first experiment, the number of red and green microspheres reaching the TBLN was similar. For simplicity, particle color homogeneity was expressed as the ratio of either the number of red to green microspheres or green to red microspheres, whichever was greater. Thus, 100 percent homogeneity represents macrophages with either all red or all green microspheres. Approximately 88 percent of the instilled PAM's had 100 percent particle color homogeneity (Fig. 5A). After 3 days in the lung, the proportion of particle-laden PAM's with 100 percent homogeneity was about 52 percent (Fig. 5B). This decrease in particle color homogeneity probably resulted from the release of particles by some PAM's in vivo, and the phagocytosis of the free particles by other PAM's. The proportion of particle-laden PAM's in the TBLN at 3 days with 100 percent particle color homogeneity was about 58 percent (Fig. 5C). The mean number of microspheres in the instilled particle-laden PAM's, the lavaged PAM's, and the macrophages from the TBLN was 26, 29, and 15, respectively.

These results suggest that PAM's can migrate from the lung alveoli to the TBLN and, in the process, carry particles with them. Had the PAM's released the microspheres in the alveolus, lung interstitium, or afferent lymphatics, mix-

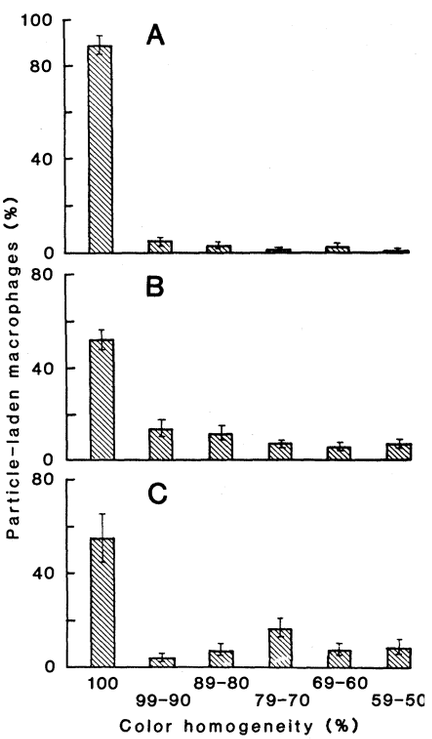


Fig. 5. Percentages of particle-laden macrophages from lungs and from TBLN's that contained various ratios of red and green microspheres. Six beagles were instilled with 5×10^{10} red microspheres in the right cardiac lung lobe and 5×10^{10} green microspheres in the right diaphragmatic lung lobe. After 4 or 14 days the instilled areas were lavaged and PAM's were pooled, pelleted, and resuspended in 2 ml of saline. The autologous PAM's were then immediately reinstilled into the left cardiac lobe. Three days later, the left cardiac lobe was lavaged, and the left TBLN removed. (A) PAM's instilled into the left cardiac lung lobe. (B) PAM's lavaged from lungs 3 days after instillation of above PAM's. (C) Macrophages from the TBLN 3 days after instillation of the particle-labeled PAM's. One hundred percent represents macrophages containing all red or all green microspheres. Bars represent the mean ± standard error of the results for six dogs.

ing of the colored microspheres would have occurred. This would have resulted in few macrophages in the TBLN with 100 percent particle color homogeneity.

The probability that particles are translocated from the lung to the TBLN by macrophages has important implications. The movement of particles by this mechanism may be important in the dissemination of macrophage-resistant microorganisms that gain entry to the host by the respiratory tract. Inhaled toxic particles could be disseminated in a similar manner (*12*).

Immune responses induced to antigens deposited in the lungs may also be dependent on this mechanism of translocation. Potential allergens are constantly inhaled. If these allergens were translocated to the TBLN's, hypersensitivities could occur. Conversely, if an infectious microorganism were deposited in the lung, translocation of the antigens of the microorganism to the TBLN's would be necessary for the induction of an immune response against the microorganism. It has been shown that Langerhans' cells participate in immune responses by carrying antigen from the skin to the draining lymph nodes (*13*).

This study and that of Corry *et al.* (*7*) indicate that PAM's can migrate from the alveolus to the TBLN's. Our results also indicate that PAM's can transport particles to the TBLN's and therefore could determine the induction of lung immune responses by regulating antigen translocation to the TBLN's.

References and Notes

1. G. M. Green, *Arch. Intern. Med.* **131**, 109 (1973).
2. J. D. Brain, *ibid.* **126**, 477 (1970).
3. P. E. Morrow and C. L. Yuille, *Fundam. Appl. Toxicol.* **2**, 300 (1982).
4. D. E. Bice, D. L. Harris, B. A. Muggenburg, *Exp. Lung Res.* **1**, 33 (1980).
5. D. E. Bice, M. A. Degen, D. L. Harris, B. A. Muggenburg, *Am. Rev. Respir. Dis.* **126**, 635 (1982).
6. C. T. Schnizlein, D. E. Bice, C. E. Mitchell, F. F. Hahn, *Arch. Environ. Health* **37**, 201 (1982); D. E. Bice, J. L. Mauderly, R. K. Jones, R. O. McClellan, *Fundam. Appl. Toxicol.*, in press.
7. D. Corry, P. Kulkarni, M. F. Lipscomb, *Am. J. Pathol.* **115**, 321 (1984).
8. A. Johansson and P. Camner, *Toxicology* **15**, 157 (1980).
9. A. G. Harmsen, D. E. Bice, B. A. Muggenburg, *J. Leukocyte Biol.* **37**, 483 (1985).
10. M. B. Snipes, B. A. Muggenburg, D. E. Bice, *J. Toxicol. Environ. Health* **11**, 703 (1983).
11. J. D. Brain, S. P. Sorokin, J. J. Godleski, in *Respiratory Defense Mechanisms*, J. D. Brain, Ed. (Dekker, New York, 1977), vol. 2, pp. 849–889.
12. A. R. Brody, M. W. Roe, J. N. Evans, G. S. Davis, *Lab. Invest.* **47**, 533 (1982).
13. I. Silberg-Sinakin, G. J. Thorbecke, R. L. Baer, S. A. Rosenthal, V. Berezowsky, *Cell. Immunol.* **25**, 137 (1976).

14. Supported by Department of Energy contract DE-ACO4-76EV01013. We thank Mike Jarpe for technical assistance and Dr. Robert Jones and the other staff members at the Inhalation Toxicology Research Institute who made helpful suggestions during the preparation of this report.

31 May 1985; accepted 3 October 1985

Myofibrils Bear Most of the Resting Tension in Frog Skeletal Muscle

Abstract. *The tension that develops when relaxed muscles are stretched is the resting (or passive) tension. It has recently been shown that the resting tension of intact skeletal muscle fibers is equivalent to that of mechanically skinned skeletal muscle fibers. Laser diffraction measurements of sarcomere length have now been used to show that the exponential relation between resting tension and sarcomere length for whole frog semitendinosus muscle is similar to that of single fibers. Slack sarcomere lengths and the rates of stress relaxation in these muscles were similar to those in skinned fibers, and sarcomere length remained unchanged during stress relaxation, as in skinned fibers. Thus, in intact semitendinosus muscle of the frog up to a sarcomere length of about 3.8 micrometers, resting tension arises, not in the connective tissue as is commonly thought, but in the elastic resistance of the myofibrils.*

ALAN MAGID
DOUGLAS J. LAW
Department of Anatomy, Duke University Medical Center, Durham, North Carolina 27710

A simple form of muscular force is the "passive" tension that results when resting muscles are stretched. The sum of the passive and active tensions is the total force acting to shorten a muscle. Passive tension is commonly attributed to elastic forces in the connective tissue and not to myofibrillar elasticity (*1–3*). This early view contrasts sharply with the more recent demonstration that the passive tension of single fibers is borne largely by the myofibrils, with a small contribution at extreme length from the collagen fibrils in the sarcolemma (*4–7*). To what extent might this also be true for whole muscles?

To answer this question, we have measured the variation of passive tension with sarcomere length (S) in intact frog semitendinosus muscles. Muscles (*8*) were stretched in increments, and the tension was permitted to relax until it became steady or nearly so, that is, until it exhibited changes of less than 1 percent per minute (*9*). For measurements of S, a narrow beam of coherent light from a He-Ne laser could be directed at any point on the muscle. The diffraction pattern from the chosen region fell upon a ground-glass screen 300 mm away. S was calculated from the positions of the diffraction fringes, marked directly on the screen or photographed with a 35-mm camera (*10*).

When a resting muscle was quickly stretched, tension rose immediately and then began to decay as soon as stretching stopped (Fig. 1A). S remained constant (changed less than ±1 percent) during stress relaxation (Fig. 1B). The extent of stress relaxation increased with each step in length. These force responses showed no sign of the delayed rise often seen when activated muscles are stretched (stretch activation), nor did they show any sign of the short-range elastic component attributed by Hill (*11*) to a few weakly attached cross-bridges. These findings indicate that the forces studied here are purely passive. One indication that this force is viscoelastic and not simply viscous is that muscles that were held stretched overnight remained taut.

The length-tension curves in four resting muscles (Fig. 2A) represent the range of variation encountered in this study. Tension increased exponentially with S in all cases. The curves in Fig. 2A are calculated from the expression (*12*)

$$\sigma = \frac{E_0}{\alpha}\,(e^{\alpha\epsilon_s} - 1) \qquad (1)$$

where σ is stress (force per cross-sectional area), E_0 is the initial elastic modulus, α is an empirical constant, and ϵ_s is

Table 1. Values of α and E_0 for the length-tension equation for resting muscles, Eq. 1. Tabulated are means ± standard error of the mean (SEM). Data for intact fibers are from Rapaport (*5*); skinned fiber data are from Magid (*7*). The value of α for whole muscle does not differ significantly from that for intact fibers or skinned fibers ($P = 0.287$, analysis of variance, rank 2 affine hypothesis). The differences among E_0 values are significant ($P < 0.001$).

Preparation	Number of samples	Exponent α	Initial elastic modulus, E_0 ($\times 10^3$ N/m^2)
Whole muscles	11	4.28 ± 0.19	2.6 ± 0.25
Intact single fibers	10	4.04*	9.8*
Skinned single fibers	12	4.13 ± 0.10	5.4 ± 0.75

*No SEM's are reported because the values come from a single analysis of pooled data.