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Pathogenesis of Tuberculosis in Mice Exposed to Low and High Doses of an Environmental Mycobacterial Saprophyte before Infection

ROGELIO HERNANDEZ-PANDO,[1] LENIN PAVÖN,[1] KUTZI ARRIAGA,[1] HECTOR OROZCO,[1] VICENTE MADRID-MARINA,[2] AND GRAHAM ROOK[3]*

Experimental Pathology Laboratory, Department of Pathology, Instituto Nacional de la Nutricion "Salvador Zubiran," Mexico City,[1] and Division of Molecular Virology, Infectious Disease Research Centre, Public Health Institute, Cuernavaca, Morelos,[2] Mexico, and Department of Bacteriology, UCL Medical School, London, United Kingdom[3]

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Mycobacteria are ubiquitous in the environment, but they are not part of the normal human microbial flora. It has been suggested that variable contact with mycobacteria can influence susceptibility to mycobacterial pathogens and the efficacy of subsequent *Mycobacterium bovis* BCG vaccination. To test this, mice were immunized with high or low doses of an environmental saprophyte, *M. vaccae*, that is intensely immunogenic as an autoclaved preparation. Two months later, they received an intratracheal challenge with *M. tuberculosis* H37Rv. Recipients of a low Th1-inducing dose (10^7 organisms) were partially protected and maintained a high ratio of interleukin 2 (IL-2)-positive to IL-4-positive cells in the perivascular, peribronchial, and granulomatous areas of the lung, whereas in unimmunized controls the IL-4-positive cells increased markedly between days 21 and 28. In contrast, recipients of the high dose (10^9 organisms), which primes Th2 as well as Th1 cytokine production, died more rapidly than unimmunized controls and showed massive pneumonia from day 7. The ratio of IL-2-positive to IL-4-positive cells in all compartments of the lung rapidly fell to 1 by day 14 for these animals. These events correlated with cytokine mRNA profiles and with increases in the local toxicity of tumor necrosis factor alpha (TNF-α), demonstrable only when a major Th2 component was present. These data indicate that cross-reactive epitopes present in an environmental saprophyte can evoke either protective responses or responses that increase susceptibility to *M. tuberculosis*. The latter are associated with the presence of a Th2 component and increased sensitivity to TNF-α.

Immunity to pathogenic mycobacteria in mice requires a Th1 cytokine pattern (interleukin 2 [IL-2], gamma interferon [IFN-γ]) accompanied by a variety of cytotoxic T cells ($CD4^+$, $CD8^+$, $CD4^-$ $CD8^-$/CD1 restricted) and by tumor necrosis factor alpha (TNF-α). Antigens that are shared by many or all mycobacterial species are important targets of this immune response. For instance, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), derived from a strain of bovine tuberculosis, is protective against leprosy (10), and the 65-kDa heat shock protein of *M. leprae* is powerfully protective against tuberculosis in mice if administered in a form that induces a type 1 response (27). Similarly, recent large epidemiological studies have supported earlier observations (28) that variable resistance to mycobacterial pathogens, and variable protective efficacy of BCG in different parts of the world, can be attributed to variable exposure to mycobacteria in the environment, quite distinct from *M. tuberculosis*, *M. leprae*, or BCG (9). This is compatible with the fact that mycobacteria are ubiquitous but not part of the normal human flora, so the nature, route, and dose of exposure to environmental saprophytes are variables that depend on where and how each individual lives. We showed previously that one such environmental saprophyte, *M. vaccae* NCTC 11659, is intensely immunogenic even when killed by autoclaving and that a low dose (10^7 organisms) administered subcutaneously to mice will evoke a relatively pure Th1 pattern of response, whereas a high dose (10^9 organisms) administered by the same route evokes a mixed Th1 plus Th2 (or Th0) pattern of cytokine release (15). Moreover, delayed-type hypersensitivity (DTH) responses are evoked in animals following both immunization protocols, but the DTH response sites in the low-dose recipients (Th1) do not undergo necrosis when 1 μg of TNF-α is injected at 24 h, whereas the response sites in the high-dose recipients (Th1 plus Th2) are sensitive to TNF-α-induced tissue damage (15). This may explain the paradoxical role of TNF-α in tuberculosis, since it may protect via macrophage activation in Th1-dominated responses but may cause tissue damage when there is an inappropriate Th2 component.

In this study, we have examined the effects of these two contrasting saprophyte-induced response patterns in a well-documented model of pulmonary tuberculosis (14). We confirm that a Th1 response to the cross-reactive mycobacterial antigens is protective, but in addition we show that the mixed (Th1 plus Th2) response evoked by the higher dose has the reverse effect and actually increases susceptibility. It has been suggested that certain types of exposure of humans to some environmental mycobacteria lead to increased susceptibility to mycobacterial disease. Further epidemiological studies to confirm this are now justified.

MATERIALS AND METHODS

Experimental model of tuberculosis infection in mice. The experimental model of tuberculosis infection in mice has been described in detail elsewhere (13, 14). Briefly, male BALB/c mice were used at 6 to 8 weeks of age. Virulent *M. tuberculosis* H37Rv was cultured in Youman's modification of the medium of Proskauer and Beck. After 4 weeks of culture, the colonies were suspended in phosphate-buffered saline (PBS) containing 0.05% Tween 80 by being shaken for 10 min with glass beads. The suspension was centrifuged for 1 min at 350 × *g* to

* Corresponding author. Mailing address: Department of Bacteriology, UCL Medical School, Windeyer Building, 46 Cleveland St., London W1P 6DB, United Kingdom. Phone: 44 171 380 9489. Fax: 44 171 636 8175. E-mail: g.rook@ucl.ac.uk.

remove large clumps of bacilli. Then a preliminary bacterial count was made by smearing the supernatant at a known ratio of volume to area and counting 10 random fields after staining by the Ziehl-Neelsen technique. The suspension was finally diluted to 10^6 bacteria in 100 μl of PBS and aliquoted at −70°C. Before use, the bacteria were recounted, and viability was checked as described previously (15).

To achieve intratracheal infection, mice were anesthetized with 18 μl of thiopental (Pentothal) given intraperitoneally, equivalent to a dose of 56 mg/kg of body weight. The trachea was exposed via a small midline incision, and 10^6 viable bacteria in 100 μl of PBS were injected. The incision was then sutured with sterile silk, and the mice were kept vertical until the effects of the anesthestic had worn off. The infected animals were maintained in groups of five in cages fitted with microisolators. Mice were killed and exsanguinated at 1, 3, 7, 14, 21, 28, 60, and 120 days. The data are pooled from two experiments, each involving infection of 150 animals, i.e., 300 animals in total. Unless otherwise indicated, five mice from each of the three experimental groups were tested or sacrificed at each time point in each experiment. There were eight time points ($5 \times 3 \times 8 = 120$ mice/experiment; two experiments). The remaining animals (10/group = 30 mice) were left untouched to provide the survival data.

Preimmunization with autoclaved *M. vaccae*. *M. vaccae* NCTC 11659 was from a batch manufactured according to Good Manufacturing Practice at the Centre for Applied Microbiological Research, Porton Down, United Kingdom (batch MV001). This is the batch currently in use for trials in human tuberculosis. The manufacturing process involved culture on Sauton's medium solidified with 1.5% (wt/vol) agar. This medium contains no antigenic substances. The organisms were suspended in borate-buffered saline (pH 8.0) at 10^{10} ml^{-1} and autoclaved at 15 lb/in^2 for 15 min before storage in glass vials at 4°C. Mice were immunized by injection of 100 μl containing 10^7 or 10^9 organisms in pyrogen-free normal saline. All immunizations were subcutaneous, at the base of the tail. Two months later, these mice and unimmunized controls were infected with *M. tuberculosis* via the intratracheal route as described above.

Preparation of tissue for histology and immunohistochemistry. For histological study, the lungs were perfused via the trachea with 100% ethanol and immersed for 24 h in the same fixative. Parasagittal sections were taken through the hilus, and these were dehydrated and embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The following five parameters were then measured in square micrometers with a Zidas Zeiss image analysis system: area of peribronchial infiltration, area of perivascular infiltration, area of granuloma, area of interstitial inflammation, and percentage of lung affected by pneumonia.

For immunohistochemistry, lung sections were mounted on silane-coated slides and deparaffinized, and the endogenous peroxidase was quenched with 0.03% H_2O_2 in absolute methanol. The lung sections were incubated for 3 h at room temperature with biotin-labelled monoclonal antibodies against IL-2 or IL-4 (Genzyme, Boston, Mass.) diluted 1/50 in PBS. Bound antibodies were detected with avidin biotin peroxidase (Vector) and counterstained with hematoxylin. For quantification, five random fields of each pulmonary lobule in two different experiments using three mice from each treatment group were evaluated at ×400 magnification. Immunohistochemically positive and negative cells located in the alveolar-capillary interstitium, perivascular and peribronchial sites of inflammation, granulomas, and pneumonic areas were counted, and the number of positive cells was expressed as a percentage of the total number of cells present.

Measurement of DTH and TNF-α sensitivity of DTH sites. Culture filtrate was harvested from *M. tuberculosis* H37Rv grown as described above for 5 to 6 weeks. Then culture filtrate antigens were precipitated with 45% (wt/vol) ammonium sulfate, washed, and redissolved in PBS. For evaluation of DTH, each mouse received an injection of 20 μg of antigen in 40 μl of PBS in the hind footpad. In order to achieve very low nonspecific background swelling, the needle perforated the skin near the "heel." Then the needle travelled under the skin so that antigen was injected over the "palm," where swelling was also measured. We have found that ensuring the absence of skin puncture wounds at the site where the DTH is read reduces background and variability. The swelling at the palm was measured with an engineer's micrometer before and 24 h after the injection. In some experiments, recombinant murine TNF-α in pyrogen-free saline was injected into the DTH site immediately after the 24-h measurement, and the swelling was measured again 20 h later (i.e., 44 h after injection of the antigen). The TNF-α was supplied by Genzyme (lot no. B6548) and contained insignificant levels of lipopolysaccharide (0.017 ng/mg of TNF-α).

RT-PCR analysis of IFN-γ and IL-4 mRNA in lung homogenates. Lungs were removed and immediately frozen in liquid nitrogen. Hilar lymph nodes and thymic tissue were eliminated, and RNA was isolated by using Trizol (Gibco BRL, Gaithersburg, Md.) as described elsewhere (7). cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and oligo(dT) priming. Reverse transcription-PCR (RT-PCR) was performed as described previously (14), and PCR products were electrophoresed on 6% polyacrylamide gels and analyzed with an image densitometer (Bio-Rad, Hercules, Calif.) coupled to a computer program (Molecular Analyst 1.4). Expression of glucose 3-phosphate dehydrogenase was used to control for RNA content and integrity.

Statistical analysis. Student's *t* test was used for all data except the survival curves, for which the numbers of deaths in each group at each time point were compared by Fisher's exact test.

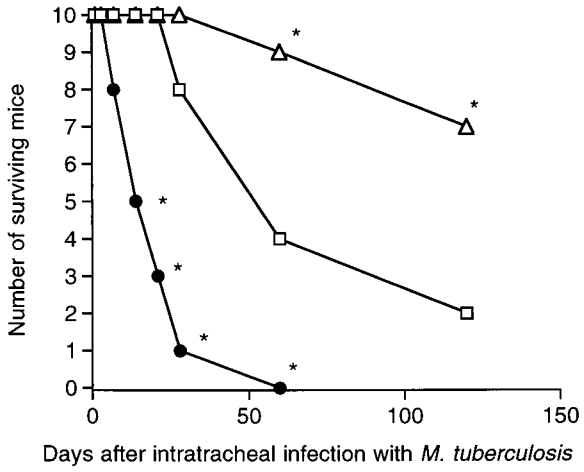

FIG. 1. Effects of priming with 10^7 (△) or 10^9 (●) autoclaved *M. vaccae* organisms or with saline (□) 2 months before intratracheal infection on the survival of BALB/c mice. Depending on the immunization protocol, the "common" epitopes can mediate responses causing either protection (△) or increased susceptibility (●). *, significant difference from result for saline controls as determined by Fisher's exact test. (Reprinted from the Annual Review of Microbiology [24] with permission from the publisher.)

RESULTS

Effect of preimmunization with *M. vaccae* on survival and pathology. Preimmunization with 10^7 autoclaved *M. vaccae* NCTC 11659 organisms 2 months before intratracheal infection caused significant prolongation of survival, as reported previously (24). Ninety percent of the mice were still alive at day 60, compared to 40% of the controls ($P = 0.029$, as determined by Fisher's exact test), and 70% were still alive at day 120, compared to only 20% of the controls ($P = 0.035$) (Fig. 1). This is the dose previously shown to evoke an optimal Th1 response, with TNF-α-insensitive DTH positivity (15).

In sharp contrast, preimmunization with 10^9 autoclaved *M. vaccae* organisms, previously shown to evoke a mixed Th1 plus Th2 response and TNF-α-sensitive DTH positivity, resulted in accelerated death so that 90% of the mice were dead by day 28 ($P = 0.003$ relative to unimmunized controls; at day 28, there were no deaths among recipients of 10^7 *M. vaccae* organisms).

The most striking correlate of these effects of preimmunization was seen in the analysis of the areas affected by pneumonia (Fig. 2a). Recipients of 10^9 *M. vaccae* organisms showed pneumonia affecting 4% of the lung as early as day 3 (Fig. 3A to C), reaching 15% by day 21, when there was still no pneumonia in the other groups. The area affected increased rapidly, reaching 28% by day 28 (Fig. 3D), when few animals survived. It is likely that it is the pneumonia that killed these animals. In control animals, pneumonia was not apparent until day 28 (7% of the lung) and then increased with time, affecting more than 30% of the lung by day 120 (Fig. 2 and 3F). Pneumonia appeared at the same time and to the same extent in recipients of 10^7 *M. vaccae* organisms, but it plateaued and failed to rise further, so less than 5% of the lung was affected in the animals sampled at day 120 (Fig. 2a and 3G).

The peribronchial and perivascular (Fig. 2b) compartments showed similar patterns of infiltration. This was apparent in unimmunized infected animals by day 1, and with minor fluctuations during the first 4 weeks, there was a steady increase throughout the experiment. In recipients of 10^7 *M. vaccae* organisms, the infiltration tended to be greater. This was apparent by day 1 and was particularly striking in the perivascular

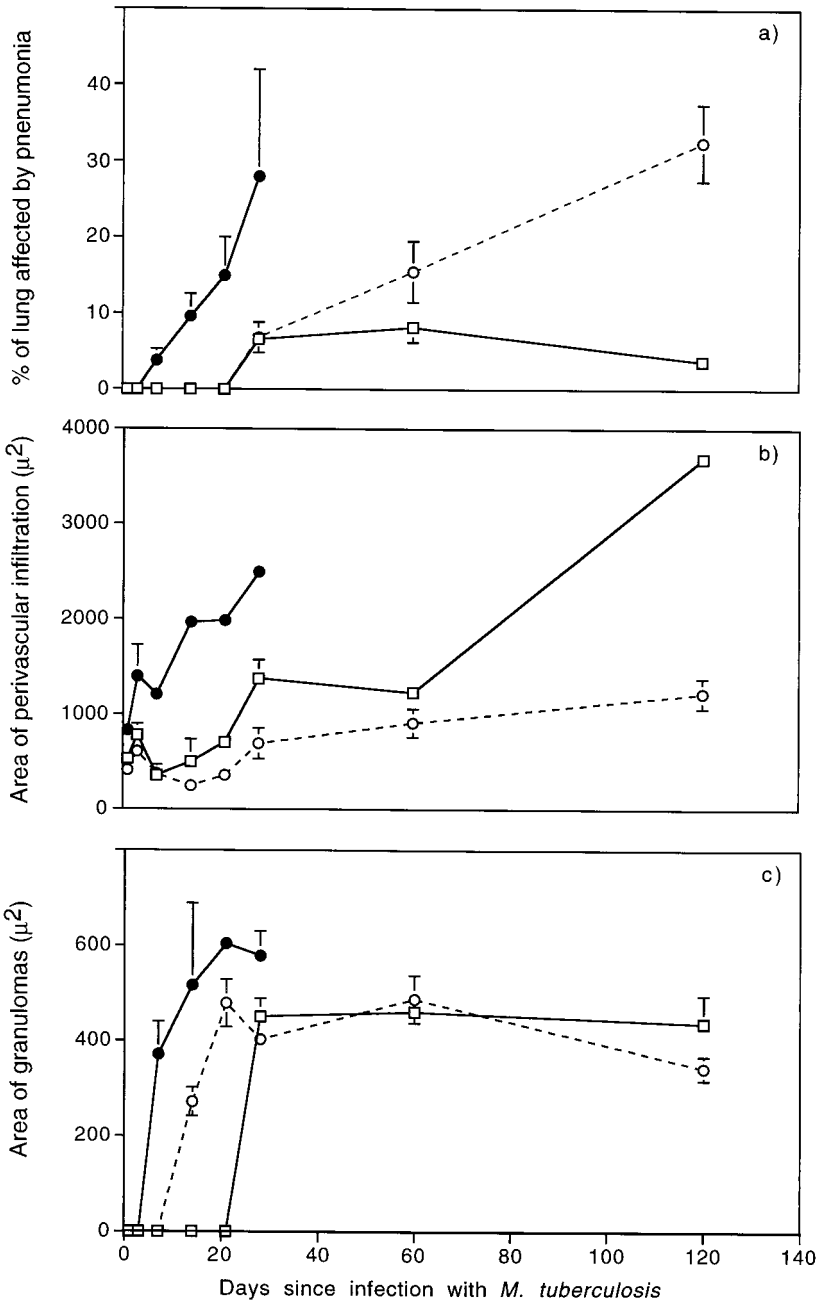

FIG. 2. Morphometric studies of the lungs of BALB/c mice at intervals after infection via the trachea with *M. tuberculosis* H37Rv, as described in the text. (a) Extent of pneumonia; (b) perivascular infiltration; (c) granuloma size. The animals had been preimmunized 2 months before infection with 10^7 (□) or 10^9 (●) autoclaved *M. vaccae* organisms. Control animals were not preimmunized (○). Data are means for 4 mice, and error bars indicate standard deviations (SD). Where no error bars are shown, the SD was too small to plot. Early onset of pneumonia in the recipients of 10^9 *M. vaccae* organisms correlated with accelerated death.

compartment of the immunized mice sampled on day 120, in which there was more than three times as much infiltrate as in the unimmunized controls. The recipients of 10^9 *M. vaccae* organisms showed much more rapid infiltration, which by 14 days was already greater than ever achieved in the control animals. In the few surviving animals at day 28, there were still massive infiltrates. No animals survived for analysis of later time points.

Interstitial infiltration was apparent in all groups by day 1 and striking by day 3. In the surviving mice (controls and recipients of 10^7 *M. vaccae* organisms), there was a further increase in interstitial infiltration between days 21 and 28, reaching a plateau that was then maintained until the end of the experiment (data not shown).

Granuloma formation was apparent in the control mice from day 14 and rose to a plateau from day 21. However, granuloma formation was delayed in the recipients of 10^7 *M. vaccae* organisms, in which granulomas were not seen until day 28, when they were similar in extent to those seen in the controls. In contrast, recipients of 10^9 *M. vaccae* organisms showed accel-

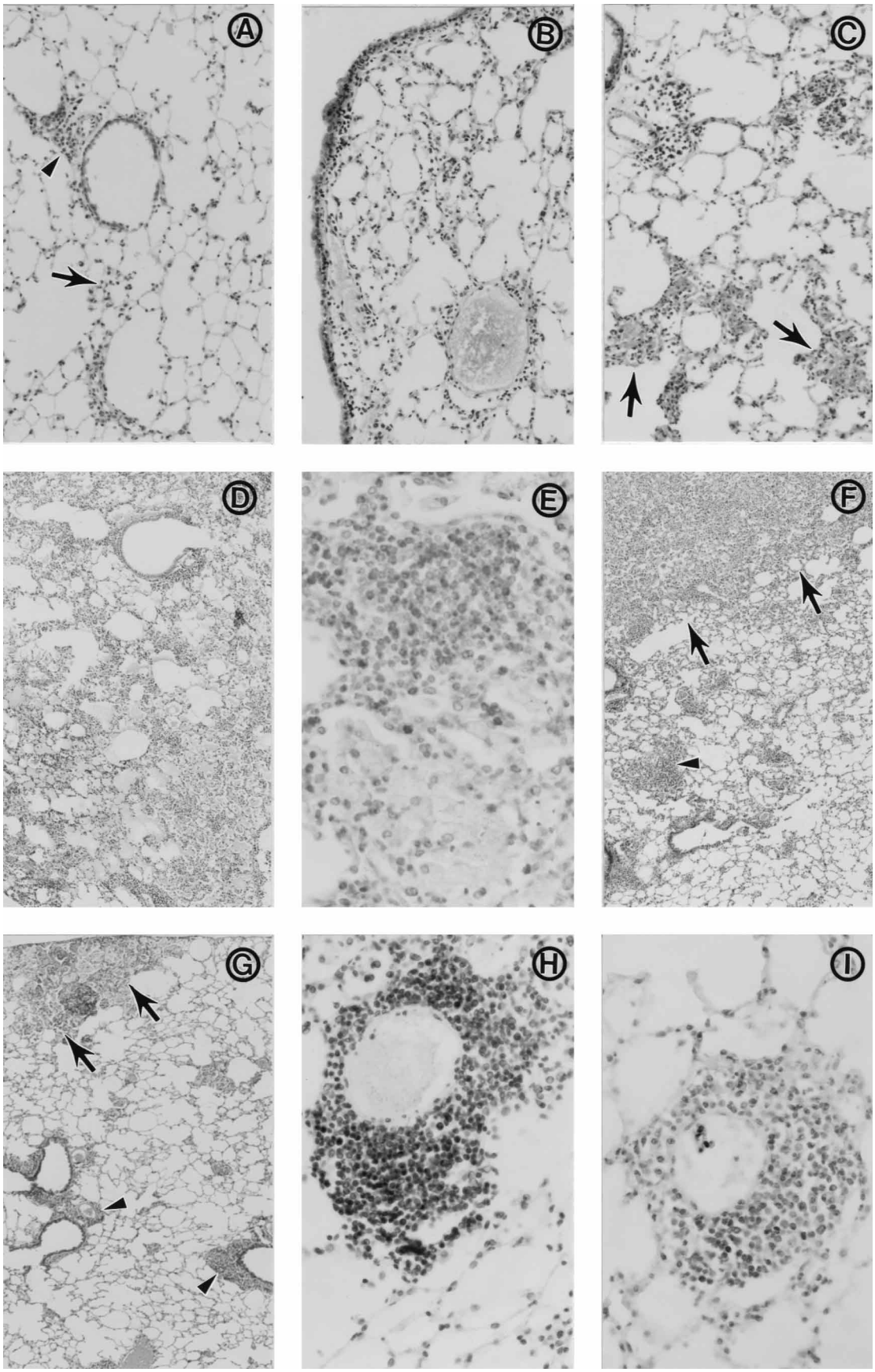
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FIG. 3. Representative histopathology and immunohistochemistry at different times during the evolution of experimental pulmonary tuberculosis in control mice and in mice presensitized with 10^7 or 10^9 *M. vaccae* organisms. (A) Light micrograph of control mouse lung 3 days after intratracheal infection. There is slight inflammatory infiltration in the walls of the blood vessels (arrowhead), bronchi, and interstitium (arrow) (hematoxylin and eosin staining; magnification, ×92). (B) Increased inflammatory response after 3 days of infection in an animal that had received 10^7 *M. vaccae* organisms 2 months before infection. The distribution of the inflammation is as in the control (hematoxylin and eosin staining; magnification, ×92). (C) Animal preimmunized with 10^9 *M. vaccae* organisms. There are exaggerated inflammation and small pneumonic patches (arrows) by 3 days. (D) Massive pneumonia and intra-alveolar proteinaceous material at 1 month in a lung from an animal preimmunized with 10^9 *M. vaccae* organisms. (E) Abundant cytoplasmic IL-4 in a pneumonic area from the lung shown in panel D (magnification, ca. ×370). (F) The lung of a control animal 4 months after infection. There are extensive areas of granuloma formation (arrowhead) and pneumonia (arrows) and perivascular inflammatory infiltrates (hematoxylin and eosin staining; magnification, ca. ×37). (G) The lung at 4 months after infection, from an animal that had been preimmunized with 10^7 *M. vaccae* organisms. In contrast to the control (F), there is little pneumonia (arrows) and there are pronounced lymphocyte cuffs around the blood vessels (arrowheads) (hematoxylin and eosin staining; magnification, ca. ×37). These cuffs contain many cells positive for IL-2 (H) but few that are positive for IL-4 (I) (magnification, ×92).

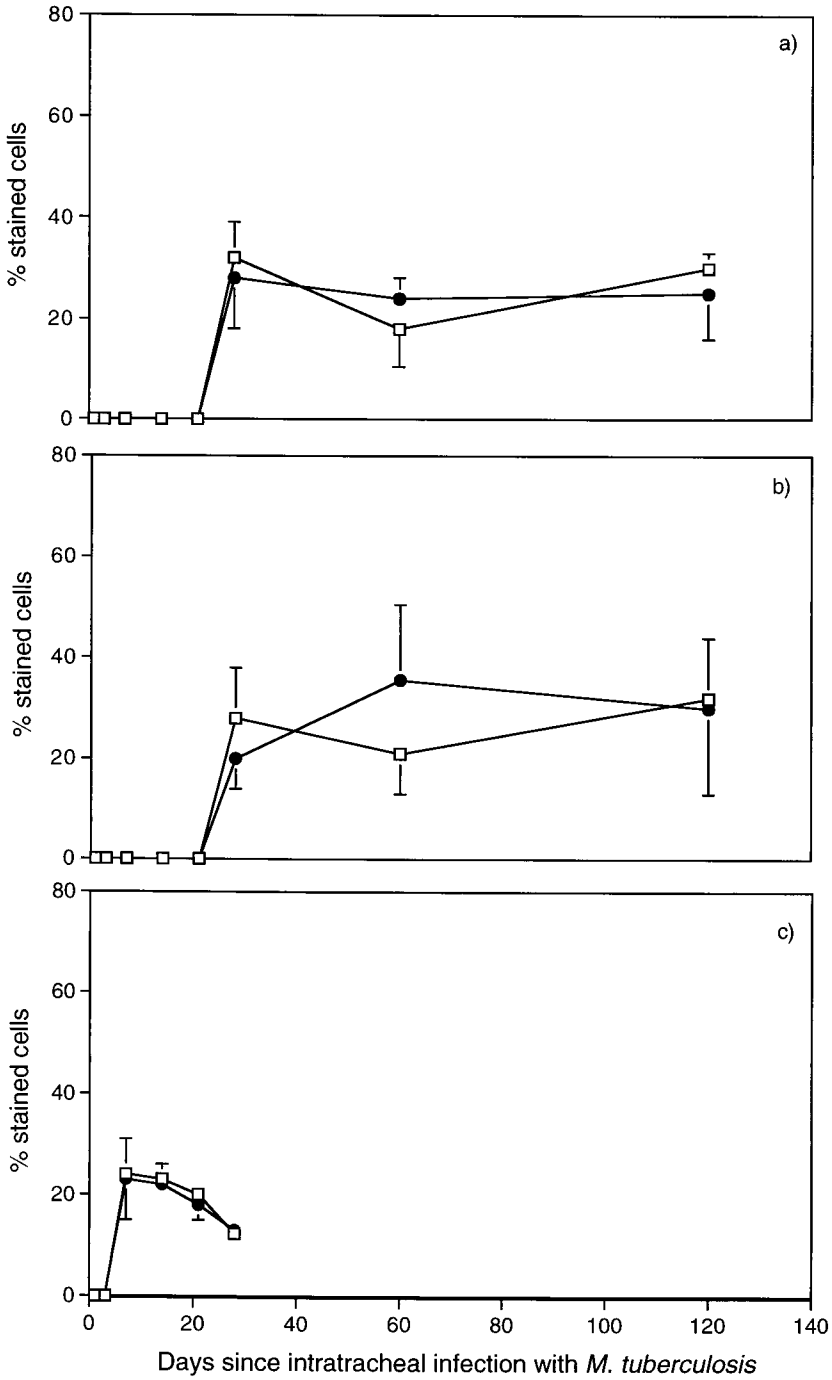

FIG. 4. Immunocytochemical analysis of IL-2-positive (□) and IL-4-positive (●) cells in the pneumonic areas of the lungs of BALB/c mice at intervals after infection via the trachea with *M. tuberculosis* H37Rv, as described in the text. Results are shown for control animals (a) and animals that had been preimmunized 2 months before infection with 10^7 (b) or 10^9 (c) autoclaved *M. vaccae* organisms and are means for four mice ± SD. Areas of pneumonia always contained as many IL-4-positive as IL-2-positive cells.

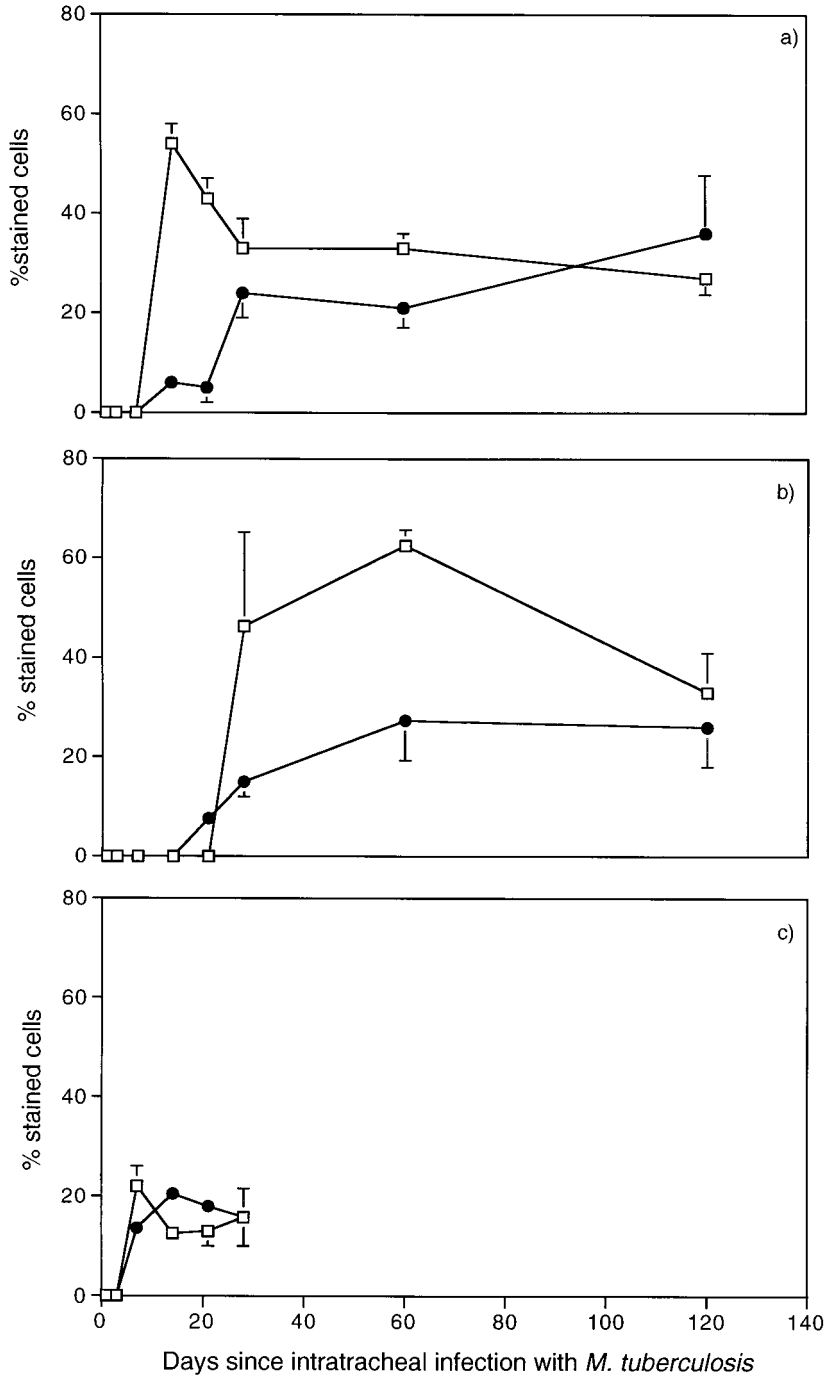

FIG. 5. Immunocytochemical analysis of IL-2-positive (□) and IL-4-positive (●) cells in the granulomatous areas of the lungs of BALB/c mice at intervals after infection via the trachea with *M. tuberculosis* H37Rv. Results are shown for control animals (a) and animals that had been preimmunized 2 months before infection with 10^7 (b) or 10^9 (c) autoclaved *M. vaccae* organisms and are means for four mice ± SD. In recipients of 10^7 *M. vaccae* organisms granuloma formation was delayed and the ratio of IL-2-positive to IL-4-positive cells stayed high longer than it did in the controls. Recipients of 10^9 *M. vaccae* organisms showed the reverse trends.

erated granuloma formation, reaching levels comparable to the maximum achieved in the other groups as early as day 7.

Immunocytochemistry and distribution of cells stained for IL-2 and IL-4. Areas of pneumonia, whenever present, contained approximately equal numbers of IL-2-positive and IL-4-positive cells (Fig. 3E and 4). This consistent presence of a Th2 component is in agreement with the fact that pneumonia represents a failure of immunity and correlates with progressive disease. As outlined above (Fig. 2), pneumonia appeared earliest in the rapidly dying animals preimmunized with 10^9 *M. vaccae* organisms, a dose known to prime a mixed Th1 plus Th2 response pattern. There was little pneumonia in the recipients of 10^7 *M. vaccae* organisms (Fig. 2a), but the few areas of pneumonia that there were contained equal numbers of

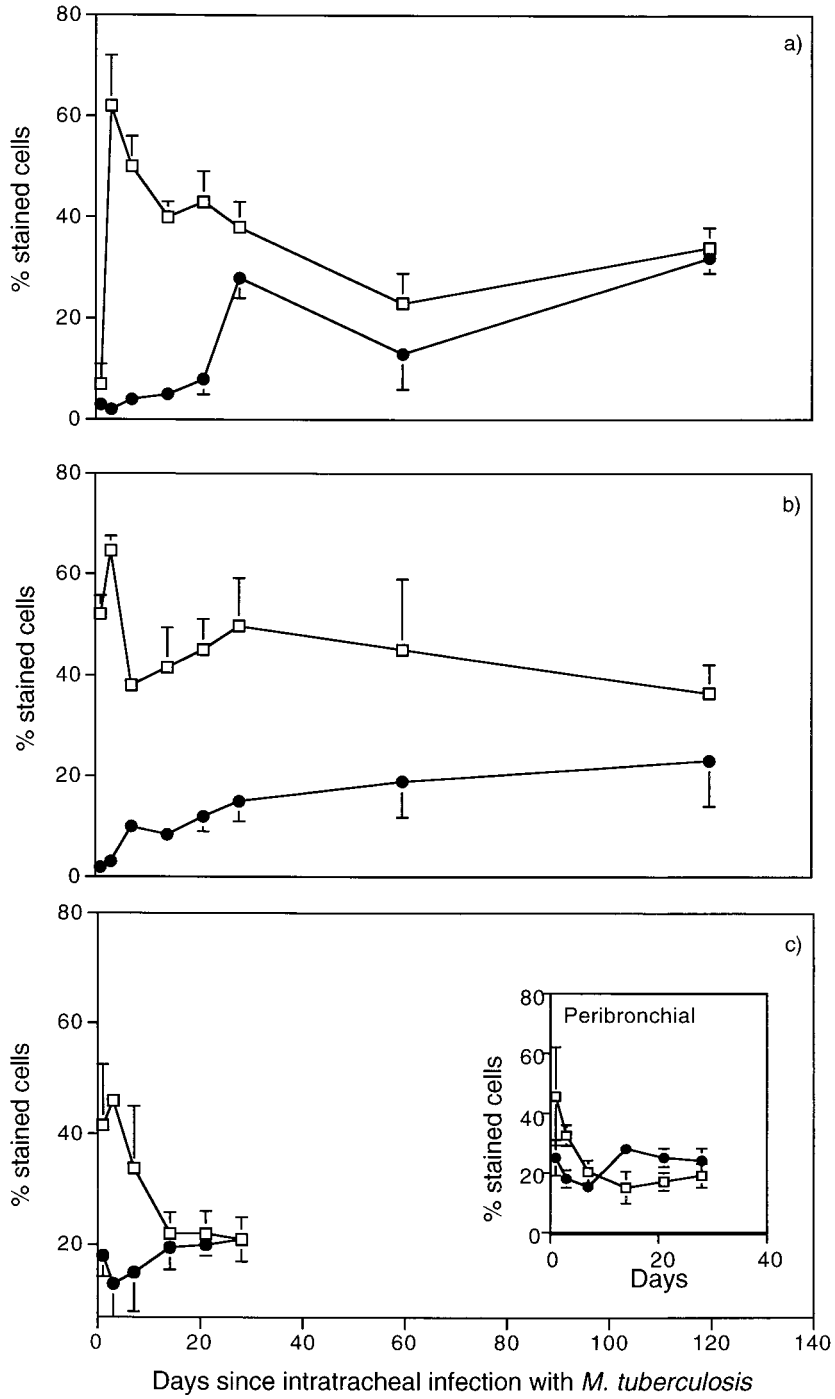

FIG. 6. Immunocytochemical analysis of IL-2-positive (□) and IL-4-positive (●) cells in the perivascular compartment of the lungs of BALB/c mice at intervals after infection via the trachea with *M. tuberculosis* H37Rv. Results are shown for control animals (a) and animals that had been preimmunized 2 months before infection with 10^7 (b) or 10^9 (c) autoclaved *M. vaccae* organisms. The insert shows similar data for the peribronchial zone. Data are means for four mice ± SD. The number of IL-4-positive cells increases between days 21 and 28 in the controls. This increase was delayed in recipients of 10^7 *M. vaccae* organisms and accelerated in recipients of 10^9 *M. vaccae* organisms.

IL-2- and IL-4-positive cells (Fig. 4b). Thus, sites of pneumonia were always sites where Th1 and Th2 cells were present in approximately equal numbers, even when there was very little pneumonia, as in the protected animals that had received 10^7 *M. vaccae* organisms.

The granulomatous areas in the recipients of 10^9 organisms also showed equal numbers of IL-4- and IL-2-positive cells (Fig. 5c), though neither population reached more than 20% of the total number of cells. In contrast, the granulomas of control animals contained mostly IL-2-positive cells (~40%) up to day 21. Then, between days 21 and 28, the percentage of IL-4-positive cells rose and the percentage of IL-2-positive

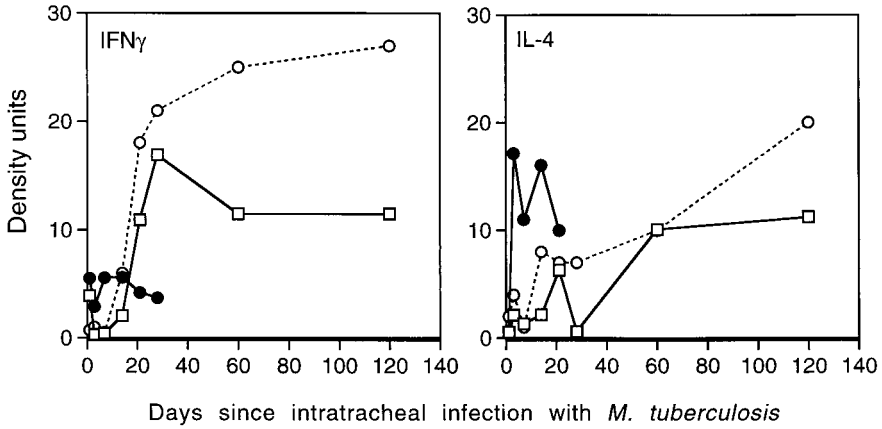

FIG. 7. Kinetics of cytokine mRNA production during experimental pulmonary tuberculosis in control mice (○) and in mice preimmunized with 10^7 (□) or 10^9 (●) autoclaved *M. vaccae* organisms 2 months before infection via the trachea with *M. tuberculosis* H37Rv. Densitometric readings are shown as described in the text. Animals preimmunized with 10^9 *M. vaccae* organisms show a rapid rise in mRNA for IL-4, but not for IFN-γ. Animals preimmunized with 10^7 *M. vaccae* organisms resemble controls but have lower levels of mRNA for IFN-γ late in the disease, possibly reflecting the reduced inflammation in their lungs at this time (see Fig. 3F and G).

cells fell, so that from day 28 the percentages of these two cell populations were similar (Fig. 5a). Mice immunized with the protective Th1-inducing dose of *M. vaccae* (10^7 organisms) showed more-sustained dominance of IL-2-positive (Fig. 3H) relative to IL-4-positive (Fig. 3I and 5b) cells, with a ratio of almost 3:1 still present at day 60.

Findings in the perivascular areas were similar. The IL-2/IL-4 ratio in the recipients of 10^9 *M. vaccae* organisms had fallen to 1 by day 14. In the controls, there was a sharp rise in percent IL-4-positive cells between days 21 and 28, so that the ratio approached 1 by day 28, whereas in the recipients of 10^7 *M. vaccae* organisms, the rise of IL-4-positive cells and the decline in IL-2-positive cells were delayed (Fig. 6).

These patterns of Th1 and Th2 infiltration are in broad agreement with the results of RT-PCR analysis of mRNA for IL-4 and IFN-γ (Fig. 7). (RT-PCR for IL-2 was not performed.)

Sensitivity to TNF-α of DTH sites evoked by soluble antigens of *M. tuberculosis*. It has been shown previously that the effect of TNF-α injected directly into the site of an ongoing DTH reaction correlates with the pattern of cytokine release that can be elicited from the spleen cells of the same mice cultured with mycobacterial antigen. TNF-α causes further swelling (15, 24) or even frank necrosis (1, 2) detectable 20 to 24 h later if the animals have a mixed Th1 plus Th2 cytokine pattern (15). On the other hand, TNF-α has no measurable effect if the animals have been immunized so as to have only a Th1 response (15). Unimmunized infected mice developed positive DTH responses to the soluble antigens of *M. tuberculosis* by day 7 ($P < 0.005$ relative to naive animals). The response remained positive through day 60 but was lost by day 120 ($P = 0.1$). However, injection of TNF-α into these DTH sites at 24 h did not evoke an increase in swelling (measured at 44 h) during the first 28 days (Fig. 8). On days 60 and 120 (i.e., after the ratio of IL-4-positive and IL-2-positive cells had approached 1), injection of TNF-α caused marked additional swelling ($P = 0.005$ relative to reading without TNF-α) (Fig. 8).

In contrast, the animals preimmunized with 10^7 *M. vaccae* organisms (Th1 inducing) were significantly DTH-positive at all times but never showed TNF-α-sensitive DTH sites, possibly reflecting the continuing high IL-2/IL-4 ratio. The recipients of 10^9 *M. vaccae* organisms, which had low IL-2/IL-4 ratios that rapidly fell to 1 (Fig. 6c), showed TNF-α-sensitive DTH sites at all times, including day 1 (Fig. 8c). This is in agreement with the previous observation that preimmunization with this dose induces a state of TNF-α-sensitive DTH responsiveness even in the absence of infection with *M. tuberculosis* (15). This sensitivity to TNF-α in the recipients of 10^9 *M. vaccae* organisms occurred even when no significant response could be elicited with antigen alone, because only the day 7 test was positive in this group compared to control values. This agrees with the toxicity of TNF-α in the macroscopically negative DTH site elicited in the control infected mice at day 120 (Fig. 8a). Recipients of 10^9 organisms usually die by day 28, but two anomalous long-term survivors in the second experiment were tested on day 60, and again, they were macroscopically DTH negative, but the site of challenge was very sensitive to TNF-α (Fig. 8c).

DISCUSSION

When mice are immunized with particulate antigens, there is a clear relationship between the dose given and the ratio of Th1 to Th2 cytokine production that is primed. Thus, very low doses prime a Th1 pattern, while larger doses prime a Th2 component as well. This has been shown with *Leishmania* (5), BCG (4), and *M. vaccae* NCTC 11659 (15). The effect is not confined to live organisms, since *M. vaccae* is used as an autoclaved suspension.

The fact that *M. vaccae* is used killed means that it can be used as a very stable and reproducible immunogen. We have used it to investigate the influence on the progress of the disease of antigens that this environmental saprophyte shares with *M. tuberculosis*. Mice were immunized 2 months before challenge to eliminate short-term effects of macrophage activation.

Immunity to tuberculosis requires a Th1 pattern of response accompanied by a range of cytotoxic cell types that probably lyse phagocytes when they fail to destroy ingested organisms (3, 19, 20, 22, 29). This may allow the organisms to be released and then taken up by fresh activated phagocytes. In tuberculous mice, in which immunity has failed, type 2 cytokines are also expressed (14, 23). We have shown here that induction of

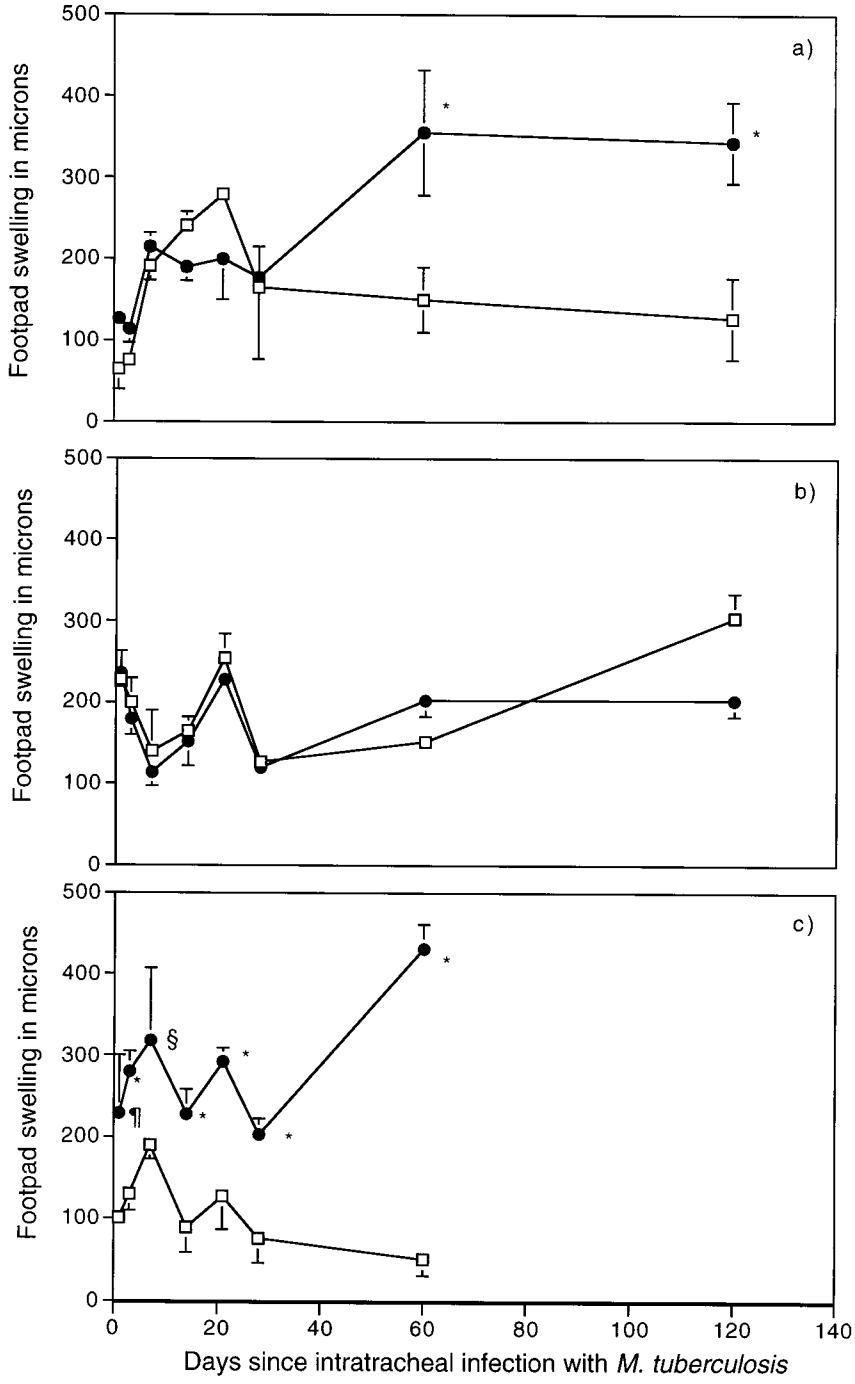

FIG. 8. DTH responses to soluble antigens of *M. tuberculosis* in tuberculous mice. Results are shown for control animals (a) and animals that had been preimmunized 2 months before infection with 10^7 (b) or 10^9 (c) autoclaved *M. vaccae* organisms. Swelling was measured at 24 h (□), and then 1 μg of recombinant murine TNF-α was immediately injected into the DTH site. Swelling was then measured again at 44 h (●). Positive DTH responses in comparison with naive animals were elicited in the control infected mice at all times between days 7 and 60. DTH was always positive in recipients of 10^7 *M. vaccae* organisms but only on day 7 in recipients of 10^9 *M. vaccae* organisms. TNF-α caused an increment in swelling in the unimmunized animals after day 28, when IL-4-positive cells are abundant, and at all times in recipients of 10^9 *M. vaccae* organisms, which have a preexisting Th2 component. TNF-α did not cause an increment in swelling in DTH sites of the recipients of the Th1-inducing dose of 10^7 organisms. The significance of enhancement of swelling due to TNF-α, relative to that of DTH sites without TNF-α, is as follows: ∗, $P < 0.005$; ¶, $P < 0.025$; and §, $P < 0.05$ (Student's *t* test).

a Th1 response to epitopes shared by *M. vaccae* and *M. tuberculosis* is able to partly vaccinate BALB/c mice, but only if the optimal Th1-inducing dose is used. This is manifested as prolonged survival, and preservation of a high ratio of IL-2-positive to IL-4-positive cells in granulomas and in the perivascular and peribronchial sites of inflammation. In contrast, preimmunization with a dose (10^9 organisms) that induces a mixed Th1 plus Th2 response leads to strikingly increased susceptibility to

the disease relative to that of unimmunized controls, with rapid induction of pneumonia and a ratio of Th1 to Th2 cells (IL-2/IL-4 ratio) that approaches 1 within 7 days.

These results show that the response to shared epitopes present in a member of the fast-growing subgenus of mycobacteria can have both positive and negative effects on the subsequent course of tuberculosis, depending on the Th1-Th2 balance of the response induced by the saprophyte before the infectious challenge. Since mycobacteria are ubiquitous and very immunogenic but not part of the normal human flora, the Th1-Th2 balance of each individual's response to these shared epitopes will depend on lifestyle and on the abundance and nature of saprophytic mycobacteria in the local ecosystems. It was suggested previously that such variable exposure might be one of the factors that predetermines susceptibility to mycobacterial disease and the subsequent efficacy of BCG vaccination (28). Our new data support this idea, as do recent analyses of data from Malawi (9). While it is now confirmed that environmental saprophytes can be protective (9), it has been postulated (28), but not proven, that they can also prime detrimental effects. The data presented here show that this is certainly the case in mice, and it is likely to be equally true in humans.

These data also cast light on the paradoxical role of TNF-α. In addition to type 1 cytokines, TNF-α is essential for immunity to tuberculosis in mice. Treatment of mice with neutralizing anti-TNF-α antibodies led to dissemination of BCG infection (18), and neutralizing antibodies or knockout of the 55-kDa TNF-α receptor led to rapid death from *M. tuberculosis* (11). Thus, TNF-α is necessary for protection, probably because it triggers killing mechanisms in IFN-γ-activated macrophages (6, 16). Nevertheless, many symptoms of tuberculosis, such as fever, weight loss, and tissue damage, resemble the known pathological effects of TNF-α. Evidence that these symptoms may indeed be caused by TNF-α in human tuberculosis has come from experiments using thalidomide, which decreases the half-life of the mRNA for this cytokine (21). Patients treated with thalidomide show rapid symptomatic relief and weight gain (17). We are therefore faced with a paradox. TNF-α is essential for immunity but may also be responsible for pathology. The results shown here provide a partial solution to this dilemma. It was shown previously, with the same immunogenic autoclaved preparation of *M. vaccae*, that TNF-α becomes toxic in the presence of a mixed Th1 plus Th2 response pattern, but not when there is a relatively pure Th1 response (15). We have now superimposed infection with virulent *M. tuberculosis* upon the same experimental system and shown that sensitivity of tuberculin-induced DTH sites to TNF-α correlates with development of pneumonia and rapid progression to death and tends to occur in groups of animals that have 20% or more IL-4-positive cells in their inflammatory lesions (for instance, in unimmunized mice 28 days or more after infection or recipients of 10^9 *M. vaccae* organisms from day 7). The same may be true in human tuberculosis. For instance, the IL-4 gene is expressed in patients' peripheral blood mononuclear cells (26), which readily release IL-4 (25), while there is a deficit in IL-2 expression (26). The same two points have emerged from studies using three-color flow cytometry to look at actual cytokine production. Most tuberculosis patients have circulating T cells that secrete IL-4 (29a), and they often have high circulating levels of IL-10 (8) and of specific immunoglobulin E antibody (IL-4 dependent) (30). It is of particular relevance that when tuberculin test sites were studied with a laser Doppler velocimeter, it was found that the extent to which blood flow was reduced in the center of the site at both 6 and 48 h was related to the level of specific immunoglobulin E antibody to *M. tuberculosis* (12). Cumulatively, these findings outweigh the observation that there is no increased expression of mRNA for type 2 cytokines in peripheral blood cells from human disease (31). The Th2 cells are clearly present in the periphery and may well be present in the interstitial and peribronchial compartments of human tuberculous lungs.

In conclusion, induction by saprophytic environmental mycobacteria of Th2 responses to shared mycobacterial epitopes can lead to accelerated tuberculosis and to increased toxicity of TNF-α in mice, and it is likely that the same phenomenon occurs in humans.

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