ELSEVIER

Life Sciences, Vol. 63, No. 13, pp. 1183-1193, 1998

0024-3205/98 $19.00 + .00

PII S0024-3205(98)00379-8

PERIBRONCHIAL LYMPHOCYTE ACTIVATION IN BLEOMYCIN-INDUCED LUNG INJURY

Izidore S. Lossos[2] Raphael Breuer[2], , Miri Shriki[2] and Reuven Or[3]

[1]Institute of Pulmonology, Hadassah University Hospital, Jerusalem, Israel;
[2]Mallory Institute of Pathology, Boston University School of Medicine, Boston, Massachusetts, USA, and
[3]Department of Bone Marrow Transplantation and Cancer Immunobiology Research Laboratory, Hadassah University Hospital, Jerusalem, Israel

(Received in final form July 14, 1998)

Summary

The role of lymphocytes in bleomycin (Bleo)-induced lung injury remains obscure. In normal hamsters, peribronchial lymphatic tissue (PBLT) has been found to contain a large population of T lymphocytes responsive to interleukin 2 (IL-2) but not to IL-4. Lung injury induced by a single intratracheal instillation of Bleo in hamsters has been ameliorated by cyclosporin A (CyA). In the present study, using this model, PBLT-derived lymphocyte function was explored for 28 days after Bleo instillation. Increase in PBLT lymphocytes occurred at five time points investigated, reaching highest values on day +7 ($p < 0.0025$). Cell proliferation in response to concanavalin A was enhanced, while IL-2 +/- the mitogen had no effect. In contrast to its inactivity in the normal hamster, in the Bleo-injured animal IL-4 alone induced T cell proliferation ($p = 0.0077$) on day +7. CyA therapy initially suppressed and delayed recovery of the number of lymphocytes and their activation. The results of this study suggest the existence of a vulnerable period in Bleo-induced lung injury and indicate that lymphocytes participate in the pathogenesis of the insult to the tissue. The unresponsiveness to IL-2 and the emergence of cellular response to IL-4 indicate immune deviation in PBLT-derived T cells.

Key Words: bleomycin-induced lung injury, cyclosporin A, peribronchial lymphatic tissue, lymphocytes, IL-2, IL-4

Correspondence to Raphael Breuer, MD, Institute of Pulmonology, Hadassah University Hospital, POBox 12 000,Jerusalem, Israel. Fax 972-2-6435897

Multiple cell types, cell products and cytokines have been implicated in bleomycin (Bleo)-induced pulmonary fibrosis (1). However, the role of T lymphocytes in the pathogenesis of Bleo-induced interstitial pulmonary fibrosis is controversial (2-7). Some studies have shown that in T cell-depleted animals the development of Bleo-induced lung injury is not preventable (2, 3), while others have suggested an active role of T lymphocytes in the regulation of the inflammatory and fibrotic process (4-7). We have previously shown that cyclosporin A (CyA), an immunosuppressive agent primarily affecting T-lymphocyte function, ameliorates Bleo-induced lung injury (8).

In the present study we sought to determine the role of the cellular immune response in Bleo-induced pulmonary injury. To that end, in vitro T-cell activation in peribronchial lymphatic tissue (PBLT) obtained from animals with or without CyA was evaluated.

Materials and methods

Animals

All animal procedures were approved by the Institutional Committee for Animal Care. Male Syrian hamsters (*Mesocricetus Auratus*; Harlan Sprague Dawley, Indianapolis, IN, USA) weighing 100-145 g, were studied. Four to five hamsters were housed per metal cage on hardwood shavings. A 12-hour light and dark cycle was maintained; the animals had access to water and rodent laboratory chow ad libitum. Animals were allowed to acclimatize to these conditions for one week after which treatments were started.

Bleomycin and cyclosporin A

Bleomycin sulfate (15 mg; Bristol Laboratories, Syracuse, NY) was dissolved in sterile 0.9% saline on the day of intrathecal (IT) instillation (1 mg/0.5 ml solution per animal, administered under light CO_2 anesthesia). CyA (Sandimmune, Sandoz, Switzerland), diluted in sterile saline, was administered intraperitoneally (IP) for six consecutive days each week at a dose of 20 mg/kg body wt/day for 4 weeks. In our preliminary studies, this dosage led to CyA serum levels within the "therapeutic window" (100 to 400 ng/ml) and was not associated with clinical toxicity (8).

Experimental design

Hamsters were randomly assigned to four weight-matched groups, as follows: a) IT Bleo on day 0 and IP CyA starting on day -1 (Bleo-CyA); b) IT Bleo and IP saline (Bleo-Sal); c) IT saline and IP CyA (Sal-CyA); d) IT and IP sterile saline (Sal-Sal).On day -1 (normal control) and on day 1, 3, 7, 14, 28 after IT instillation, 4-5 animals of the Bleo-CyA, Bleo-Sal and Sal-CyA groups, and 2 animals of the Sal-Sal group were killed by transection of the

abdominal aorta while under pentobarbital anesthesia (80 mg/kg, IP).

The lungs with trachea en bloc, PBLT and spleen were excised as previously described (9). Spleen and PBLT were strained through sterile silk screens into petri dishes containing Hank's balanced salt solution (Biological Industries, Beth Haemek, Israel). After centrifugation (10 min, 1000 rpm, room temperature) the pellets were suspended in RPMI 1640 (Biological Industries, Beth Haemek) supplemented with 10% inactivated fetal calf serum (Biological Industries, Beth Haemek), 2 mM L-glutamine and 1% penicillin-streptomycin solution (Sigma, St Louis, MO, USA). Cells were allowed to adhere to sterile plastic T-60 bottles (Nunc, Denmark) overnight in a CO_2 incubator (5%) at 37° C. Non-adherent cells were removed, centrifuged (10 min, 1000 rpm, room temperature) and resuspended at a concentration of 2.5×10^6 cells/ml in supplemented RPMI medium. All non-adherent cells consisted of mononuclear cells (MNC), containing more than 90% lymphocytes as determined by cytospin cell staining (Diff Quick Stain, Baxter Inc., USA).

Mononuclear cells from PBLT and spleen were counted in a standard AO hemocytometer. Trypan blue (0.1%) dye exclusion was used to determine cell viability. The number of PBLT and splenic MNC of each animal was expressed as cell number per animal.

Lymphocyte proliferation

PBLT lymphocytes from the animals killed on day -1, +1, +7, +14 and +28 were assayed for their response to concanavalin A (ConA; Miles-Yeda, Rehovot, Israel), human recombinant interleukin 2 (rIL-2), and for their response to a combination of the two stimulants. Proliferation assays in the presence of human recombinant interleukin 4 (rIL-4) +/-ConA were carried out on cells extracted on days +7 and +14. The numbers of animals examined at each time point for each group are presented in the figure legends. The rIL-2 (supplied as proleukin, 3×10^6 Cetus units equivalent to 18 million IU; kindly provided by EuroCetus/Chiron, Amsterdam, the Netherlands) was initially diluted with water for injection and subsequently rediluted with dextrose 5%. The human rIL-4 was supplied as 1.5 μg (in powder form) equivalent to 3×10^4 units bioactivity (Genzyme, Cambridge, MA, USA).

Triplicates of 2.5×10^5 cells in 100 μl were plated into flat-bottomed 96-well microtiter plates (Greiner, Labortechnik, Germany). ConA, rIL-2, rIL-4, either single or in combinations, were dissolved at a concentration of 5 μg/ml, 10 U/ml, and 100 U/ml, respectively, in supplemented RPMI 1640 and were added to cells in 100 μl aliquots, as described elsewhere (9). Cells cultured in medium alone were used as control. Cells were

incubated for 120 hrs in a 5% CO_2 incubator at 37°C and pulsed with 10 μl [^{3}H]thymidine (Rotem Industries, Beersheba, Israel) for 24 hrs, conditions previously found to result in maximal lymphocyte proliferation (9). Cells were harvested onto glass fiber filter strips (Tamar, Jerusalem, Israel) and immersed in 1.0 ml scintillation fluid (National Diagnostics, Atlanta, GA, USA) contained in 5-ml scintillation vials. Samples were counted in a LS7500 Beckman scintillation counter (Beckman Instruments, Fullerton, CA, USA). Results are expressed as stimulation index (SI) i.e., the ratio between the counts per minute of experimental and unstimulated cells

Statistical analysis

The ANOVA test was used to analyze the effect of time on lymphocyte number and proliferation. Comparisons between the effects of IT Bleo or Sal were performed by using the Student t-test. Since lymphocyte number per animal or per gram animal wt led to similar results, data are presented as number of lymphocytes per animal. Probability values of <0.05 were considered statistically significant.

Results

Quantitative analysis of lymphocytes in PBLT and spleen

The quantity of PBLT lymphocytes of Sal-Sal animals did not change over time, not differing from that of untreated animals (data not shown). In the Sal-CyA group the PBLT lymphocytes elevated on day +1 without significant changes over time. Compared with the Sal-Sal group, however, this increase became significant on day 14 ($p = 0.004$) (Fig. 1). The Bleo-Sal group showed a 13-fold increase in lymphocyte number on day 1 and a 36-fold increase on day 7 ($p < 0.001$). Compared with the Sal-Sal group there was a marked increase in the number of lymphocytes at all five time points ($p < 0.0025$). Treatment of Bleo-instilled hamsters with CyA caused a decrease in PBLT lymphocytes at days 1 and 7 in comparison with Bleo-Sal animals ($p = 0.0074$ and $p = 0.02$, respectively) (Fig. 1). The quantity of PBLT lymphocytes in Bleo-CyA was higher than in the controls (Sal-Sal) at all time points.

No changes occurred in the number of splenic lymphocytes in the Bleo-Sal, Sal-CyA and Sal-Sal groups. The Bleo-CyA displayed a two-fold rise on day 3 compared with the Sal-Sal animals (data not shown).

PBLT lymphocyte proliferation in response to ConA and rIL-2

To determine whether PBLT-derived lymphocytes are activated by Bleo and to evaluate the effect of CyA therapy, the activity of PBLT-derived T cells was examined by means of their spontaneous

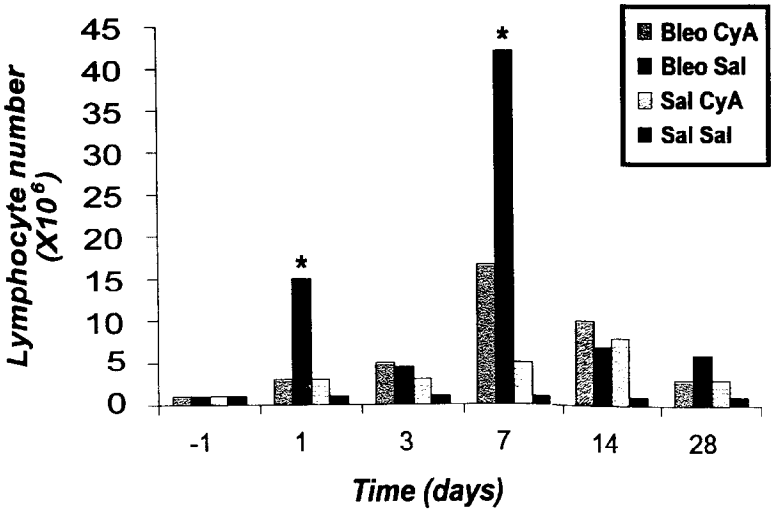

Fig. 1

Number of PBLT lymphocytes at the indicated days prior to or following intratracheal treatment. Bleo-Sal animals showed a marked increase ($p < 0.0025$) in lymphocyte number at all time points compared with untreated animals (day -1). Treatment with CyA impeded the Bleo-induced increase in lymphocytes. Values represent the mean of 4-5 animals per treatment group at each time point.

*$p < 0.05$ compared with Bleo-CyA.

proliferation and their response to ConA, ConA + rIL-2, and rIL-2 alone. Unstimulated cells showed similar proliferative response in Bleo-Sal, Bleo-CyA and Sal-Sal animals at all time points (data not shown), not differing in this respect from the results on day -1. In Bleo-Sal animals, ConA and ConA + rIL-2 elicited increased cell proliferation on day +1, which peaked on day +7 ($p = 0.02$), subsequently decreasing to baseline levels (Fig. 2A, 2B).

In the Bleo-CyA group the proliferative response to ConA and ConA + rIL2 was delayed, reaching a maximum on day +14 (Fig. 2A, 2B), and returning to baseline on day +28. No response to rIL-2 alone was observed in either Bleo group at all time points tested (data not shown). The mean SI values of ConA +/- rIL-2 were lower in the Bleo-CyA group than in the Bleo-Sal animals on day 1 and day 7 (Fig. 2A, 2B). This effect of CyA was statistically significant on day +1 in the absence of rIL-2. The addition of rIL-2 to ConA failed to significantly increase lymphocyte proliferation in the Bleo-Sal and Bleo-CyA groups at any of the time points tested.

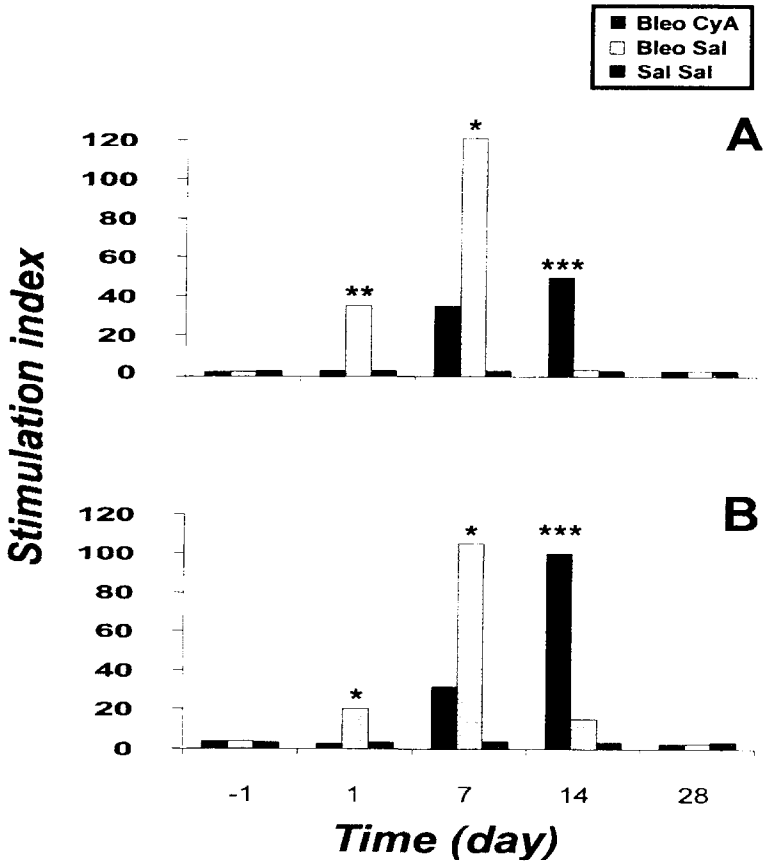

Fig. 2

Effect of ConA (A) + IL-2 (B) on PBLT lymphocytes of Bleo-Sal and Bleo-CyA animals. PBLT cells (2.5 x 10^6/ml) were incubated with ConA (5 μg/ml) with/without IL-2 (10 U/ml). Unstimulated cells served as control. ^{3}H-thymidine was added 24 h before harvesting on day 6. Values represent the mean stimulation index of 4-5 animals at each time point.

*$p < 0.05$ compared with day -1.

**$p < 0.05$ compared with Bleo-CyA.

***$p < 0.05$ compared with Bleo-Sal.

PBLT lymphocyte proliferation in response to rIL-4

Based on the observation that after the IT instillation of Bleo, rIL-2 +/- ConA had no effect on cell proliferation, and coupled with the maximal mitogenic response on day +7, the effect of rIL-4 on PBLT-derived T cells was evaluated twice, on days +7 and +14. rIL-4 alone induced in vitro stimulation of PBLT-derived lymphocytes on day +7 after exposure to Bleo (Bleo-Sal versus Sal-Sal $p = 0.0065$; Fig. 3).

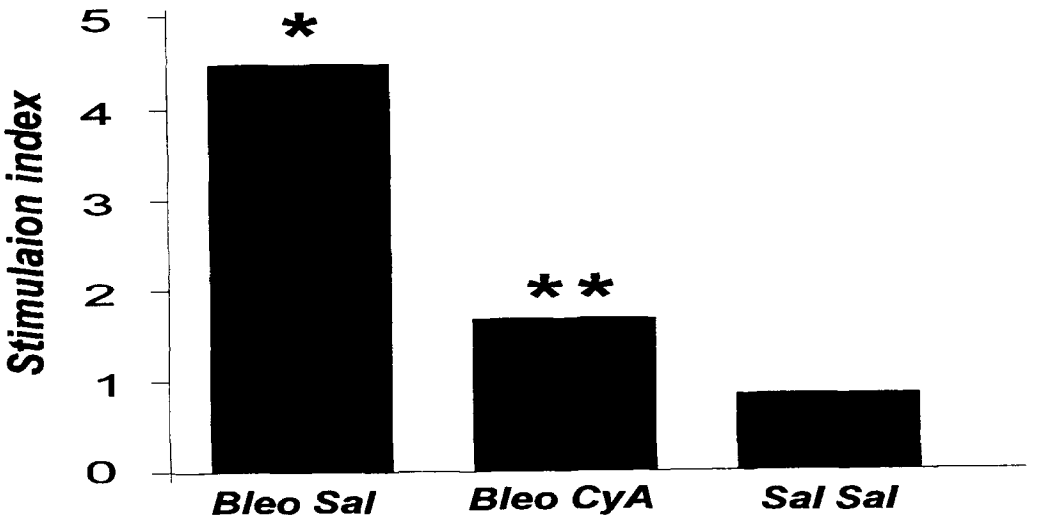

Fig. 3

Effect of IL-4 (100 U/ml) on PBLT-derived lymphocytes (2.5×10^6/ml) in Bleo-Sal, Bleo-CyA and Sal-Sal animals at day +7 following intratracheal Bleo or Sal instillation. Unstimulated cells served as controls. ^{3}H-thymidine was added 24 h before harvesting on day 6. Values represent the mean stimulation index of 3-4 animals in each group.
*$p < 0.05$ compared with Sal-Sal.
**$p < 0.05$ compared with Bleo-Sal

CyA administration to Bleo-treated animals caused a significant suppression of the response to rIL-4 ($p = 0.0077$; Fig. 3). No proliferative response to this cytokine was observed in the Sal-Sal and untreated animals. The acquisition of IL-4 responsiveness detected on day +7 declined and had disappeared by day +14 (3 experiments). This cellular behavior indicates a narrow based cone-shaped response curve for the experimental period.

Discussion

The results of the present study suggest a link between PBLT-derived T cell activation and the pathogenesis of Bleo-induced lung injury. Nevertheless, although after Bleo instillation both number and in vitro proliferative response to ConA of PBLT-derived lymphocytes was increased, spontaneous proliferation was not affected. The specificity of T cell response was tested by culturing these cells with either IL-2 or IL-4, each correlating with a functionally distinctive subpopulation. Bleo instillation

caused the appearance of rIL-4-responsive PBLT lymphocytes, a situation that had not been found to exist in untreated hamsters (9). In addition, CyA, which is known to ameliorate Bleo-induced pulmonary injury (8, 10), lessened the Bleo-induced increase in PBLT lymphocytes and delayed their proliferative response.

The Bleo-induced increase in the PBLT lymphocyte population may have resulted from either local cell proliferation or lymphocyte influx. Lymphocyte activation as a consequence of Bleo instillation may induce T cell progression into the proliferative phase or, on the other hand, the compound may provide a signal toward a competent state (11) that requires additional stimulation by a second growth factor. Our study suggests that the latter is the case, in view of the similarity between Bleo-treated and intact animals regarding lack of spontaneous lymphocyte proliferation, and the increased response to each ConA and rIL-4 in the lung injured groups. Whether Bleo alone is capable of inducing T cell proliferation in vitro was examined in PBLT and splenic lymphocytes from normal and Bleo-treated animals using four different concentrations of the drug ranging between 10^{-3}-10^{-6} units/ml. In these experiments, Bleo was incapable of providing signals leading to proliferation a fact that, on the other hand, does not negate the eventual function of the drug as an inducer of T cell competence.

In contrast to the responses in untreated animals (9), addition of rIL-2 to ConA in Bleo-treated animals failed to increase T cell proliferation. The inability of the cells to respond to rIL-2 might be the result of maximal stimulation obtained by ConA, or it might indicate that expression of the rIL-2 receptor is down regulated during the immediate post lung injury period despite the increased number of lymphocytes. Emergence of rIL-4 responsiveness concomitant with the injury favors the second explanation, although the two suppositions are not mutually exclusive.

Previous studies have shown that stimulation of T-cell proliferation does not occur exclusively through IL-2/IL-2-receptor interactions, but can also be induced by the IL-4/IL-4-receptor interaction in an independent manner (12). The pathways of differentiation of $CD4^+$ and $CD8^+$ T cells into T helper 1 (Th1)/Th2 and T cytotoxic 1 (Tc1)/Tc2 subsets has been recognized (13), where Th1/Tc1 produce mainly IL-2 and interferon gamma, while Th2/Tc2 secrete chiefly IL-4, IL-5 and IL-10 (13-15). PBLT lymphocytes from normal hamsters respond predominantly to IL-2 (Th1/Tc1 pattern(s)) (9). Here we have shown that evolution of Bleo-induced lung injury is associated with unresponsiveness to IL-2 and concomitant acquisition of, albeit short-term, responsiveness to IL-4 i.e., immune deviation of PBLT lymphocytes toward Th2/Tc2 pattern(s). The maximal response to IL-4 on day +7 points to the inherent complexity of balance shifts between Th1 and Th2 T cell subpopulations. It has been argued that Th1/Th2-dominated responses might be achieved by preferential expansion of

discrete populations or, alternatively, by a functional shift in the distribution of the cells toward one or the other extreme through upregulation of the specific receptor (16). Involvement of cytokines from tissue-infiltrated inflammatory cells in the pathophysiology of fibrosis has been suggested. Among these, IL-4 has been found to stimulate collagen gene expression and collagen synthesis, appointing this cytokines as a prime candidate in the development of the fibrotic process (17, 18).

The role of Th2/Tc2 type cells in Bleo-induced lung injury is still unclear. These cells may be autoprotective or autoreactive, and therefore either abate or enhance the lung injury. Bleo causes influx of eosinophils in bronchoalveolar lavage fluid and lung parenchyma, which reaches a peak on the seventh day after administration of the drug (8, 19). The eosinophilia is brought about by an increase in both IL-5 mRNA expression and IL-5 secretion, and their peaking on day +7 (20). The IL-4 responsiveness documented in the present study and the findings by Thrall et al (19) and Kermani-Gharace and Phan (20), emphasize the involvement of Th2 cells in the immunopathogenesis of lung fibrosis. In addition, the culmination of the immunological events on day +7 provide proof for the cascade of events taking place during Bleo injury. An elevation in IL-4 mRNA and IL-4 receptor mRNA during the course of Bleo-induced lung injury has been suggested (21). IL-4 expression in the lung may be critical to the fibrinogenic process, as this cytokine stimulates collagen production by fibroblasts (17, 18) and may generate fibroblast proliferation (22).

We have previously shown that CyA attenuates Bleo-induced lung injury (8), probably by decreasing the number of PBLT lymphocytes and delaying their activation. This implies the existence of an early vulnerable period, during which T cells play an active role in the establishment of Bleo-induced pulmonary injury. This biphasic effect of CyA must be ascribed to the irreversible induction of T lymphocyte competence and progression in this animal model of Bleo-induced lung fibrosis. Selective effects of CyA on Th1 and Th2 subsets have been reported (23). It should be noted, however, that the effects of CyA do not remain limited to lymphocytes, but have been shown to inhibit lipopolysaccharide-induced MHC antigen expression and to block the extracellular production of the tumor necrosis factor (24, 25).

Whether all or part of these above activities affect CyA-induced amelioration of Bleo-injury awaits further investigation. A variety of inflammatory cytokines (e.g., transforming growth factor B, tumor necrosis factor) have been implicated in the pathogenesis of Bleo-induced pulmonary injury (1). However, the role of cell-mediated immune processes in the evolvement of interstitial pulmonary disorders is controversial (2-7, 26). Janick-Buckner et al. (3) demonstrated that depletion of specific T-cell subpopulations by cytotoxic monoclonal antibodies had no demonstrable effect on a Bleo-induced increase in hydroxyproline

levels in C57BL/6J mice. In contrast, these authors discovered in the same murine model an increased number of lymphocytes expressing the IL-2 receptor in the cell population obtained by broncho-alveolar lavage (27).

Furthermore, decrease in hydroxyproline has been noted in thymectomized Bleo-treated rats (5), in rats following treatment with anti-lymphocyte globulin (4), and in mice receiving anti-CD3 monoclonal antibody (28). In addition, treatment of Bleo-resistant BALB/c mice with cyclophosphamide has been shown to result in enhanced fibrosis (7). Relative resistance to Bleo was reinstated when mice thus treated received splenocytes. The authors therefore proposed that cyclophosphamide-sensitive suppressor T cells might regulate the immune response of BALB/c mice to Bleo-induced injury (7). Our study supports T-cell-mediated pathogenesis of Bleo-induced lung inflammation and fibrosis. Further studies are required to elucidate the link between Th1/Th2, Tc1/Tc2 subsets, anti-IL-4 (in vitro and in vivo), other lymphocytes including their cytokine production and the development of interstitial pulmonary injury.

Acknowledgments

This study was supported in part by the Robert A. Rosenblum Research Fund and by the Charlotte and Louis Kaitz Boston University School of Medicine-The Hebrew University-Hadassah Medical School Exchange Program, and the Israeli Ministry of Health.

References

1. P.F. PIGUET, Int. Rev. Exp. Pathol. **34b** 173-181 (1993).
2. S.V. SZAPIEL, N.A. ELSON, J.D. FULMER, G.W. HUNNINHAKE and R.G. CRYSTAL, Am. Rev. Respir. Dis. **120** 893-899 (1979).
3. D. JANICK-BUCKNER, G.E. RANGES and M.P. HACKER, Toxicol. Appl. Pharmacol. **100** 474-484 (1989).
4. R.S. THRALL, J.R. MCCORMICK, S.H. PHAN, R.H. JACK and P.A. WARD, Am. Rev. Respir. Dis. **119** (Suppl) 83 (1979) (abstr).
5. R.S. THRALL, E.J. LOVETT III, R.W. BARTON, J.R. MCCORNICK, S.H. PHAN and P.A. WARD, Am. Rev. Respir. Dis. **121** (Suppl) 99 (1980) (abstr).
6. D.J. SCHRIER, S.H. PHAN and B.M. MCGARRY, Am. Rev. Respir. Dis. **127** 614-617 (1983).
7. D.J. SCHRIER and S.H. PHAN, Am. J. Pathol. **116** 270-278 (1984).
8. I.S. LOSSOS, R. OR, R.H. GOLDSTEIN, M.W. CONNER and R. BREUER, Exp. Lung Res. **22** 337-349 (1996).
9. R. OR, I.S. LOSSOS, E. HIRSCHFELD and R. BREUER, Exp. Lung Res. **22** 245-253 (1996).
10. L.E. SENDELBACH, R.C. LINDENSCHMIDT and H.P. WITSCHI, Toxicol. Lett. **26** 169-173 (1985).
11. R. OR, J. KOVAR, J. DOMENICO and E.W. GELFAND, Clin. Immunol. Immunopathol. **62** 314-320 (1992).

12. R. OR, H. RENZ, N. TERADA and E.W. GELFAND, Clin. Immunol. Immunopathol. **64** 210-217 (1992).
13. T.R. MOSMANN and S. SAD, Immunol. Today **17** 138-146 (1996).
14. S. TREMBLEAU, T. GERMANN, M.K. GATELY and L. ADORINI, Immunol. Today. **16** 383-386 (1995).
15. R.A. SEDER and W.E. PAUL, Annu. Rev. Immunol. **12** 635-673 (1994).
16. A. KELSO, Immunol. Today **16** 374-379 (1995).
17. P. GILLERY, C. FERIN, J.F. NICILAS, F. CHASTANG, B. KALIS, J. BANCHGEREAU and F.X. MAQUHART, FEBS Lett. **302** 231-234 (1992).
18. C. FERTIN, J.F. NICOLAS, P. GILLERY, B. KALIS, J. BANCHEREAU and F.X. MAQUART, Cell. Molec. Biol. **37** 823-829 (1991).
19. R.S. THRALL, J.R. MCCORMICK, R.M. JACK, R.A. MCREYNOLDS and P.A. WARD, Am. J. Pathol. **95** 117-130 (1979).
20. KERMANI-GHARACE M and PHAN SH, Am. J. Respir. Cell Mol.Biol. **16** 438-447 (1997).
21. C.M. BAECHER-ALLAN and R.K. BARTH, Regional Immunol. **5** 207-217 (1993).
22. R.P. PHIPPS, D.P. PENNY, P.C. KENG and G. SEMPOWSKI, Am. Rev. Respir. Dis. **145** A1732 (1992).
23. P.A. BRETSCHER and C. HAVELE, Eur. J. Immunol. **22** 349-355 (1992).
24. P.F. HALLORAN, J. URMSON, S. FARKAS, R.A. PHILLIPS, G.FULOP, S. COCKFIELD and P. AUTENRIED, Transplantation **46** 685-725 (1988).
25. D.T. NGUEN, M.K. ESKANDARI, L.E. DEFORGE, C.L. RAIFORD, S.L. KUNKEL and D.G. REMICK, J. Immunol. **144** 3822-3828 (1990).
26. R.S. THRALL and R.W. BARTON, Am. Rev. Respir. Dis. **129** 279-283 (1984).
27. D. JANICK-BUCKNER, G.E. RANGES and M.P. HACKER, Toxicol. Appl. Pharmacol **100** 465-473 (1989).
28. S.K. SHARMA, J.A. MACLEAN, C. PINTO and R.L. KRADIN, Am.J. Respir. Crit. Care Med. **154** 193-200 (1996).