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T-cell lung granulomas induced by sepharose-coupled *Mycobacterium tuberculosis* protein antigens: immunosuppressive phenomena reversed with cyclophosphamide and indomethacin

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SUMMARY

We induced lung granulomas in BALB/c mice by intratracheal instillation of Sepharose beads coated with a *Mycobacterium tuberculosis* protein extract. Granulomas composed of macrophages and lymphocytes were induced. The granulomatous reaction reached its peak 3–7 days after challenge and lasted for approximately 1 month. Immunolabelling of tissue sections and bronchial washings revealed that granulomas were predominantly composed of T lymphocytes with the cytotoxic-suppressor phenotype ($CD8^+$). Granulomas were associated with a significant decrease in anti-mycobacterial immunity manifested by a drop in delayed-type hypersensitivity reactions and antibody titres. The immunosuppressive phenomena were abolished with cyclophosphamide or indomethacin. Control granulomas induced with methylated bovine serum albumin (BSA) were smaller and composed by similar numbers of $CD4^+$ and $CD8^+$ cells. BSA granulomas did not alter antibody titres but they decreased delayed-type hypersensitivity to BSA which was restored to normal with indomethacin but not with cyclophosphamide. Our findings show that mycobacterial proteins anchored to Sepharose beads are granulomatogenic and that they preferentially recruit $CD8^+$ cells which, together with locally produced prostaglandins, down-modulate cell-mediated and humoral immunity to mycobacterial antigens.

INTRODUCTION

The failure of recent bacillus Calmette-Guérin (BCG) vaccination trials[1] together with its re-emergence in developed countries, show that tuberculosis is not yet under control as formerly believed.[2] It seems now that the control of this infection will require a much better understanding of the interactions between *Mycobacterium tuberculosis* and its hosts. When *M. tuberculosis* infects an appropriate host, it triggers complex and varied effects: these include the well-known adjuvant effect of cell wall components, non-specific activation of the monocyte–macrophage system, and strong antibody and cell-mediated immunity responses.[3] Mycobacteria might also trigger immunosuppressive[4] and autoimmune phenomena[5] and in tissues, *M. tuberculosis* evokes a distinctive type of inflammatory response, the granuloma, which is the histologic hallmark of tuberculosis.

The granulomatous reaction seems to have a dual effect. In some individuals it may result in tissue damage including necrosis and fibrosis while in others it is related to selfhealing and immune resistance.[6] In this regard, it is known that individuals anergic to purified protein derivative (PPD) with advanced tuberculosis exhibit poorly developed granulomas.[7] Moreover, in patients with the acquired immunodeficiency syndrome mycobacterial granulomas are poorly developed or absent.[8] Further observations linking granulomas and cell-mediated immunity are the lack of this response in experimental animals deprived of thymus[9] and immunohistochemical findings documenting predominant T-cell participation in granuloma cell composition.[10,11] Therefore, it would be worthwhile to learn about the granulomatogenic substances in the tubercle bacillus and the events taking place locally within the granuloma, for this would further our understanding of antimycobacterial immunity. This work was aimed to assess the granulomatogenic potential of mycobacterial proteins, the cells involved in granuloma formation and some associated immune phenomena. For this purpose, Sepharose beads with covalently linked *M. tuberculosis* culture filtrate proteins were injected intratracheally into the lungs of BALB/c mice.

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MATERIALS AND METHODS

Animals

All experiments were performed on BALB/c male mice bred in

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our own facilities. Mice were given routine animal chow and chlorinated water *ad libitum* until they were used, usually at 6–8 weeks of age.

Growth of Mycobacterium tuberculosis *and immunization of mice*

Mycobacterium tuberculosis strain H37/Rv was grown in Proskauer and Beck medium. After 5–6 weeks the culture medium was filtered to eliminate bacilli and the culture filtrate proteins (CFP) were precipitated with 45% $(NH_4)_2SO_4$. Phenylmethylsulphonyl fluoride (Sigma Chemical Co., St louis, MO) was added as enzymatic inhibitor before dialysis in phosphate-buffered saline (PBS). Methylated bovine serum albumin (mBSA) was purchased from Sigma. Mice were immunized with two subcutaneous and intraperitoneal injections of CFP or mBSA, 1 week apart. For the first immunization, 50 μg of the antigen were emulsified in complete Freund's adjuvant and for the second immunization 25 μg of the antigen were dissolved in saline.

Coupling of antigens to Sepharose beads and induction of pulmonary granulomas

Thirty micrograms of CFP or mBSA were coupled to 15 000 cyanogen bromide-activated Sepharose 4-B beads (Sigma) following the manufacturers' instructions. Ten days after the second immunization, pulmonary granulomas were induced by intratracheal injection of 100 μl of PBS containing about 15 000 beads coated with CFP (Mtb-beads) in mice sensitized with CFP. Mice sensitized with mBSA were injected intratracheally a similar number of mBSA-coated beads (mBSA-beads). In control experiments mice sensitized with either CFP or mBSA received intratracheally about 15 000 Sepharose beads whose active groups had been blocked with 0·2 M, pH 8 glycine (uncoated beads). For the intratracheal injection of beads, mice were anaesthetized with 56 mg/kg body weight sodium penthotal. The trachea was exposed by a small incision in the neck region and beads were injected through a catheter placed into the trachea. Mice were maintained erect until the effects of anaesthesia passed. Under these conditions animal mortality was kept very low.

Obtention of cells by bronchoalveolar lavage (BAL)

Mice were anaesthetized and killed by exsanguination cutting the axillary blood vessels. To collect inflammatory cells by bronchial washing, the thoracic cavity was opened, a catheter was placed into the trachea and the lungs were washed six times with 1 ml of PBS. The cell suspension was centrifuged at 600 ***g*** for 10 min and the cells were counted in a haemocytometer. Smears stained with Giemsa were used for differential cell counts. For lymphocyte phenotyping, smears were fixed in cold acetone for 5 min, rinsed and incubated for 30 min with biotin-labelled monoclonal antibodies to murine CD4 and CD8 (Becton Dickinson, San Jose, CA) diluted 1/25 in PBS. Bound antibodies were detected with a streptavidin–horseradish peroxidase conjugate (Zymed Laboratories, San Jose, CA) diluted 1/10 in PBS. Peroxidase was revealed with H_2O_2 and 4-chloro-1-naphthol.

Preparation of tissue sections for histologic examination and immunohistochemistry

Mice were killed at 1, 3, 7, 14, 21 and 28 days after bead injection. For histologic studies the lungs were perfused with 10% buffered formalin for 24 hr and embedded in paraffin. Sections of 8 μm thickness taken through the hilus were stained with haematoxylin & eosin. For immunohistochemical studies, the lungs were perfused with OCT compound (Miles Laboratories, Elkhart, IN), frozen in liquid nitrogen, and sectioned with a cryostat. Slides were fixed for 1 min in cold acetone and stored at −70° in sealed boxes until use. Biotin-labelled monoclonal antibodies used for phenotyping of BAL cells were used for the immunohistochemical analysis of lung tissue. In addition, immunohistochemistry with peroxidase-labelled antisera to mouse IgG and IgM (Cappel Laboratories, Durham, NC) was performed. Slides were counterstained with methyl green. Macrophages were identified with non-specific esterases staining.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot

The culture filtrate proteins precipitated with $(NH_4)_2SO_4$ were extensively dialysed, concentrated and separated by SDS–PAGE under reducing conditions in a discontinuous buffer system on slab gels of 10% acrylamide. Gels were mounted in an electrontransference chamber (Idea Scientific, Corvallis, PA) and transferred to nitrocellulose sheets (BioRad Laboratories, Richmond, CA). Unbound sites on the paper were blocked with 3% BSA in PBS–Tween 20. Stripes were incubated with mice sera diluted 1/100 for 1 hr at 37°. After rinsing, bound antibodies were revealed with an antimouse IgG goat antiserum labelled with peroxidase (Cappel Laboratories) which was revealed with H_2O_2 and 4-chloro-1-naphthol for 15 min.

Measurement of delayed type hypersensitivity (DTH) and of antibody titres by enzyme-linked immunosorbent assay (ELISA)

Each mouse received a footpad intradermal injection of 25 μg of CFP or mBSA in 25 μl of PBS. For control, the contralateral food pad was injected with PBS only. Footpads were measured with an engineer's caliper before and 24 hr after intradermal injection of antigen. Control foot pad thickness was subtracted from that of test mice and expressed in microns. For ELISA, sera were processed in one session and two replicates per serum were included. Wells were sensitized with 0·5 μg of CFP or mBSA in 100 μl of carbonate buffer, pH 9·6, overnight at 37°. Blocking of possible remaining active sites in the plastic was done with 3% BSA in PBS–Tween 20 for 1 hr at 37°. Wells sensitized with mBSA were blocked with 1% skimmed powdered milk in PBS–Tween 20 for 1 hr at 37°. Test sera were applied to the wells diluted 1/1000 in PBS–Tween 20 for 1 hr at 37°. After rinsing, a peroxidase-labelled goat antibody to mouse IgG diluted 1/1000 in PBS–Tween 20 was applied to the wells.

Pharmacological manipulation with cyclophosphamide and indomethacin

The aim of these experiments was to investigate if prostaglandins or cells sensitive to cyclophosphamide (CY) were involved in the drop of antibody and DTH responses observed in mice with lung granulomas. It is well known that indomethacin (IND) blocks the synthesis of prostaglandins[12] and that low CY doses abrogate a mechanism tending to down-regulate

DTH responses without affecting antibody responses.[13] One day before intratracheal challenge each mouse received a single intraperitoneal injection of 20 mg CY (Sigma) per kilogram of weight dissolved in 100 μl saline. Indomethacin (Sigma) was first dissolved in 0·1 M NaOH in saline. Each mouse was administered intraperitoneally 5 mg of IND per kilogram of weight, daily during the complete experimental procedure.

Statistics

Variance analysis was used to evaluate differences between groups.

RESULTS

SDS–PAGE and immunoblot of mycobacterial proteins used to coat Sepharose beads

As previously reported[14] Coomassie Blue-stained SDS–PAGE gels of the culture filtrates used to coat Sepharose beads showed about 55 protein bands (not shown). In order to estimate the efficiency of the procedure to couple CFP to Sepharose, mice were immunized with Mtb-beads in incomplete Freund's adjuvant and sera were processed for immunoblot. There was recognition of 19 bands indicating that many of the CFP antigens adhered to the beads and that they were inducing an antibody response in immunized mice. The diffuse band characteristic of antibodies against lipoarabinomannan was not observed (not shown).

Histologic changes

The extent of inflammation around 10–20 beads per animal was estimated in μm^2 with an eyepiece micrometer (Zeiss Jena, Germany). Around Mtb-beads there was an intense granulomatous-type inflammation (Fig. 1) which reached its peak (Fig. 2) 3 days after challenge ($22 \times 10^3 \mu m^2 \pm 2{\cdot}3$ SD). Thereafter, inflammation declined progressively, to virtually

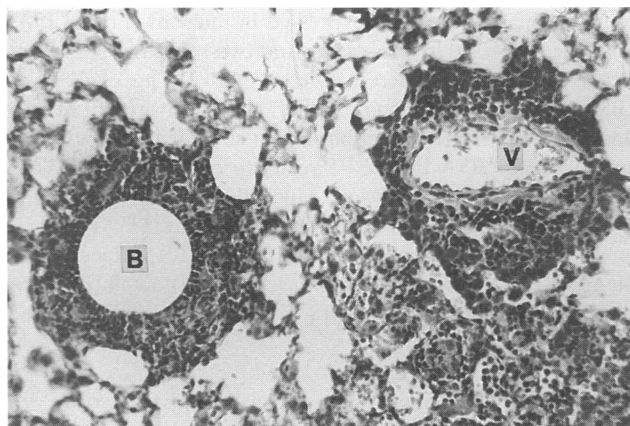

Figure 1. Photomicrograph of a 3-day granuloma of a mouse challenged with Sepharose beads coated with a protein extract obtained from *M. tuberculosis* culture filtrates. Macrophages and lymphocytes surround the bead (B). On the right there is a medium-size vein (V) with intramural and perivascular mononuclear inflammation. Hematoxylin & eosin, ×52.

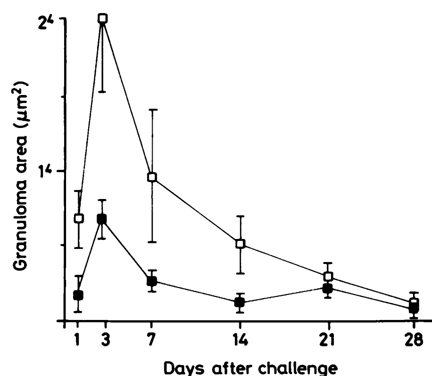

Figure 2. Surface area of granulomatous inflammation around beads coated with mycobacterial proteins (□) or with methylated bovine serum albumin (■). The results of five or six experiments with two or three mice are expressed as the mean and standard deviation.

disappear at the end of the study (28 days). At 24 hr, the earliest period examined, most cells were non-specific esterase-positive macrophages in direct contact with the beads. At 3 days and after, lymphocytes were predominant and macrophages were displaced to the periphery of the granuloma. Multinucleated giant cells and polymorphonuclear leucocytes were not found. The inflammatory response to mBSA-beads was less intense but similar in cell composition and time course (Fig. 2). Mtb-granulomas were significantly bigger than mBSA granulomas at 3 ($P < 0{\cdot}05$), 7 ($P < 0{\cdot}001$) and 14 days ($P < 0{\cdot}4$) post-challenge. Other changes observed were the infiltration of vein walls by lymphocytes and macrophages (Fig. 1), paracortical hyperplasia of hilar lymph nodes and an apparent increase in bronchus-associated lymphoid tissue. These changes were more intense in mice challenged with Mtb-beads than with mBSA-beads and they followed a time course similar to that of the granulomatous reaction. Uncoated beads elicited a poor reaction with a single layer of non-specific esterase-positive macrophages embracing the beads.

Analysis of BAL cells on Giemsa-stained smears

In unchallenged control mice the total number of cells per mouse was 600×10^3; of these, 95% were macrophages and 5% were lymphocytes. In mice challenged with Mtb-beads the number of cells was conspicuously increased (Fig. 3). At the peak of the granulomatous reaction (3 days), there were $4{\cdot}35 \times 10^6$ cells (87% lymphocytes, 11% macrophages, 2% polymorphonuclear leucocytes). Thereafter, cellularity declined to reach control values at 3 weeks postchallenge. In animals challenged with mBSA beads inflammation was much less intense and similar numbers of lymphocytes and macrophages were observed (Fig. 3).

Phenotyping of T lymphocytes by immunohistochemistry of lung sections and BAL cells

In mice challenged with Mtb-beads, $CD8^+$ cells predominated over $CD4^+$ lymphocytes at all times (except at 24 hr), in both bronchial washings and tissue sections (Fig. 4 and Fig. 5). This difference was significant in tissue sections at days 3 ($P < 0{\cdot}002$) and 7 ($P < 0{\cdot}003$) and in bronchial washings at day 7

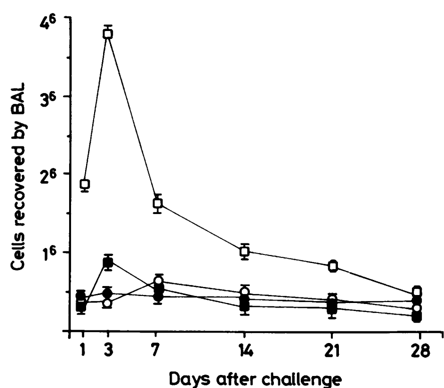

Figure 3. Differential cell counts made on Giemsa-stained smears of lung cells recovered by BAL of mice with lung granulomas. At least 100 cells per mouse were counted. Lymphocytes, Mtb granulomas (□); macrophages, Mtb granulomas (○); lymphocytes, mBSA granulomas (●); macrophages mBSA granulomas (■). The results are expressed as the mean and standard deviation.

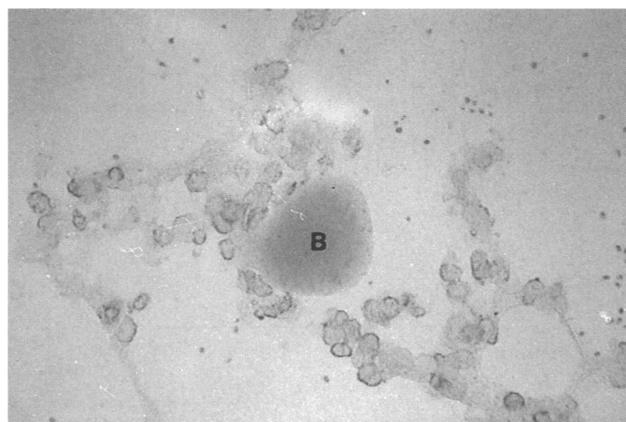

Figure 5. Immunohistochemistry of a 7-day lung granuloma induced by mycobacterial proteins. Frozen sections were incubated with a monoclonal antibody to $CD8^+$. Most cells around the bead (B) show membrane labelling, × 204.

($P < 0{\cdot}002$) after challenge. A similar CD4/CD8 ratio was observed in the interstitium and blood vessels. In mice challenged with mBSA-beads, similar numbers of $CD4^+$ and $CD8^+$ cells were observed at all times in both tissue sections and BAL cells (Fig. 4). Immunohistochemistry of lung sections with anti-IgG and anti-IgM antibodies showed no participation of antibody-producing cells in granuloma formation.

Mtb-bead granulomas decrease antibody and DTH responses to mycobacterial antigens. Reversal with cyclophosphamide and indomethacin

Induction of lung granulomas with beads coated with mycobacterial proteins was associated with a drop in IgG antibody titres and in DTH to CFP (Fig. 6 and Fig. 7). The fall in antibody titres was sudden and statistically significant at 3 and 7 days postchallenge ($P < 0{\cdot}05$ and $P < 0{\cdot}02$, respectively) which were the periods of the maximal granulomatous

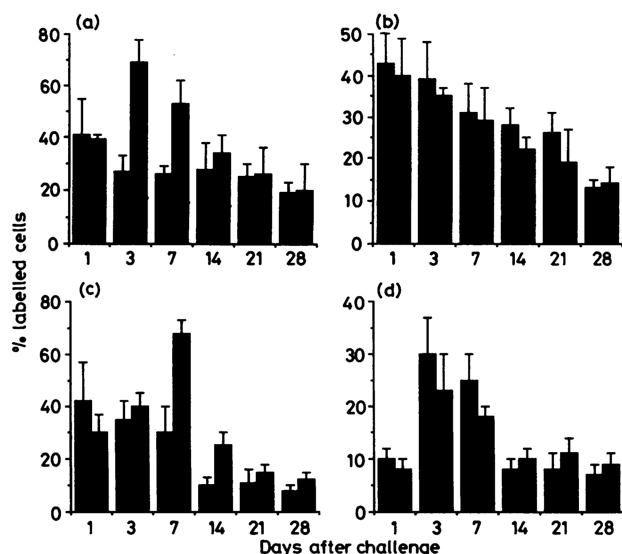

Figure 4. Percentages of $CD4^+$ (black bars) and $CD8^+$ (stripped bars) T-cell subsets in tissue sections (a) and BAL cells (c) of Mtb-bead granulomas and in tissue sections (b) and BAL cells (d) of mBSA granulomas. At least 100 BAL cells per mouse were counted. The cells of at least 10 randomly chosen granulomas per mouse were counted. The results of two to four experiments on two or three mice are expressed as the mean and standard deviation.

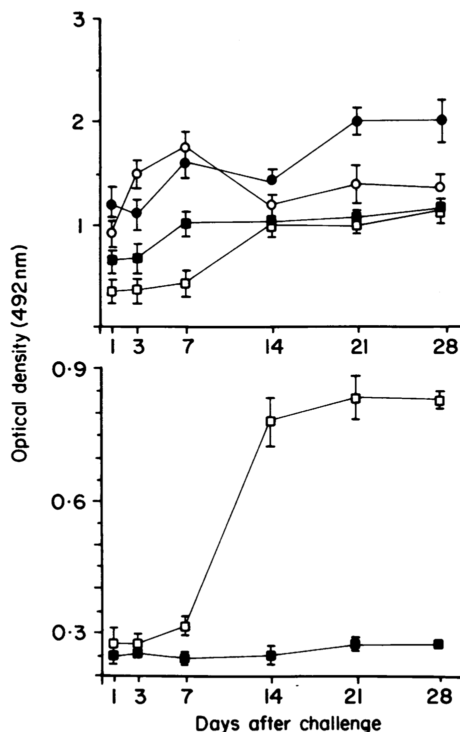

Figure 6. Anti-mycobacterial antibody titres (top panel) by ELISA in mice with Mtb-bead granulomas (□) and the effects of pharmacological manipulation with cyclophosphamide (○) or indomethacin (●) on the antibody response. As a control, antibody titres were measured in mice primed with CFP and injected intratracheally with uncoated beads (■). Anti-mBSA antibody titres (bottom panel) in mice with mBSA-bead granulomas (□) and in control mice primed with mBSA and injected intratracheally with uncoated beads (■).

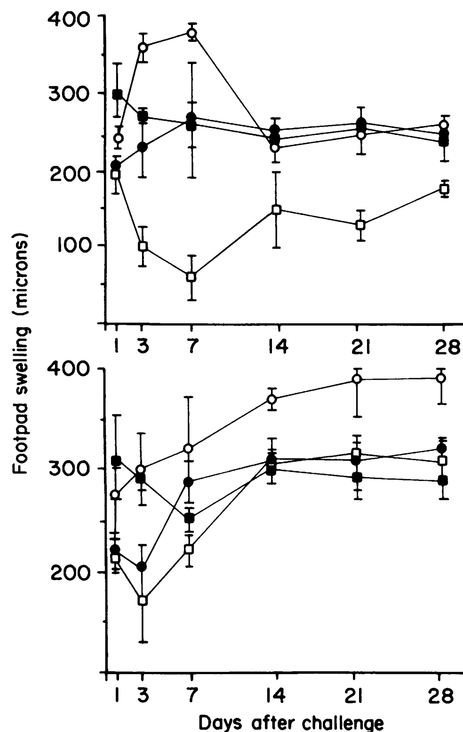

Figure 7. Delayed-type hypersensitivity (DTH) response to mycobacterial antigens (top panel) in mice with Mtb-bead granulomas (□) and after treatment with cyclophosphamide (■) or indomethacin (○). Control footpad swelling of mice which were injected with uncoated beads is shown (●). DTH response to mBSA (bottom panel) in mice with mBSA granulomas (□) and after pharmacological manipulation with indomethacin (○) or cyclophosphamide (●). Control mice primed with mBSA in complete Freund's adjuvant were inoculated intratracheally with uncoated beads (■).

response. The possibility that beads behaved as 'sponges' adsorbing antibody was ruled out by showing that overnight incubation of sera with 15000 Mtb-beads did not deplete antibody to the extent they did *in vivo* (not shown). The drop in DTH was also higher at 3 and 7 days postchallenge ($P < 0{\cdot}04$ and $P < 0{\cdot}02$, respectively). Treatment of mice with CY or IND restored antibody and DTH responses to the values observed in control mice challenged with uncoated beads (Fig. 7). With both drugs the increase in antibody titres and DTH was statistically significant at days 3 and 7. In mice with mBSA granulomas, antibody titres to mBSA increased sharply after challenge, well above the levels seen in control mice which received uncoated beads (Fig. 6). With mBSA granulomas, DTH reactions fell significantly at days 1, 3 and 7 postchallenge (Fig. 7). This drop was reversed with IND ($P < 0{\cdot}02$) but not with CY. Pharmacological manipulation with CY and IND also altered the granulomatous response and the cell composition of bronchial washings. With both drugs there was a striking reduction in Mtb granuloma size (not shown) and CY, but not IND, altered BAL cell-composition, markedly lowering the number of lymphocytes (not shown).

DISCUSSION

The present work shows that *M. tuberculosis* proteins coupled to Sepharose beads induce granuloma-like inflammatory reactions in the lungs of BALB/c mice and that these reactions down-regulate antimycobacterial immunity. Granulomas were characterized as cell-mediated immunity reactions since they were predominantly composed of T cells with the cytotoxic/suppressor phenotype ($CD8^+$), while granulomas induced with mBSA were composed of similar numbers of $CD4^+$ and $CD8^+$ cells. Immunohistochemical investigations of T cells within tuberculosis granulomas have yielded dissimilar results. In some studies a predominance of $CD4^+$ cells over $CD8^+$ cells have been reported[11] while in others the $CD8^+$ lymphocyte has been the most abundant cell.[10,15]

Although the $CD4^+$ lymphocyte is widely considered as the key cell in the regulation of antimycobacterial immunity,[6,16–18] our current observations and other recent findings show that $CD8^+$ cells might also be important. A striking $CD8^+$ T-cell response of human and mouse lymphocytes to mycobacteria has been reported.[5,16,18] Furthermore, passively transferred $CD8^+$ lymphocytes are protective and *in vivo* depletion of these cells with monoclonal antibodies facilitates infection.[18,19] The antimycobacterial potential of $CD8^+$ cells could be related to the secretion of interferon-γ and the lysis of macrophages primed with mycobacteria.[16] In addition, $CD8^+$ cells may participate in the immunosuppressive phenomena of tuberculosis.[20] In this regard, it is of interest that the $CD8^+$-rich granulomas induced with mycobacterial proteins coupled to Sepharose beads were associated with down-regulation of antimycobacterial immunity. Indeed, following intratracheal instillation of Mtb-beads, there was a fall in antibody titres while the specific antibody response of mice with mBSA granulomas increased sharply as expected from a recall response. Of more significance perhaps was the anergy associated with granuloma formation, this manifested by a sustained and significant fall of DTH to mycobacterial antigens.

Although hypersensitive granulomas of sarcoidosis can be associated with down-regulation of DTH responses,[21] a similar role for tuberculosis granulomas has not been acknowledged in the literature. This phenomenon is of interest for the relevance it could bear on the pathogenesis of tuberculosis and because it contradicts the common view that granuloma formation indicates enhanced immune resistance to mycobacteria.[6,22] These findings should be added to others showing that immunosuppression contributes to the maintenance of tuberculosis. As shown by skin and *in vitro* tests, patients with advanced tuberculosis are frequently anergic to mycobacterial antigens.[7] Similar observations have been made in mice infected experimentally with mycobacteria.[23] Macrophagic cells which are activated to release factors that inhibit CD4 responses and cytokine production participate in the immunosuppressive phenomena of tuberculosis.[24–26] It has been reported that $CD8^+$ T cells activated by mycobacterial components might also inhibit lymphocyte blastogenesis.[20] In the development of down-regulation of immunity in tuberculosis, disturbances in the T-helper-cell balance (Th1/Th2) should be also considered. In lepromatous leprosy high mRNA of Th2 cytokine has been found.[26] It is also of interest that $CD8^+$ cells have shown to be an important source of the Th2 cytokine interleukin-4.[27] With regard to the mycobacterial substances involved, it has been shown by *in vitro* studies that cell-wall carbohydrates[20,24] and lipids including

lipoarabinomannan[28] are immunosuppressive. An additional contribution of this work, therefore, is to show that mycobacterial proteins can also down-regulate antimycobacterial immunity.

Cyclophosphamide-sensitive cells and prostaglandins in the immunosuppression triggered by the granulomatous response to Sepharose-coupled mycobacterial proteins participate. Indeed, treatment with CY and IND restored depressed antibody and DTH responses to normal. It has been reported that prostaglandin E_2 activates T cells to release factors that suppress T- and B-cell mitogenesis[12] and that low doses of CY such as those used in this work can augment DTH reactions without affecting antibody production.[13] The CY effects have been related to depletion of a suppressor T-cell population.[13]

Although a role for prostaglandins in the suppression of immunity observed in some bacterial and fungal diseases has been proposed,[12] information concerning a similar role for prostaglandins in tuberculosis is scarce. It has been reported that IND increases DTH and blastogenic responses to *Mycobacterium bovis*[29] and that *in vitro* stimulation of macrophages by BCG causes an increase in prostaglandin production.[30]

Finally, concerning the down-modulation of antimycobacterial immunity observed in these experiments, it must be considered that, regardless of the antigens involved, very efficient mechanisms operate in the lung that down-regulate immune responses.[31]

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