INFECTION AND IMMUNITY, Feb. 1997, p. 623–629 Vol. 65, No. 2
0019-9567/97/$04.00+0

Adjuvant Modulation of Immune Responses to Tuberculosis Subunit Vaccines

ERIK B. LINDBLAD,[1] MARTIN J. ELHAY,[1] REGINA SILVA,[2] RUI APPELBERG,[2] AND PETER ANDERSEN[1]*

The TB Research Unit, Statens Seruminstitut, 2300 Copenhagen S., Denmark,[1] *and Centro de Citologia Experimental, 4150 Porto, Portugal*[2]

Received 1 August 1996/Returned for modification 25 September 1996/Accepted 5 November 1996

Mice were immunized with experimental subunit vaccines based on secreted antigens from *Mycobacterium tuberculosis* in a series of adjuvants, comprising incomplete Freund's adjuvant (IFA), dimethyl dioctadecyl ammoniumbromide (DDA), RIBI adjuvant, Quil-A saponin, and aluminum hydroxide. Immune responses induced by these vaccines were characterized by in vitro culture of primed cells, PCR analysis for cytokine mRNA, detection of specific immunoglobulin G isotypes induced, and monitoring of protective immunity to tuberculosis (TB). The study demonstrated marked differences in the immune responses induced by the different adjuvants and identified both IFA and DDA as efficient adjuvants for a TB subunit vaccine. Aluminum hydroxide, on the other hand, induced a Th2 response which increased the susceptibility of the animals to a subsequent TB challenge. DDA was further coadjuvanted with either the Th1-stimulating polymer poly(I-C) or the cytokines gamma interferon, interleukin 2 (IL-2), and IL-12. The addition of IL-12 was found to amplify a Th1 response in a dose-dependent manner and promoted a protective immune response against a virulent challenge. However, if the initial priming in the presence of IL-12 was followed by two booster injections of vaccine without IL-12, no improvement in long-term efficacy was found. This demonstrates the efficacy of DDA to promote an efficient immune response and suggests that IL-12 may accelerate this development, but not change the final outcome of a full vaccination regime.

At present, the only available vaccine against human tuberculosis (TB) is the *Mycobacterium bovis* BCG vaccine, a live vaccine based on an attenuated strain of *M. bovis*. The efficacy of this vaccine has been shown to vary significantly in different populations, despite its general ability to induce satisfactory acquired resistance to tuberculosis in animal models (37). In field trials conducted in some countries, including Colombia and India, where tuberculosis presents a major health problem, BCG vaccination has demonstrated a relatively low efficacy and was not able to induce satisfactory protection against the disease. In other countries, the protection achieved after BCG vaccination reached levels as high as 70 to 80% (8). In recent years, the emergence of drug-resistant strains of *Mycobacterium tuberculosis* and the increased incidence of TB among human immunodeficiency virus-positive patients in both developing and western countries (29, 34–36) has added to the already serious TB problem worldwide. This has provided new impetus to the search for an improved vaccine with high efficacy in different populations, something which may be effected by a standardized subunit vaccine.

Subfractions of *M. tuberculosis* have been suggested as candidates for inclusion in a subunit vaccine against TB, and particular interest has been focused on actively secreted proteins present in *M. tuberculosis* culture filtrates (1, 26). Previous work by Andersen and Heron (3) demonstrated that secreted antigens could provide protective immunity to TB by showing that fractions of culture filtrate proteins were the target antigens of T cells responsible for the recall of protective immunity. Recently, the study of secreted antigens has been intensified, and significant levels of acquired resistance have been reported by several investigators after vaccination with subunit vaccines based on culture filtrate components (4, 27, 30).

In a live BCG vaccine, there is no need for an adjuvant. However, an additional adjuvant component is important when vaccines are to be prepared from purified protein fractions (5, 7, 23). CD4 cells have been divided into two major types based on their cytokine gene transcription and secretion (25). Th1 cells are characterized by their secretion of interleukin 2 (IL-2) and gamma interferon (IFN-γ), whereas Th2 cells produce IL-4, IL-5, and IL-10. Adjuvants may induce a T-cell response skewed in either the Th1 or the Th2 direction (16), and this may have a profound influence on the outcome of such a vaccination.

In the first experiments by Andersen (4), short-term culture filtrate (ST-CF) administered with the adjuvant dimethyl dioctadecyl ammoniumbromide (DDA) was demonstrated to induce long-lived acquired immunity against *M. tuberculosis* mediated by CD4 cells. The purpose of the present study was to investigate further the potential of ST-CF in experimental vaccination of mice against TB and investigate the influence of the adjuvant component. The study demonstrated marked differences in the immune responses induced by the different adjuvants and suggested that although various coadjuvants, such as IL-12, may accelerate the development of a Th1 response, a vaccination regime based on DDA or incomplete Freund's adjuvant (IFA) on its own induces an efficient immune response. These results are discussed in the context of optimizing a novel TB subunit vaccine.

MATERIALS AND METHODS

Mice. C57BL/6J female mice 8 to 12 weeks old were bred at Bomholtegaard breeding center, Ry, Denmark.

Bacteria. *M. tuberculosis* H37Rv cells were grown at 37°C on Löwenstein-Jensen medium or in suspension in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose.

* Corresponding author. Mailing address: The TB Research Unit, Bacterial Vaccine Department, Statens Seruminstitut, Artillerivej 5, 2300 Copenhagen S., Denmark.

Challenge and bacterial enumeration in organs. Mice were infected with 5×10^5 *M. tuberculosis* cells in a volume of 0.1 ml of phosphate-buffered saline (PBS) by intravenous injection via the lateral tail vein. At the appropriate time point, mice were killed by cervical dislocation, and the organs were removed for bacterial enumeration. Whole organs were homogenized in PBS and plated in serial 10-fold dilutions on Middlebrook 7H10 medium. After 3 weeks of incubation at 37°C, the numbers of bacteria were determined and subtracted from those obtained in control (unimmunized) animals. The resulting values will be referred to as $\log_{10}$ resistance.

Preparation of ST-CF. Preparation of ST-CF antigen was undertaken as described by Andersen et al. (2). In short, *M. tuberculosis* (8×10^6 CFU/ml) was grown in enriched Sauton medium at 37°C on an orbital shaker for 7 days. The supernatants were filtered through a 0.22-μm-pore-diameter sterility filter (Milliguard Housing System; Millipore Corp., Bedford, Mass.) and concentrated 100-fold on an Amicon YM 3 membrane (Amicon, Danvers, Mass.). The preparation was stored frozen at −80°C.

Experimental vaccines. The following adjuvants were tested in combination with ST-CF. A parallel series of adjuvant controls containing no antigen were set up. The amounts of reagent(s) per dose (200 μl) of vaccine are given.

The DDA mixture contained 250 μg of DDA (Eastman Kodak, Inc., Rochester, N.Y.) plus 25 μg of ST-CF; the DDA was heated in aqua ad injectabilia until micelle formation at 80°C, cooled to room temperature, and mixed with antigen.

The $Al(OH)_3$ mixture contained aluminum hydroxide gel adjuvant equivalent to 500 μg of aluminum (Alhydrogel; 2%; Superfos Biosector, Kvistgård, Denmark), which was added to 25 μg of ST-CF.

The IFA mixture contained 100 μl of IFA (Statens Seruminstitut, Copenhagen, Denmark) and 25 μg of ST-CF. A water-oil emulsion was prepared by repeated passage and emulsification of equal parts of oil adjuvant and aqueous antigen with a syringe.

The RIBI mixture contained 25 μg of ST-CF. The RIBI adjuvant was prepared in accordance with the supplier's (R700, RIBI Adjuvant System; Ribi Inc.) guidelines.

The Quil-A mixture contained 20 μg of Quil-A saponin adjuvant (Superfos Biosector, Kvistgård, Denmark) and 25 μg of ST-CF; the Quil-A was resuspended in PBS and sterile filtered prior to being mixed with antigen.

A second series of vaccines were tested in which DDA and IFA were coadjuvanted with either poly(I-C) or different cytokines. These immunomodulators were tested in a range of doses, with poly(I-C) (Calbiochem Corp., La Jolla, Calif. [lot 677593]) at 25 to 250 μg, recombinant IFN-γ (rIFN-γ) (GIBCO BRL) at 0.1 to 1.0 μg, rIL-2 (Becton Dickenson/Collaborative Biomedical Products) at 0.02 to 0.36 μg, and rIL-12 at 0.125 to 2.0 μg. Murine rIL-12 (lot no. R8993P) was a kind gift of M. Gately, Hoffmann-La Roche.

All experimental vaccines contained thimerosal at a final concentration of 0.01%.

Immunizations. The mice were injected subcutaneously in the back three times with a weekly interval between each injection. Each dose (0.2 ml) was split into two doses, which were injected on the right and left sides of the spine.

The first inoculum was placed at the base of the tail, and the subsequent booster injections were placed further up the back. A single dose of BCG Danish 1331 was injected subcutaneously at the base of the tail; no booster injections were administered.

For the investigation of immune responses induced in the regional lymph nodes, three animals in each group were immunized in the footpads of the hind legs. These animals were sacrificed on day 7.

Lymphocyte cultures. Lymphocytes were obtained by preparing single-cell suspensions from either lymph nodes (inguinal and popliteal nodes) or from spleens by dispersion of the tissue through a sterilized stainless steel mesh as described previously (1). Isolated cells were cultured in microtiter wells containing 2×10^5 cells in a volume of 200 μl of RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin-streptomycin, 1 mM glutamine, and 5% (vol/vol) fetal calf serum. ST-CF was used to stimulate cells in a concentration of 4 μg/ml, and concanavalin A in a concentration of 1 μg/ml was used in all experiments as a positive control for cell viability. Cellular proliferation was investigated by pulsing cultures after 48 h of incubation (1 μCi of [^{3}H]thymidine per well). After a further 22 h of incubation, plates were harvested and processed for liquid scintillation counting (Beta counter; LKB). All tests were carried out in triplicate. Supernatants for the investigation of cytokines were harvested from parallel cultures after 48 h of incubation.

Cytokine assays. Cytokines in the culture supernatants were quantified with commercially available enzyme-linked immunosorbent assay (ELISA) screening kits (IL-5; Endogen, Boston, Mass.; IFN-γ, Life Biotechnology). Samples were tested in duplicate (IL-5) or triplicate (IFN-γ) analyses. Values below 50 pg/ml were considered negative.

Detection of mRNA. Lymph node cells (10^6) were lysed in 1 ml of RNAzol B (CINNA Biotecx Laboratories, Houston, Tex.), and total RNA was purified according to the method of Chomczynski and Sacchi (12). The RNA pellets were dried and dissolved in pyrocarbonic acid diethyl ester-distilled water. All RNA samples were stored at −80°C until assayed. The integrity of RNA was checked by agarose gel electrophoresis. For the detection of cytokine gene expression, RNA was first reverse transcribed into cDNA, which was then subjected to PCR amplification with specific primers for individual cytokines by the GeneAmp PCR system 9600 (Perkin-Elmer Cetus) as described previously (10). Samples of cDNA were amplified by PCR with specific sets of primers for hypoxanthine phosphoribosyltransferase (HPRT) to standardize each sample. The PCR products were hybridized with specific [γ-^{32}P]ATP-labeled oligonucleotide probes for the cytokines in Southern blots, after adjustment for HPRT levels (10).

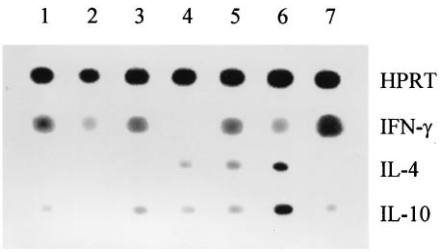

FIG. 1. Cytokine mRNA signals monitored directly in the regional lymph node after immunization with experimental TB vaccines. RT-PCR analysis for cytokine mRNA (IFN-γ, IL-4, and IL-10) was conducted with lymph node cells taken 7 days after primary immunization. Lanes: 1, saline; 2, IFA; 3, DDA; 4, RIBI; 5, saponin; 6, $A1(OH)_3$; 7, BCG.

ST-CF specific IgG isotype ELISA. ELISA plates (NUNC Maxisorp, type 96F) were coated with ST-CF (0.12 μg/well) overnight at 4°C. Free binding sites were blocked by 1% bovine serum albumin–PBS. Mice sera were analyzed in twofold dilutions. Specific antibodies of different isotypes were detected by horseradish peroxidase-conjugated rabbit anti-mouse reagent (immunoglobulin G1 [IgG1], SERT 103P; IgG2a, SERT 104P [both from SEROTEC Ltd., Oxford, United Kingdom]; IgG, P260 [DAKO]).

Reactivity to mycobacterial antigens in these polyclonal reagents was blocked by preincubation with ST-CF, and the reagents were used in a dilution of 1:200. All mouse sera were titrated, and the readout was standardized to a reference serum from mice hyperimmunized with ST-CF with IFA.

RESULTS

Characterization of immune responses induced by the experimental vaccines. (i) Early responses in the regional lymph node. Mice were immunized with the panel of experimental vaccines in the footpad of the hind limbs. At day 7, the mice were sacrificed, and lymphocytes were isolated from the inguinal and popliteal nodes. The influence of the adjuvant component on the early local immune responses was monitored by reverse transcription-PCR (RT-PCR) for cytokine mRNA. This analysis was done directly with cells isolated from the regional lymph node to allow a nonbiased picture of the responses actively occurring in vivo. The results of the mRNA detection for IFN-γ, IL-4, and IL-10 are given in Fig. 1. HPRT detection is included as a control. The BCG vaccine induced a pronounced signal for IFN-γ-specific mRNA, whereas more

TABLE 1. Antigen-specific cellular reactivity[a] monitored with the regional lymph nodes after immunization with experimental TB vaccines

Vaccine	Cellular reactivity[b]	
	cpm (10^3)	IFN-γ (pg/ml)
Saline–ST-CF	9.54 ± 0.27	395 ± 47.6
IFA–ST-CF[c]	28.40 ± 1.19	1,065 ± 299.0
DDA–ST-CF	34.30 ± 2.63	295 ± 24.6
RIBI–ST-CF	30.20 ± 0.38	760 ± 48.1
Saponin–ST-CF	7.71 ± 0.38	321 ± 21.0
$A1(OH)_3$–ST-CF	28.20 ± 0.28	465 ± 42.5
BCG	6.13 ± 0.68	405 ± 36.1
Control	1.57 ± 0.00	175 ± 67.5

[a] The in vitro analyses were performed with lymphocytes isolated from the popliteal and inguinal lymph nodes 7 days after immunization.

[b] Values are given as means ± standard errors of triplicate analyses. Note that cellular reactivity for IL-5 was <50 for each vaccine (duplicate analyses).

[c] This experiment was repeated, and the values represent the compiled data.

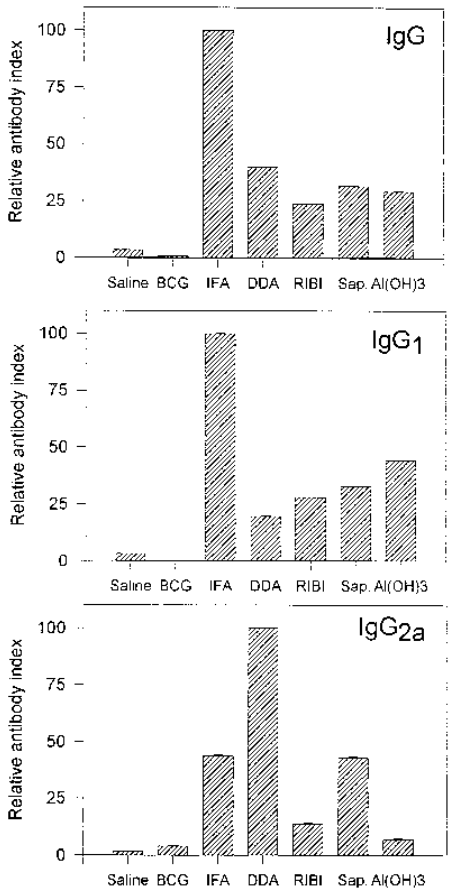

FIG. 2. IgG isotype responses induced by experimental TB vaccines. Mice were immunized three times with ST-CF in different adjuvant combinations or received a BCG vaccination. Five weeks after the primary vaccination, serum from five mice in each group was pooled, and specific IgG, IgG1, and IgG2A levels were determined by ELISA. Values shown are calculated as index values against the group showing the highest individual response (equivalent to 100%). Sap., saponin.

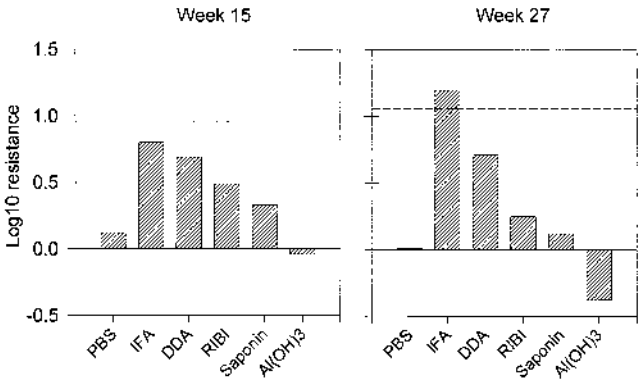

FIG. 3. Acquired resistance to TB induced by experimental subunit vaccines based on ST-CF and different adjuvants. Mice were challenged with virulent *M. tuberculosis*, and CFU were enumerated 15 and 27 weeks after primary immunization to monitor longlived immunity. The values depicted in the figure are $\log_{10}$ resistance expressed in the spleen. Values are corrected for possible nonspecific resistance induced by the adjuvant alone. The level of protection conferred by BCG vaccine is indicated by the dashed line.

moderate reactions were found in the groups that received DDA and Quil-A and, surprisingly, in the nonadjuvanted PBS–ST-CF group. Low-level signals were found in the IFA and aluminum groups too. High levels of IL-4- and IL-10-specific mRNA were only found in the group immunized with aluminum hydroxide-adjuvanted vaccines. Low, but detectable levels were found in the saponin and RIBI groups. Neither IL-4- nor IL-10-specific mRNA was seen in this assay when the groups were immunized with IFA. No IL-4 message was seen in the DDA group. To monitor antigen-specific T-cell responses primed by the different immunizations, isolated lymphocytes were cultured in vitro, and cytokine release and cellular proliferation in response to stimulation with ST-CF were investigated (Table 1). A potent proliferative response was observed in the cultures from mice having received IFA, DDA, RIBI, or aluminum hydroxide. These were all significantly larger than in the ST-CF–saline controls ($P < 0.001$ by analysis of variance and Tukey-Kramer multiple comparisons test), whereas lymphocytes from mice who received Quil-A–ST-CF vaccine gave a weaker response, but the response was still significantly larger than that of the negative control animals ($P < 0.05$). The proliferative response of the BCG group was found not to differ significantly from that of the control. The culture supernatants were analyzed for the presence of IFN-γ and IL-5. IL-5 could not be detected, whereas IFN-γ could be detected in low concentrations in all culture supernatants. Only minor and statistically insignificant differences ($P > 0.05$) were found in the release promoted by the different adjuvants at this early time point.

(ii) IgG isotype titers. The levels of IgG1 and IgG2A are known to reflect the stimulation of Th2 and Th1 cells, respectively. The induction of specific antibodies of these subclasses during an immune response therefore represents a readout suited to give an impression of the overall Th1/Th2 balance. An ELISA was set up for detection of ST-CF-specific IgG, IgG1, and IgG2A antibodies in the serum of mice at week 5 after a full vaccination regime. The results are presented as a relative antibody index for each of the subclasses (Fig. 2). The response from each group is shown as percentage of that of the group with the highest response. The reciprocal midpoint titers for the groups given as the 100% references were as follows: IgG (IFA–ST-CF), 800; IgG1 (IFA–ST-CF), 750; and IgG2A (DDA–ST-CF), 150. As shown, all of the experimental vaccines induced a humoral response. The highest IgG response was observed for the IFA-adjuvanted vaccine. BCG, in contrast, did not raise a humoral response. Most of the vaccines induced a mixed response with both IgG1 and IgG2A subclasses. DDA, however, gave a response strongly skewed toward IgG2A, whereas the opposite relationship existed for the aluminum hydroxide adjuvant, which gave a response consisting primarily of the IgG1 subclass.

Acquired resistance provided by the experimental vaccines. The relative potency of the experimental vaccines was investigated at two different time points after primary vaccination (15 and 27 weeks), in parallel with a standard BCG vaccine. The prolonged vaccination challenge interval was chosen to allow the monitoring of long-lived immunological memory. The protective efficacy was tested by the administration of an intravenous challenge, and bacterial numbers were enumerated in the spleens of animals killed 2 weeks later. The vaccine efficacies were calculated as specific $\log_{10}$ resistance, and the figures have been corrected for small differences in the level of nonspecific resistance provoked by the different adjuvants by subtraction of the adjuvant-only controls. Fifteen weeks after vaccination, the resistance provided by the different vaccines differed markedly and ranged from no resistance to high levels of resistance (Fig. 3). The most efficient vaccine was BCG,

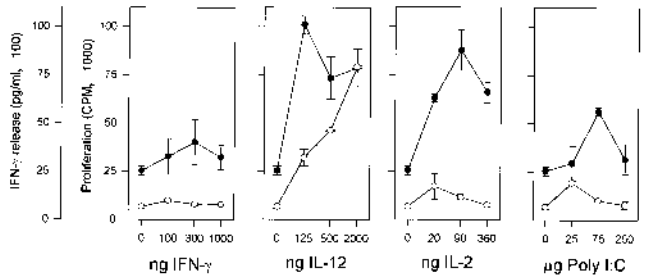

FIG. 4. Influence of coadjuvants on the immune response induced by experimental TB vaccines. ST-CF–DDA-based vaccine was coadjuvanted with increasing amounts of either cytokines (IFN-γ, IL-12, or IL-2) or a Th1-stimulating polymer, poly(I-C) (Poly I:C). Lymphocytes were isolated from the regional lymph nodes 7 days after a single vaccination and restimulated in vitro with ST-CF. Cellular proliferation (●) and IFN-γ release (○) were monitored. Results have been depicted as means of triplicate analyses ± standard errors. The proliferative and IFN-γ responses in unstimulated cultures in each case were below 6,510 cpm and 1,155 pg/ml, respectively.

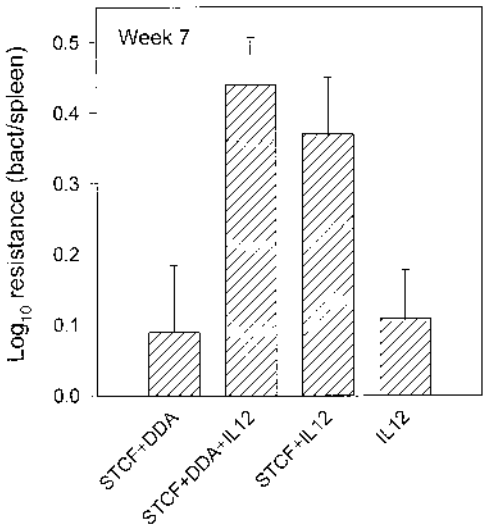

FIG. 5. Acquired resistance to TB induced by experimental subunit vaccines based on ST-CF, DDA, and the coadjuvant IL-12. Mice received one subcutaneous immunization and were challenged with virulent *M. tuberculosis* 7 weeks later. The values depicted in the figure are $\log_{10}$ resistance expressed in the spleen. Bact, bacteria.

which reduced the number of CFU in the spleen by 0.95 log. No significant difference, however, was found between BCG and vaccines based on IFA ($P = 0.33$; *t* test, unpaired and two tailed), and only a marginal difference was found with DDA ($P = 0.054$). Both the RIBI adjuvant and Quil-A conferred a modest resistance, significantly lower than the efficacy seen with the BCG vaccine. The experimental vaccine based on aluminum hydroxide provided no resistance to TB; on the contrary, the animals given the aluminum hydroxide vaccine demonstrated a tendency towards a slightly increased number of CFU. A similar pattern was found in mice rested 24 weeks before challenge, and CFU were enumerated at week 27. The highest level of protection was found in the group that had been vaccinated with ST-CF and IFA. This group had increased resistance compared to at week 15 ($P = 0.009$). The groups vaccinated with BCG and ST-CF–DDA had similar levels of resistance at weeks 15 and 27, whereas immunity had waned in the groups vaccinated with ST-CF and RIBI or Quil-A. Animals immunized with the aluminum-adjuvanted vaccine were at this late time point rendered significantly more susceptible to TB challenge than control animals ($P = 0.002$).

Influence of different coadjuvants on responses primed by an experimental vaccine. The DDA-adjuvanted vaccines evoked high levels of protective immunity which were stable throughout the test period. However, whereas a pronounced IFN-γ mRNA signal was expressed in the lymph node itself, only low levels of IFN-γ were induced by this adjuvant when regional lymph node cells were tested in vitro on day 7. The possibility that this adjuvant could be improved by the addition of various immunomodulators to stimulate a more potent Th1 response was therefore investigated.

Groups of three mice each were set up. These mice received vaccines containing micelles of DDA added to a mixture of ST-CF and various coadjuvants. The polymer poly(I-C), which has been shown to skew the immune response in the Th1 direction by stimulating macrophages to release IFN-α and IL-12 (24), was tested in doses from 25 to 250 μg. The cytokines IFN-γ, rIL-2, and rIL-12 were tested in various doses chosen to cover a physiologically active dose range. On day 7 after immunization, the mice were sacrificed, lymph node cells were isolated, and cellular proliferation and the presence of IFN-γ in ST-CF-stimulated cultures were monitored (Fig. 4).

Priming in the presence of poly(I-C) was shown to stimulate both cellular proliferation and some increase in IFN-γ release. The optimum dosage for stimulating cellular proliferation differed from the dosage (25 μg) inducing maximal increase of the IFN-γ response (Fig. 4). A significant increase in IFN-γ was obtained, although the scale chosen throughout Fig. 4 renders this difference less obvious. Recombinant IFN-γ at a dose of 300 ng induced a slight potentiation of the T-cell activity, whereas little effect on IFN-γ release in the cultures was found. For the proliferative response, the optimal effect of adding rIL-2 to the vaccines was found at 90 ng, and only a limited influence on IFN-γ release was found. A dramatic amplification of the IFN-γ response was found with rIL-12. The optimum dosage was 2,000 ng/dose, and a clear dose-response relationship was found.

Influence of IL-12 on resistance provided by the experimental vaccines. ST-CF–DDA coadjuvanted with IL-12 induced high levels of IFN-γ production by primed T cells, and whether this effect was reflected in the level of resistance to virulent challenge with *M. tuberculosis* was therefore investigated. C57BL/6J mice were immunized once with 100 μg of ST-CF adjuvanted with DDA, DDA–IL-12, or IL-12 alone. A one-immunization protocol was chosen in this experiment to allow sensitive monitoring of the possible influence of the coadjuvant. After 7 weeks, the animals were challenged with virulent *M. tuberculosis*. Vaccination with one dose of the ST-CF–DDA vaccine did not prime an efficient immune response and led to low levels of resistance (0.09 $\log_{10}$ resistance) (Fig. 5). However, inclusion of IL-12 in this vaccine resulted in a significant protective immune response, which reduced the number of CFU by 0.44 log. Interestingly, IL-12 did not need to be delivered with DDA to have an adjuvant effect, because IL-12–ST-CF on its own reduced the bacterial numbers in the spleen by 0.37 log. This effect was specific, because IL-12 alone did not significantly protect without ST-CF.

This line of investigation was continued with a vaccination regimen in which mice were vaccinated three times at weekly intervals in accordance with the practice for vaccination used for the testing of the different adjuvants. To push the initial differentiation of T cells in a Th1 direction, IL-12 at a dose of 0.5 μg per animal was added to ST-CF adjuvanted with IFA or DDA for the primary immunization only. The two subsequent

TABLE 2. Influence of IL-12 on cellular reactivity and acquired resistance induced by experimental TB vaccines

Exptl vaccine[c]	Cellular reactivity[a]		Bacterial no. (log_{10} CFU)[b]
	cpm (10^3)	IFN-γ (pg/ml)	
DDA–ST-CF–IL-12	37.4 ± 1.9	4,613 ± 32	5.04 ± 0.04
DDA–ST-CF	41.1 ± 1.2	5,336 ± 347	5.00 ± 0.06
IFA–ST-CF–IL-12	13.2 ± 4.1	801 ± 202	4.80 ± 0.13
IFA–ST-CF	23.8 ± 1.5	840 ± 232	4.85 ± 0.11

[a] Cellular reactivity was monitored with lymphocytes isolated from the spleen 5 weeks after the last of three immunizations. Values are given as means ± standard errors of triplicate analyses of cells pooled from three mice.

[b] Bacterial numbers are given as log_{10} CFU of *M. tuberculosis* isolated from the spleen 8 weeks after the last of three immunizations. Each group consisted of five mice, and the values are given as means ± standard errors.

[c] IL-12 was added to the primary injection only.

immunizations contained ST-CF and the adjuvant without IL-12. Five weeks after the final immunization, splenocytes were isolated, and antigen-specific cellular proliferation and IFN-γ production were measured (Table 2). A more potent proliferative response was found in spleen cells from the DDA group than those from mice immunized with IFA adjuvanted with ST-CF, and the group released approximately fivefold-higher levels of IFN-γ. Surprisingly, inclusion of IL-12 in the primary immunization had no influence on the immune response to ST-CF, as measured by cellular proliferation and IFN-γ, in either of these adjuvant groups. Similarly, there was no difference in log_{10} numbers of CFU isolated from the spleen, whether or not IL-12 was added to the first vaccination.

DISCUSSION

In this study, we have used a series of well-characterized adjuvants to stimulate the immune response in mice to CF proteins from *M. tuberculosis*. The range of adjuvants included aluminum hydroxide, which is at present the most widely used adjuvant in practical vaccination (21), as well as a selection of adjuvants, some of which were previously reported to be good stimulators of cell-mediated immunity (9, 20).

The influence of adjuvants will be at the site of administration and in the draining lymph nodes. Part of their function will be the stimulation and release of mediators, such as cytokines, in lymphoid tissue. The cytokines that are present during the priming of an acquired immune response are crucial in determining whether the response will be either a Th1 or a Th2 type immune response (25). It has been suggested that this segregation takes place in the local lymphoid tissue, shortly after the initial immunization (11, 28, 32, 43). In the light of this, it was considered of importance to investigate the cytokine profile of T cells taken from the local draining lymph nodes on day 7 after priming. All adjuvants tested were found to prime a cellular response to ST-CF monitored as antigen-specific proliferation of lymphocytes isolated from the regional lymph nodes. These cellular responses were, however, only associated with the release of low levels of soluble cytokines (Table 1). In the present study, an RT-PCR analysis demonstrated significant levels of mRNA for cytokines expressed in the lymph nodes. A clear mRNA signal for IL-4 and IL-10 was found in the animals immunized with the aluminum hydroxide-adjuvanted vaccines, whereas IFN-γ-specific mRNA, on the other hand, was at its highest levels in the BCG group and was less pronounced in the DDA, saponin, and IFA groups. T cells taken from lymph nodes from animals immunized with ST-CF in the absence of adjuvants gave an unexpected readout for IFN-γ. This result may suggest that ST-CF in itself has some immunomodulatory ability. The discrepancy between in vivo expression and in vitro detection of these cytokines can be explained by differences in the sensitivity of the methods used, because some cytokines may have a very short half-life in vitro. On the other hand, it seems likely that adjuvants at very early time points induce nonspecific cytokine signals in the lymph node not detectable in the assay for antigen-specific lymphoproliferation. Early cytokine signals such as IL-1, tumor necrosis factor alpha, IFN-γ, and IL-12 are products of macrophages and NK cells (6, 14, 22, 42), and recently this list has been expanded by the discovery of early IL-4 production from $CD4^+$ $CD8^+$ NK1.1 cells (45). These signals may be present in the lymphoid tissue long before full differentiation of T cells has occurred.

The polarized expression of mRNA for IL-4 and IFN-γ in the aluminum hydroxide and BCG groups is in agreement with findings from previous studies (16). Mycobacterial cell walls contain components with potent adjuvant activity, such as trehalose dimycolate and muramyl dipeptides (19), and have been reported to select for human Th1-type T cells that produce IFN-γ (18, 31). Aluminum hydroxide, on the other hand, stimulates preferentially a humoral response mediated by Th2 cells (16, 21).

The experimental vaccines based on adjuvants expected to stimulate a Th1 response demonstrated various degrees of efficacy, monitored as increased resistance to subsequent challenge with TB. Strikingly, the use of aluminum hydroxide as an adjuvant primed an immune response which increased the susceptibility of the animals to virulent challenge. This difference was highly significant when long-lived immunity was monitored at week 27 ($P = 0.002$), whereas a similar tendency failed a test for significance 15 weeks after vaccination ($P = 0.557$). A possible adverse effect of a Th2 response in TB may draw parallels to the sensitive balance between Th1 and Th2 cells decisive for the outcome of disease in leprosy. Amplification by PCR of mRNA extracted from skin biopsies isolated from tuberculoid leprosy patients (with strong cell-mediated immunity but low bacterial numbers), demonstrated mRNA coding for IFN-γ and IL-2. Lepromatous leprosy patients (with low cell-mediated immunity but high bacterial numbers) were, in contrast, characterized by mRNA coding for IL-4, IL-5, and IL-10 (44). Along the same line, the mouse model of *Leishmania* infection has provided convincing evidence for the importance of the Th1/Th2 balance (33). A direct correlation of the Th subset and the outcome of infection was obtained from studies with Th1 and Th2 cell lines specific for *Leishmania major* (33). Transfer of a Th1 cell line secreting IL-2 and IFN-γ protected mice against infection, whereas a Th2 cell line secreting IL-4 exacerbated infection. The present study, and in particular the increased susceptibility after administration of the aluminum-adjuvanted vaccine, suggests that a similar role for Th2 may exist in the TB infection.

IFA is a highly potent adjuvant and an excellent model adjuvant in experimental immunology. In this study, it was demonstrated that as the only adjuvant tested, vaccines adjuvanted with IFA exhibited an increased level of resistance from 15 to 27 weeks postvaccination. At this time point, the animals exhibited the same high level of acquired resistance as the BCG-vaccinated animals, and this gradual increase in vaccine efficacy could reflect a continuous stimulus from the slowly metabolized mineral oil adjuvant. However, IFA is unlikely to be considered for practical vaccination because of its profile of adverse reactions (17). The establishment of long-lived stable immunological memory with both the DDA- and the IFA-adjuvanted vaccines in the present study is not in agreement

with findings from another recent long-term study of immunity provided by TB subunit vaccines. Roberts et al. (30) immunized mice with culture filtrate proteins in IFA and demonstrated a waning of the immunity over time and only modest levels of protection after 20 weeks. An explanation of these contradictory results is difficult to provide, but it is noteworthy that Roberts et al. used proteins derived from late culture filtrates as opposed to the defined ST-CF used in the present study. It may be that such filtrates lack the necessary amounts of certain critical components secreted at early time points (e.g., ESAT-6, the recently identified key target for long-lived memory T cells [39]). Another possibility is that the route of challenge (aerosol versus intravenous) influences the manifestation of protective immunity. This possibility is currently being investigated in our laboratory.

DDA has previously been used without toxic effects in human vaccination trials (38, 41). Previous studies from this laboratory demonstrated that an experimental vaccine composed of ST-CF and DDA was able to induce an efficient long-term immunity to TB (4). DDA stimulates predominantly Th1 cells, but as reported previously, significant levels of IgG1 antibodies are induced in immunized mice, indicating some Th2 contribution. In the present study, we have tried to modulate this immune response into a clear-cut Th1 response by the administration of an IFN-γ-inducing polymer or cytokines. In agreement with the function of IL-12 in the differentiation of Th1 cells (40), we found that this cytokine was a very efficient coadjuvant capable of stimulating the production of markedly increased levels of IFN-γ from primed T cells. However, attempts to improve the immunization by adding IL-12 to the first dose of vaccine followed by two booster immunizations with normal vaccine had no influence on the resulting level of Th1 activity or immunity to TB. This indicates that the addition of IL-12 may exert a transient effect and that DDA on its own may provide ample triggering of the pathways responsible for the Th1 profile. Recent failure to boost Th1 activity significantly, even by repeated injections of IL-12 around each booster vaccination, strongly supports this suggestion (unpublished results). IFN-γ knockout mice develop a severe disseminated form of tuberculosis (13, 15). This underlines the importance of IFN-γ in controlling TB, and although still subject to discussion, this molecule is becoming widely recognized as a correlate of protective immunity. In the present study, no significant difference was found in the protective efficacies promoted by DDA and IFA, despite the differences in amounts of IFN-γ released by lymphocytes primed by the two vaccines. Taken together, these findings indicate that it is possible to modulate the immune response to *M. tuberculosis* culture filtrate antigens to induce high levels of IFN-γ as well as lasting protective immunity in mice. However, depending upon the choice of adjuvant, it is possible to induce levels of IFN-γ in vitro which do not correlate to the levels of acquired protective immunity. Hence, adjuvant-induced IFN-γ is not a clear correlate for protective immunity, and the significance of this cytokine may be more complex than previously suggested.

ACKNOWLEDGMENTS

This study was supported by grants from the World Health Organization Global Programme for Vaccine Development (IMMYC), The Danish Research Council, The Danish Research Center for Biotechnology, and the European Community (Project no. TS3*/CT94/0113).

We thank Birgitte Smedegaard and Annette Hansen (Department of Bacterial Vaccines) and Lene Rasmussen (Department of Mycobacteria) for excellent technical assistance.

REFERENCES

1. **Andersen, P., D. Askgaard, L. Ljungqvist, M. W. Bentzon, and I. Heron.** 1991. T-cell proliferative response to antigens secreted from *Mycobacterium tuberculosis*. Infect. Immun. **59:**1558–1563.
2. **Andersen, P., D. Askgaard, L. Ljungqvist, J. Bennedsen, and I. Heron.** 1991. Proteins released from *Mycobacterium tuberculosis* during growth. Infect. Immun. **59:**1905–1910.
3. **Andersen, P., and I. Heron.** 1993. Specificity of a protective memory immune response against *Mycobacterium tuberculosis*. Infect. Immun. **61:**844–851.
4. **Andersen, P.** 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. Infect. Immun. **62:**2536–2544.
5. **Audibert, F. M., and L. D. Lise.** 1993. Adjuvants: current status, clinical perspectives and future prospects. Immunol. Today **14:**281–284.
6. **Bancroft, G. J., R. D. Schreiber, and E. R. Unanue.** 1991. Natural immunity: a T-cell-independent pathway of macrophage activation, defined in the *scid* mouse. Immunol. Rev. **124:**5–24.
7. **Barry, D. W., E. Staton, and R. E. Mayner.** 1974. Inactivated influenza vaccine efficacy: diminished antigenicity of split-product vaccines in mice. Infect. Immun. **10:**1329–1336.
8. **Bloom, B. R., and P. E. M. Fine.** 1994. The BCG experience: implications for future vaccines against tuberculosis, p. 531–557. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. ASM Press, Washington, D.C.
9. **Bomford, R.** 1980. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Clin. Exp. Immunol. **39:**435–441.
10. **Castro, A. C., P. Minóprio, and R. Appelberg.** 1995. The relative impact of bacterial virulence and host genetic background on cytokine expression during *Mycobacterium avium* infection of mice. Immunology **85:**556–561.
11. **Chatelain, R., K. Varkila, and R. L. Coffman.** 1992. IL-4 induces a Th2 response in *Leishmania major*-infected mice. J. Immunol. **148:**1182–1187.
12. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by guanidine isothiocyanate-chloroform extraction. Anal. Biochem. **162:**156–159.
13. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon-γ gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
14. **D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trichieri.** 1993. Interleukin-10 inhibits human lymphocyte IFN-γ production by suppressing natural killer cell stimulatory factor/interleukin-12 synthesis in accessory cells. J. Exp. Med. **178:**1041–1048.
15. **Flynn, J. A., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for interferon-γ in resistance to *M. tuberculosis* infection. J. Exp. Med. **178:**2249–2254.
16. **Grün, J. L., and P. H. Maurer.** 1989. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. Cell. Immunol. **121:**134–145.
17. **Gupta, R. K., E. H. Relyveld, E. B. Lindblad, B. Bizzini, S. Ben-Efraim, and C. K. Gupta.** 1993. Adjuvants—a balance between toxicity and adjuvanticity. Vaccine **11:**293–306.
18. **Haanen, J. B. A. G., R. de Waal Malefijt, P. C. M. Res, E. M. Kraakman, T. H. M. Ottenhoff, R. R. P. de Vries, and H. Spits.** 1991. Selection of human T-helper type 1-like T-cell subset by Mycobacteria. J. Exp. Med. **174:**583–592.
19. **Jolles, P., and A. Paraf.** 1973. Chemical and biological basis of adjuvants. Mol. Biol. Biochem. Biophys. **13:**11–52.
20. **Kraaijeveld, C. A., H. Snippe, M. Harmsen, and B. J. Bautahar-Trouw.** 1980. Dimethyldioctadecylammonium bromide as an adjuvant for delayed type hypersensitivity and cell-mediated immunity against Semliki Forest virus in mice. Arch. Virol. **65:**211–217.
21. **Lindblad, E. B.** 1995. Aluminum adjuvants, p. 21–35. *In* D. E. S. Stewart-Tull (ed.), The theory and practical application of adjuvants. John Wiley & Sons, New York, N.Y.
22. **Loppnow, H., L. Brade, H. Brade, E. T. Rietschel, S. Kusumoto, T. Shiba, and H.-D. Flad.** 1986. Induction of human interleukin 1 by bacterial and synthetic lipid A. Eur. J. Immunol. **16:**1263–1267.
23. **Lövgren, K.** 1987. Construction and potential of the ISCOM as immunogen. Ph.D. thesis. National Veterinary Institute, Uppsala, Sweden.
24. **Manetti, R., F. Annunziato, L. Tomasevic, V. Gianno, P. Parronchi, S. Romagnani, and E. Maggi.** 1995. Polyinosinic acid:polycytidylic acid promotes T helper type 1-specific immune responses by stimulating macrophage production of interferon-α and interleukin-12. Eur. J. Immunol. **25:**2656–2660.
25. **Mosmann, T. R., and R. L. Coffman.** 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145–173.
26. **Orme, I. M., P. Andersen, and W. H. Boom.** 1993. T cell response to *Mycobacterium tuberculosis*. J. Infect. Dis. **167:**1481–1497.
27. **Pal, P. G., and M. A. Horwitz.** 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. Infect. Immun. **60:**4781–4792.

28. **Powrie, F., S. Menon, and R. L. Coffman.** 1993. Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity *in vivo*. Eur. J. Immunol. **23:**2223–2229.
29. **Relkin, F., C. P. Aranda, S. M. Garay, R. Smith, K. A. Berkowitz, and W. N. Rom.** 1994. Pleural tuberculosis and HIV infection. Chest **105:**1338–1341.
30. **Roberts, A. D., M. G. Sonnenberg, D. J. Ordway, S. K. Furney, P. J. Brennan, J. T. Belisle, and I. M. Orme.** 1995. Characteristics of protective immunity engendered by vaccination of mice with purified culture filtrate protein antigens of *Mycobacterium tuberculosis*. Immunology **85:**502–508.
31. **Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom.** 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science **254:**279–282.
32. **Scharton-Kersten, T., and P. Scott.** 1995. The role of the innate immune response in Th1 cell development following *Leishmania major* infection. J. Leukocyte Biol. **57:**515–522.
33. **Scott, P.** 1988. The role of Th1 and Th2 cells in experimental cutaneous leishmaniasis. Exp. Parasitol. **68:**369–372.
34. **Selwyn, P. A., D. Hartel, V. A. Lewis, E. E. Schoenbaum, S. H. Vermund, R. S. Klein, A. T. Walker, and G. H. Friedland.** 1989. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. N. Engl. J. Med. **320:**545–550.
35. **Selwyn, P. A., B. M. Sckell, P. Alcabes, G. H. Friedland, R. S. Klein, and E. E. Schoenbaum.** 1992. High risk of active tuberculosis in HIV-infected drug users with cutaneous anergy. JAMA **268:**504–509.
36. **Shafer, R. W., K. D. Chirgwin, A. E. Glatt, M. A. Dadhouh, S. H. Landesman, and B. Suster.** 1991. HIV prevalence, immunosuppression and drug resistance in patients with tuberculosis in an area endemic for AIDS. AIDS **5:**399–405.
37. **Smith, D. W.** 1985. Protective effect of BCG in experimental tuberculosis. S. Karger, Basel, Switzerland.
38. **Stanfield, J. P., D. Gall, and P. M. Bracken.** 1973. Single-dose antenatal tetanus immunisation. Lancet **ii:**215–219.
39. **Sørensen, A. L., S. Nagai, G. Houen, P. Andersen, and Å. B. Andersen.** 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infect. Immun. **63:**1710–1717.
40. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13:**251–276.
41. **Veronesi, R., A. Correa, and D. Alterio.** 1970. Single dose immunization against tetanus. Promising results in human trials. Rev. Inst. Med. Trop. **12:**46–54.
42. **Wallis, R. S., M. Amir-Tahmasseb, and J. J. Ellner.** 1990. Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: the monocyte Western blot. Proc. Natl. Acad. Sci. USA **87:**3348–3352.
43. **Wolf, S. F., J. Bliss, K. Stokes, and J. Sypek.** 1995. Activity of IL-12 as adjuvant in promoting Th1 and Th2 recall responses. Res. Immunol. **146:** 486–493.
44. **Yamamura, M., K. Uyemura, R. J. Deans, K. Weinberg, T. H. Rea, B. R. Bloom, and R. L. Modlin.** 1991. Defining prospective responses to pathogens: cytokine profiles in leprosy lesions. Science **254:**277–279.
45. **Yoshimoto, T., and W. E. Paul.** 1994. $CD4^{pos}$, $NK1.1^{pos}$ T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. J. Exp. Med. **179:**1285–1295.

Editor: S. H. E. Kaufmann