INFECTION AND IMMUNITY, Jan. 1993, p. 142–145
0019-9567/93/010142-04$02.00/0

Vol. 61, No. 1

T-Lymphocyte Response in a Guinea Pig Model of Tuberculous Pleuritis

SUSAN W. PHALEN* AND DAVID N. McMURRAY

Department of Medical Microbiology and Immunology, College of Medicine, Texas A&M University Health Science Center, College Station, Texas 77843

Received 21 May 1992/Accepted 14 October 1992

The ability to induce tuberculous pleuritis in *Mycobacterium bovis* BCG-vaccinated guinea pigs was investigated as a model of human disease. A pleural effusion of 5 to 10 ml was obtained 6 to 7 days after the bilateral pleural injection of a suspension of heat-killed *M. tuberculosis* cells. Histological lesions were indicative of granulomatous pleuritis. Comparative studies of T lymphocytes obtained from pleural fluid and peripheral blood revealed increased antigen-driven lymphoproliferation and E rosette formation in pleural effusion lymphocytes. The $CD2^+$ T-lymphocyte population appeared to be expanded or concentrated in pleural fluid, suggesting a compartmentalization of antigen-reactive T lymphocytes. These data demonstrate that experimental tuberculous pleuritis with effusion, closely resembling the human disease, can be produced in BCG-vaccinated guinea pigs.

Tuberculosis is one of the world's most prevalent infections, with 7 million to 9 million new cases diagnosed and approximately 3 million deaths occurring annually (4). One important clinical manifestation of tuberculosis is pleuritis; this condition occurs in approximately 10% of untreated tuberculin skin test converters, making *Mycobacterium tuberculosis* a significant cause of exudative pleural effusions (6, 7).

Tuberculous pleuritis develops in patients with an intermediate degree of resistance to *M. tuberculosis* (6). Incomplete sequestration of a subpleural focus of infection and subsequent destruction of pulmonary parenchyma allows caseous material to be discharged into the pleural space (1, 6, 9). The host responds to the *M. tuberculosis* antigens by developing a delayed-type hypersensitivity within 3 to 8 weeks and pleuritis within 6 to 12 weeks (6).

Untreated tuberculous pleuritis has a variable course. Although a few patients succumb to infection, most regain health without specific chemotherapy (2). However, 60% of patients who have apparently recovered develop pulmonary or extrapulmonary tuberculosis within 5 years (1, 6). It is therefore important that tuberculous pleuritis be diagnosed and treated early in its course (12, 21).

Cell-mediated immunity may play a significant role in the pathogenesis of tuberculous pleuritis. In human tuberculous pleuritis, the lymphocyte population in pleural fluid differs from that in peripheral blood with respect to the proportions of T lymphocytes and their responsiveness to tuberculin (2, 13, 14, 16).

An animal model would allow a more precise definition of the cellular changes that occur when a tuberculous focus ruptures into the pleural space. Only a few studies of experimental tuberculous pleuritis have been reported previously. Antony et al. (1) and Windstrom et al. (20) investigated pleural effusion cell populations in the New Zealand White rabbit and the outbred guinea pig, respectively. Experimentally induced pleural effusions could provide a unique source of highly purified antigen-reactive T lymphocytes as well as antibodies, lymphokines, and other soluble factors which are participating in a successful antimycobacterial immune response. The purpose of this study was to develop an animal model which closely simulates human tuberculous pleuritis and to characterize the cellular infiltrate.

MATERIALS AND METHODS

Experimental animals. Both pathogen-free, inbred strain 2 guinea pigs (Veterinary Resources Division, University of Texas System Science Park, Bastrop, Tex.) and outbred, albino guinea pigs [Hartley-COBS, Crl:(HA)BR; Charles River Breeding Laboratories, Inc., Wilmington, Mass.] were used in this study. The animals were individually housed in polycarbonate cages and were provided with commercial guinea pig chow (Ralston Purina, St. Louis, Mo.) and tap water ad libitum.

Vaccination. Each animal received a subcutaneous injection of 0.1 ml of *Mycobacterium bovis* BCG vaccine (Danish 1331; Statens Seruminstitut, Copenhagen, Denmark) in the left inguinal region. The lyophilized BCG vaccine was prepared by being reconstituted in 0.9% sterile saline; it delivered approximately 10^3 viable organisms per animal. The viability of the vaccine was determined by plating appropriate dilutions on M7H10 agar (Difco Laboratories, Detroit, Mich.).

Induction of tuberculous pleural effusion. At 5 to 6 weeks postvaccination, a pleural effusion was induced in each animal. Cultures of *M. tuberculosis* H37Rv were harvested into sterile saline after several weeks of growth on M7H10 agar medium and killed by immersion in a water bath at 80°C for 2 h. A suspension was prepared by homogenizing the cells in 0.9% sterile saline in a Pyrex Ten Broeck tissue grinder. Dilutions of the suspension were made in 0.9% sterile saline until a protein concentration of approximately 0.125 mg/ml, as determined by a protein assay (Bio-Rad Laboratories, Richmond, Calif.), was obtained. Each guinea pig was anesthetized with an intramuscular injection of 30 mg of ketamine hydrochloride (Ketaset; Fort Dodge Laboratory, Fort Dodge, Iowa) per kg of body weight and 2.5 mg

* Corresponding author.

of xylazine (Rompun; Fort Dodge Laboratory) per kg. A total of 2.0 ml of the heat-killed *M. tuberculosis* suspension was injected bilaterally into the pleural space. A 20-gauge 1-in. (2.54-cm) needle was inserted 1.5 cm cranially and 1.5 cm laterally to the xiphoid process. The needle was advanced just through the intercostal muscles and passed directly dorsally into the pleural space. Negative pressure was exerted on the syringe to ensure that the lung was not penetrated.

Necropsy. Guinea pigs were anesthetized with an intramuscular injection of 30 mg of Ketaset per kg and 2.5 mg of Rompun per kg 7 days following induction of pleural effusion. A peripheral blood sample of 8 to 10 ml was immediately obtained by cardiac puncture with a heparinized syringe. Each animal was killed by cardiac injection of 1 ml of sodium pentobarbital (Fort Dodge Laboratory). The abdomen was opened via a median incision, and pleural fluid was drawn from the thoracic cavity through the diaphragm with a heparinized syringe. The throrax was opened above the midline, and sections of lung, diaphragm, and mediastinal tissue were removed for gross and histopathological examination.

Histopathology. Pleural tissues were fixed in neutral buffered formalin, sectioned, and then stained with hematoxylin and eosin for histopathological examination. Formalin-fixed sections and smears of aspirated pleural exudate were stained by the Kinyoun cold acid-fast technique to determine the presence of tubercle bacilli. Giemsa stain and Wright blood stain were used on smears of peripheral blood and aspirated pleural exudate for the differential leukocyte count.

Lymphocyte blastogenesis. Blood and pleural fluid lymphocytes were obtained by density gradient centrifugation (3). Briefly, peripheral blood was diluted with three times its volume, and pleural fluid was diluted with an equal volume of Hanks' phosphate-buffered saline-EDTA. Each sample of diluted pleural fluid and blood was layered over Ficoll-Histopaque (Sigma, St. Louis, Mo.) and centrifuged, and the lymphocytes at the interface were removed. The cells were washed three times with Hanks' phosphate-buffered saline and then resuspended in 1.0 ml of tissue culture medium (RPMI 1640 with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; Irvine Scientific, Santa Ana, Calif.] supplemented with 10% fetal bovine serum (Irvine Scientific), antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml; Irvine Scientific), 20 mM L-glutamine, and 0.01 mM 2-mercaptoethanol.

The number of viable cells was determined by trypan blue exclusion with a hemacytometer. The final cell concentration of both peripheral blood and pleural fluid was adjusted to 2×10^6 cells per ml. Aliquots were stimulated with concanavalin A (10 μg/ml; Sigma), purified protein derivative (PPD; 25 and 12.5 μg/ml), and recombinant *M. bovis* 65-kDa and *M. tuberculosis* 71-kDa mycobacterial proteins (10 μg/ml; kindly provided by The Laboratory of Bacteriology, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands, under contract to the United Nations Development Program/World Bank/World Health Organization). Each well of a 96-well flat-bottom microtiter plate received 2×10^5 cells in 0.1 ml of medium with 0.1 ml of mitogen or antigen; each concentration was tested in triplicate.

The cultures were incubated for 4 days at 37°C in a 5% CO_2 environment, labeled with 1.0 μCi of tritiated thymidine per well for the last 6 h, and harvested. The cellular uptake of thymidine was quantified in a liquid scintillation counter. The results were expressed as mean counts per minute (cpm) of stimulated cultures minus mean cpm of unstimulated cells from the same source.

E rosette assay. The number of E rosette-forming (CD2) T lymphocytes isolated from peripheral blood and pleural fluid was determined by using the rabbit erythrocyte rosette assay for guinea pig T lymphocytes (19). Lymphocyte suspensions were obtained as described above for blastogenesis. Fresh rabbit blood was stored in Alsever's solution at 4°C and then centrifuged just before use to remove the plasma. The cells were washed twice in phosphate-buffered saline, resuspended in medium, counted in a hemacytometer, and adjusted to a concentration of 10^8 cells per ml. Rosettes were prepared by incubating 200 μl of lymphocyte suspension and 200 μl of rabbit erythrocytes at 37°C for 30 min. The cells were centrifuged at $200 \times g$ at 4°C and incubated at 4°C overnight. The pellet was gently resuspended, and the rosettes were counted in a hemacytometer. A rosette-forming lymphocyte was defined as a lymphocyte with three or more adherent rabbit erythrocytes. Proportions of lymphocytes that formed rosettes were expressed as a percentage of total viable lymphocytes.

Statistical analysis. Mean differences (paired when appropriate) between the responses of T lymphocytes in peripheral blood and pleural effusions were tested for statistical significance by Student's *t* test. The accepted level of probability was set at 95% for all tests.

RESULTS

Induction of pleural effusion. Experimental animals were sacrificed 7 days following the bilateral intrapleural injection of 2.0 ml of heat-killed *M. tuberculosis* H37Rv cells. A serosanguineous pleural effusion of 5 to 10 ml was produced in 86% of inbred strain 2 and outbred albino guinea pigs. Analysis of pleural fluid was complicated by inconsistent lymphocyte separation. In some instances, a distinct interface could not be detected after layering over Ficoll-Histopaque and centrifugation. This resulted in decreased sample sizes for some experiments ($n < 7$).

Pathology. Gross examination revealed granulomas in lung, diaphragm, and mediastinal tissues of all animals with effusions. Histopathological testing revealed a moderate to severe granulomatous pleuritis, myositis, and interstitial pneumonia. Sections of diaphragm revealed granulomas containing multinucleated giant cells, histiocytes, and plasma cells along the periphery.

Differential leukocyte counts. Throughout the experiment, a greater percentage of lymphocytes was observed in samples of pleural fluid than in samples of peripheral blood (Fig. 1), with a concomitant decrease in the percentage of polymorphonuclear leukocytes in pleural fluid. Lymphocytes made up 53.2% ± 21.4% of the leukocytes in peripheral blood and 66.4% ± 12.7% of the leukocytes in pleural fluids. The percentages of polymorphonuclear cells in blood and pleural fluid were 44.5% ± 21.5% and 30.8% ± 12.3%, respectively. A paired-comparisons *t* test showed that 79% of the animals had a greater percentage of lymphocytes in pleural fluid ($P < 0.02$). These data suggest that compartmentalization of lymphocytes occurred at the site of disease.

Enumeration of E rosette-forming T lymphocytes. The mean percentages of E rosette-forming $CD2^+$ T lymphocytes in samples of peripheral blood and pleural effusion lymphocytes from BCG-vaccinated guinea pigs were compared (Fig. 1). The percentage of E rosette-forming lymphocytes in peripheral blood samples was 27.6% ± 16.4%, while pleural

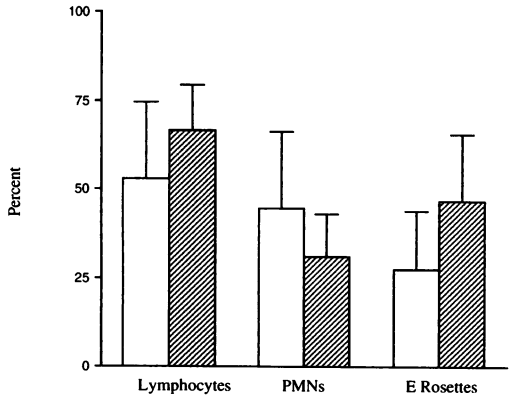

FIG. 1. Percentages of lymphocytes, polymorphonuclear leukocytes (PMNs), and E rosettes in peripheral blood (□) and pleural effusions (▨) of BCG-vaccinated guinea pigs sacrificed 1 week after induction of tuberculous pleural effusions by intrapleural injection of heat-killed *M. tuberculosis* cells. The mean ± standard deviation of the mean of 9 to 19 animals per data set is shown.

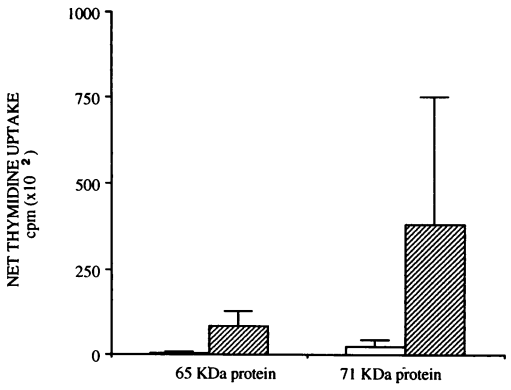

FIG. 3. The 65- and 71-kDa recombinant mycobacterial protein-induced proliferation in vitro of peripheral blood (□) and pleural effusion (▨) lymphocytes of BCG-vaccinated guinea pigs. The animals were sacrificed 1 week after induction of tuberculous pleural effusions by intrapleural injection of heat-killed *M. tuberculosis* cells. The mean ± standard deviation of the mean of four to five animals per data set is shown.

fluid samples had a mean percentage of 46.6% ± 18.8%. A paired-comparisons *t* test revealed that 67% of the animals had greater numbers of E rosette-forming lymphocytes in pleural fluid than in blood ($P < 0.03$). These data are indicative of a sequestration of mature T lymphocytes expressing the CD2 phenotype at the site of disease activity.

Lymphoproliferative response to PPD. A significantly increased in vitro proliferative response to PPD was seen in lymphocytes in pleural effusion from BCG-vaccinated guinea pigs (Fig. 2). Mean lymphoproliferative responses to 25 μg of PPD per ml were 86,888 ± 54,839 cpm for pleural effusion lymphocytes and 23,410 ± 19,376 cpm for peripheral blood lymphocytes ($P < 0.01$). Mean lymphoproliferative responses to 12.5 μg of PPD per ml were 78,990 ± 50,936 cpm and 21,574 ± 17,572 cpm from pleural fluid and peripheral blood samples, respectively ($P < 0.01$).

Lymphoproliferative response to the recombinant 65-kDa mycobacterial protein. Comparative proliferative responses of peripheral blood and pleural effusion lymphocytes from BCG-vaccinated guinea pigs to the recombinant 65-kDa mycobacterial protein are shown in Fig. 3. A trend toward increased in vitro mitogenic stimulation of pleural effusion lymphocytes was observed, but it was not statistically significant. The mean lymphoproliferative response of pleural fluid samples was 8,476 ± 4,385 cpm, but peripheral blood samples did not respond as well (565 ± 408 cpm).

Lymphoproliferative response to the recombinant 71-kDa mycobacterial protein. Comparative proliferative responses of peripheral blood and pleural effusion lymphocytes from BCG-vaccinated guinea pigs to the recombinant 71-kDa mycobacterial protein are also shown in Fig. 3. The mean proliferative response of pleural fluid lymphocytes was 37,873 ± 37,230 cpm, compared to 2,372 ± 1,890 cpm for peripheral blood lymphocytes. The comparative response data are not statistically significant. However, a trend toward an increased in vitro lymphoproliferative response of effusion cells when stimulated with this mycobacterial heat shock protein compared with that of peripheral blood lymphocytes was demonstrated. It was also noted in this experiment that the 71-kDa mycobacterial protein elicited a greater in vitro blastogenic response than the 65-kDa antigen when the responses of the same cells to the two proteins were compared.

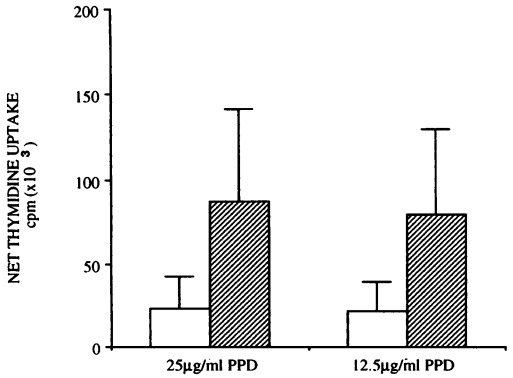

FIG. 2. PPD-induced proliferation (25 and 12.5 μg of PPD per ml) in vitro of peripheral blood (□) and pleural effusion (▨) lymphocytes of BCG-vaccinated guinea pigs sacrificed 1 week after induction of tuberculous pleural effusions by intrapleural injection of heat-killed *M. tuberculosis* cells. The mean ± standard deviation of the mean of 16 animals per data set is shown.

DISCUSSION

Pleural effusion of 5 to 10 ml was predictably obtained in BCG-vaccinated guinea pigs by the bilateral intrapleural injection of 2.0 ml of heat-killed *M. tuberculosis* cells. Histopathologic study of pleural tissues revealed a granulomatous pleuritis in every instance of successful induction of pleural effusion. A thickened visceral pleura with focal granulomas consisting of fibrous connective tissue, macrophages (some containing engulfed acid-fast bacteria), and polymorphonuclear leukocytes was seen.

The guinea pig has been used successfully in an experimental model of pulmonary tuberculosis (5, 17), and the present study was based on that model. Several characteristics of the pleural effusion model which was developed in this study are similar to the human disease; they therefore increase our confidence in the biological relevance of the model. This is illustrated in the results of the differential leukocyte counts and E rosette experiments. Normal differ-

ential leukocyte values in normal adult humans range from 25 to 70% polymorphonuclear leukocytes and from 20 to 49% lymphocytes (15). A diagnostic feature of tuberculous pleural effusion is the cellular predominance of lymphocytes (1, 2, 6, 18). In the current study, the mean percent distribution of lymphocytes was uniformly higher in pleural fluid than peripheral blood; that is, 79% of the animals exhibited higher lymphocyte counts in the pleural fluid. These data compare favorably with those seen in human tuberculous pleural effusions and suggest that lymphocytes are expanded and/or that lymphocytes are attracted to and sequestrated at the site of disease.

The results of the E rosette assay offered important confirmatory evidence of the similarities between the guinea pig model and human disease. It has been shown in studies of human tuberculous pleuritis that pleural fluids contain 47 to 95% E rosette-forming T lymphocytes (8, 10, 16). In the present experiment, samples of pleural fluid contained more E rosette-forming T lymphocytes than peripheral blood samples did. These data suggest the expansion or accumulation of $CD2^+$ T lymphocytes at the site of disease, as has been described for human tuberculous pleural effusions.

The current study assessed the comparative lymphoproliferative effects of PPD and the recombinant 65- and 71-kDa mycobacterial heat shock proteins on pleural effusion and peripheral blood T lymphocytes from BCG-vaccinated guinea pigs. Greater proliferative responses were consistently seen in the pleural effusion samples. Throughout the experiment, the PPD-driven lymphoproliferation was more pronounced than that exhibited by the recombinant mycobacterial proteins, which is indicative of the complex antigenic composition of PPD. Of the two mycobacterial heat shock proteins, the 71-kDa antigen elicited a greater lymphoproliferative response. Heat shock proteins produced by pathogens in response to the host environment may serve as important immunodominant antigens (22). However, because of their highly conserved nature, these proteins may share epitopes with heat shock proteins expressed by host cells (11). The greater response to the mycobacterial 71-kDa heat shock protein may be due to shared epitopes between it and analogous proteins from other bacteria or host cells. This may suggest the recognition of the protein by an already expanded population of T lymphocytes. It was clear that the pleural effusion T lymphocytes were consistently stimulated to a greater degree than those of peripheral blood, although this was not statistically significant. Similar results have been reported in lymphoproliferation studies of human tuberculous effusions (6, 8, 16). These results indicate that antigen-reactive T lymphocytes are either sequestered in pleural effusion fluid or preferentially expanded at the site of disease.

Taken together, the experimental evidence presented above suggests that a relevant animal model of tuberculous pleuritis has been developed. This model can provide a source of highly purified antigen-reactive lymphocytes and soluble immunoregulatory factors, which can be used to facilitate the further study of mycobacterial immunoregulatory and modulating mechanisms.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grant AI-15495 from the National Institute of Allergy and Infectious Diseases.

We thank Jane Lantz for secretarial assistance.

REFERENCES

1. **Antony, V. B., J. E. Repine, R. N. Harada, J. T. Good, and S. A. Sahn.** 1983. Inflammatory responses in tuberculous pleuritis. Acta Cytol. **27**:355–361.
2. **Barnes, P., S. D. Mistry, C. L. Cooper, C. Pirmez, T. H. Rea, and R. Modlin.** 1989. Compartmentalization of a CD4 lymphocyte subpopulation in tuberculous pleuritis. J. Immunol. **142**: 1114–1119.
3. **Bartow, R. A., and D. N. McMurray.** 1989. Vaccination with *Mycobacterium bovis* BCG affects the distribution of Fc receptor-bearing T lymphocytes in experimental pulmonary tuberculosis. Infect. Immun. **57**:1374–1379.
4. **Centers for Disease Control.** 1989. A strategic plan for the elimination of tuberculosis in the United States. Morbid. Mortal. Weekly Rep. **38**:269–271.
5. **Cohen, M. K., R. A. Bartow, C. L. Mintzer, and D. N. McMurray.** 1987. Effects of diet and genetics on *Mycobacterium bovis* BCG vaccine efficacy in inbred guinea pigs. Infect. Immun. **55**:314–319.
6. **Ellner, J. J., P. F. Barnes, R. S. Wallis, and R. L. Modlin.** 1988. The immunology of tuberculous pleurisy. Semin. Respir. Infect. **3**:335–342.
7. **Epstein, D. M., L. R. Kline, S. M. Albelda, and W. T. Miller.** 1987. Tuberculous pleural effusions. Chest **91**:106–109.
8. **Fujiwara, H., and I. Tsuyuguchi.** 1986. Frequency of tuberculin-reactive T-lymphocytes in pleural fluid and blood from patients with tuberculous pleurisy. Chest **89**:530–532.
9. **Grange, J. M.** 1988. Mycobacteria and human disease, p. 119–137. Edward Arnold, London.
10. **Jakubusek-Przylipiak, M., G. Janicka, A. Zagorecka, and W. Pregowski.** 1980. Leucocyte migration inhibition and rosette tests in pleural effusion. Eur. J. Respir. Dis. **61**:67–70.
11. **Kaufmann, S. H. E.** 1990. Heat shock proteins and the immune response. Immunol. Today **11**:129.
12. **Levine, H., P. B. Szanto, and D. W. Cugell.** 1968. Tuberculous pleurisy. Arch. Intern. Med. **122**:329–332.
13. **Moisan, T., A. J. Chandrasekhar, J. Robinson, J. McKenna, and G. Marti.** 1978. Distribution of lymphocyte subpopulations in patients with exudative pleural effusions. Am. Rev. Respir. Dis. **117**:507–511.
14. **Rossi, G. A., B. Balbi, and F. Manca.** 1987. Tuberculous pleural effusions. Am. Rev. Respir. Dis. **136**:575–579.
15. **Rubin, E., and J. L. Farber.** 1988. Pathology, p. 616. J. B. Lippincott Co., Philadelphia.
16. **Shiratsuchi, H., and I. Tsuyuguchi.** 1984. Analysis of T cell subsets by monoclonal antibodies in patients with tuberculosis after *in vitro* stimulation with purified protein derivative of tuberculin. Clin. Exp. Immunol. **57**:271–278.
17. **Smith, D. W., D. N. McMurray, E. H. Wiegeshaus, A. A. Grover, and G. E. Harding.** 1970. Host-parasite relationships in experimental airborne tuberculosis. IV. Early events in the course of infection in vaccinated and nonvaccinated guinea pigs. Am. Rev. Respir. Dis. **102**:937–949.
18. **Spieler, P.** 1979. The cytologic diagnosis of tuberculosis in pleural effusions. Acta Cytol. **5**:374–379.
19. **Stadecker, M., G. Bishop, and H. Wortis.** 1973. Rosette formation by guinea pig thymocytes and thymus derived lymphocytes with rabbit red blood cells. J. Immunol. **111**:1834–1837.
20. **Windstrom, O., and B. S. Nilsson.** 1982. Pleurisy induced by intrapleural BCG in immunized guinea pigs. Eur. J. Respir. Dis. **63**:425–434.
21. **Yam, L. T.** 1967. Diagnostic significance of lymphocytes in pleural effusions. Ann. Intern. Med. **66**:972–982.
22. **Young, D., R. Lathigra, R. Hendrix, D. Sweetser, and R. A. Young.** 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA **85**:4276–4280.