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Efficacy of culture filtrate protein preparations from Indian isolates of *M. tuberculosis* to activate T cells derived from healthy donors

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SUMMARY

SETTING: While culture filtrate proteins (CFPs) of *Mycobacterium tuberculosis* appear to be good vaccine candidates for tuberculosis, only CFPs derived from certain popular laboratory strains of *M. tuberculosis* have been studied for this purpose.

OBJECTIVE: To compare the relative efficacies of CFP preparations from two laboratory strains and four contemporary clinical isolates of *M. tuberculosis* to induce T-cell activation.

DESIGN: CFPs were isolated from six strains of *M. tuberculosis* and were used to induce 1) T-cell proliferation, 2) IFN-γ secretion, and 3) IL-12 secretion from peripheral blood derived mononuclear cell (PBMC) preparations from 33 healthy donors.

RESULTS: Significant amounts of IL-12 were spontaneously secreted by PBMC preparations; CFP preparations from two clinical isolates (JNU-7 and JNU-51) significantly boosted this response. All six CFP preparations induced IFN-γ secretion by PBMCs, but those from two contemporary strains of *M. tuberculosis* (JNU-7 and JNU-22) were most effective in this regard. The effect of CFPs from JNU-7 and JNU-22 was significantly better than those from the laboratory strains (H37Ra and Erdman). Similar results were obtained with the T-cell proliferation parameter.

CONCLUSION: These results suggest that CFPs derived from selected clinical isolates of *M. tuberculosis* may outperform those of standard laboratory strains, and may therefore be a better source of potential candidates for a tuberculosis vaccine.

KEY WORDS: tuberculosis; IFN-γ; IL-12; T-cell proliferation; Indian isolates; culture filtrate antigens

HUMAN TUBERCULOSIS caused by *Mycobacterium tuberculosis* is responsible for approximately 8 million new cases and 3 million deaths annually, making it one of the most serious health problems in the world.[1] The only available vaccine used against tuberculosis is the live bacille Calmette Guérin (BCG) vaccine, whose protective efficacy ranges from 0 to 80% in different clinical trials.[2,3] More effective anti-tuberculosis vaccines are therefore urgently needed. The identification of mycobacterial antigens which induce protective immunity is crucial for developing such vaccines. While dead mycobacteria and mycobacterial sonicate antigens induce robust immune response (antibody titers and delayed type hypersensitivity [DTH] reaction), this immunity correlates poorly with actual protection.[4] The administration of live *M. tuberculosis* induces protective immunity in mice, and lymphocytes from the immunized animals show a preference for reactivity with a set of mycobacterial antigens secreted by *M. tuberculosis* in culture, i.e., culture filtrate antigens (CFPs).[5,6] In addition, the induction of protective immunity by CFPs in conjunction with cytokines has been demonstrated in animal models.[7–9] These results have raised hopes for a CFP-based vaccine for tuberculosis.

Biochemical and immunological characterizations of CFP preparations from *M. tuberculosis* have been reported, although the CFP analyzed were generally derived from standard laboratory strains of *M. tuberculosis* such as H37Ra, H37Rv and Erdman, which were originally isolated from tuberculosis patients almost a century ago. When searching for an effective CFP-based vaccine for tuberculosis, it seems imperative that the selected antigen(s) should be a constituent of, or released by, the currently prevalent strains of *M. tuberculosis*. Initial results from our group and those of others suggest that contemporary isolates of *M. tuberculosis* may differ significantly from standard laboratory strains. Significant genetic variations within different isolates of *M. tuberculosis* have been shown in restriction fragment length polymorphism (RFLP) studies.[10,11] In addition, our own results show

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significant differences in reverse phase high performance liquid chromatography (HPLC) profiles of CFPs from different clinical isolates of *M. tuberculosis*.[12] We reasoned that if there are differences between CFPs of different strains of tuberculosis, we may find *M. tuberculosis* strains whose CFPs are relatively more potent in inducing protective immune responses.

In the present study, we compared CFP preparations from two standard strains (H37Ra and Erdman) and four contemporary Indian isolates of *M. tuberculosis*. We used the induction of T-cell proliferation and secretion of IFN-γ (interferon gamma) and IL-12 (interleukin 12) as parameters to assess the relative efficacy of these CFP preparations. Our results show that CFPs from two of the Indian isolates were significantly more potent than the CFPs of the standard laboratory strains in inducing activation of T cells of normal human donors, as assessed by the induction of T-cell proliferative response and secretion of IFN-γ. These results suggest that the search for a CFP-based protective vaccine against tuberculosis should not necessarily be restricted to CFPs of standard *M. tuberculosis* strains and may be extended to CFPs derived from currently prevalent strains of *M. tuberculosis*.

MATERIALS AND METHODS

Strains

The H37Ra strain of *M. tuberculosis* was provided by Dr Katoch (Japanese Association for Leprosy Mission Aided Institute [JALMA], Agra, India). The Erdman strain of *M. tuberculosis* (TMC 107) was obtained from the collection at Colorado State University. Several clinical isolates (JNU-4, JNU-7, JNU-22 and JNU-51) of *M. tuberculosis* were originally obtained from the New Delhi TB Center and Lala Ram Swarup TB Hospital, New Delhi, and adapted to grow in culture in our laboratory. Bacteria were grown at 37°C on Löwenstein-Jensen medium or on an orbital shaker in Sauton's medium with 0.05% Tween 80.

Preparation of culture filtrate proteins

CFPs were prepared as described previously,[9] with minor modifications. Sauton's medium (4.0 g L-aspargine, 2.0 g citric acid, 0.5 g potassium dihydrogen phosphate, 0.5g magnesium sulphate, 0.05 g ferric ammonium nitrate, 60 ml glycerol dissolved in 1 liter distilled water and pH adjusted to 7.3, without Tween 80) was inoculated with 5×10^6 colony forming units/ml of *M. tuberculosis*, and suspensions were grown as shaken cultures. After 9 days, cultures were centrifuged at $1800 \times g$ per minute for 30 minutes at 4°C, and filtered through Whatman filter paper 3M followed by filtration through a 0.45 μm membrane. The filtrate was then concentrated by ultrafiltration on an Amicon YM10 membrane (Amicon, Columbus, OH). The protein content was determined by the Coomassie brilliant blue method,[13] using bovine serum albumin (BSA) as standard.

Medium and reagents

Human peripheral blood derived mononuclear cells (PBMCs) were cultured in RPMI 1640 supplemented with 10% heat inactivated pooled human AB serum, 20 mM HEPES (N-[2-hudroxyethyl] piperazine-N′-[2-ethanesulfonic acid]), 300 μg/ml glutamine, 2×10^{-5}M 2-mercaptoethanol and 60 μg/ml gentamycin (complete medium [CM]). All other tissue culture grade reagents were purchased from Sigma Chemicals (St Louis, MO).

Preparation of PBMCs

All PBMC preparations were derived from blood samples provided by normal healthy donors (courtesy of the Indian Red Cross Society, New Delhi), over a period of 6 months (January to June 1999). To obtain PBMCs, peripheral blood was drawn from donors by venipuncture, collected into heparinized syringes and processed within 60 minutes of drawing. Buffy coats derived from these blood samples were diluted two-fold with plain RPMI 1640 medium, layered on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and centrifuged at $500 \times g$ for 30 minutes at 4°C. Interface cells were washed three times, including a low speed centrifugation step at $100 \times g$ to remove contaminating platelets, and suspended in CM.

T-cell proliferation assays

PBMCs (1×10^5/200 μl CM) were cultured in round-bottom 96-well plates (Costar, Cambridge, MA), with or without CFPs (1 μg/ml), at 37°C in 5% CO_2 for 5 days. [^{3}H] thymidine (0.5μ Ci/well, Amersham Pharmacia, Piscataway, NJ) was added 18 hours before termination of the culture. Plates were harvested by an automated cell harvester (PHD cell harvester, Cambridge Technology, MA) onto glass fiber discs and [^{3}H] thymidine incorporation was measured by liquid scintillation counting (Beckman beta scintillation counter, Beckman Coulter, Fullerton, CA).

Cytokine ELISA

Human PBMCs (1×10^5/200 μl CM) were cultured with or without CFPs (1 μg/ml), and culture supernatants were harvested on day 5. IFN-γ and IL-12 levels in the culture supernatants were estimated by using a sandwich ELISA kit (Genzyme Diagnostics, Cambridge, CA). Each sample was analyzed in triplicate according to the manufacturer's instructions. The amounts of cytokines, in picograms per milliliter, were calculated from a standard curve for human recombinant IFN-γ and IL-12.

Statistical analysis

For each set of data, arithmetic mean and standard error were calculated. The significance of differences

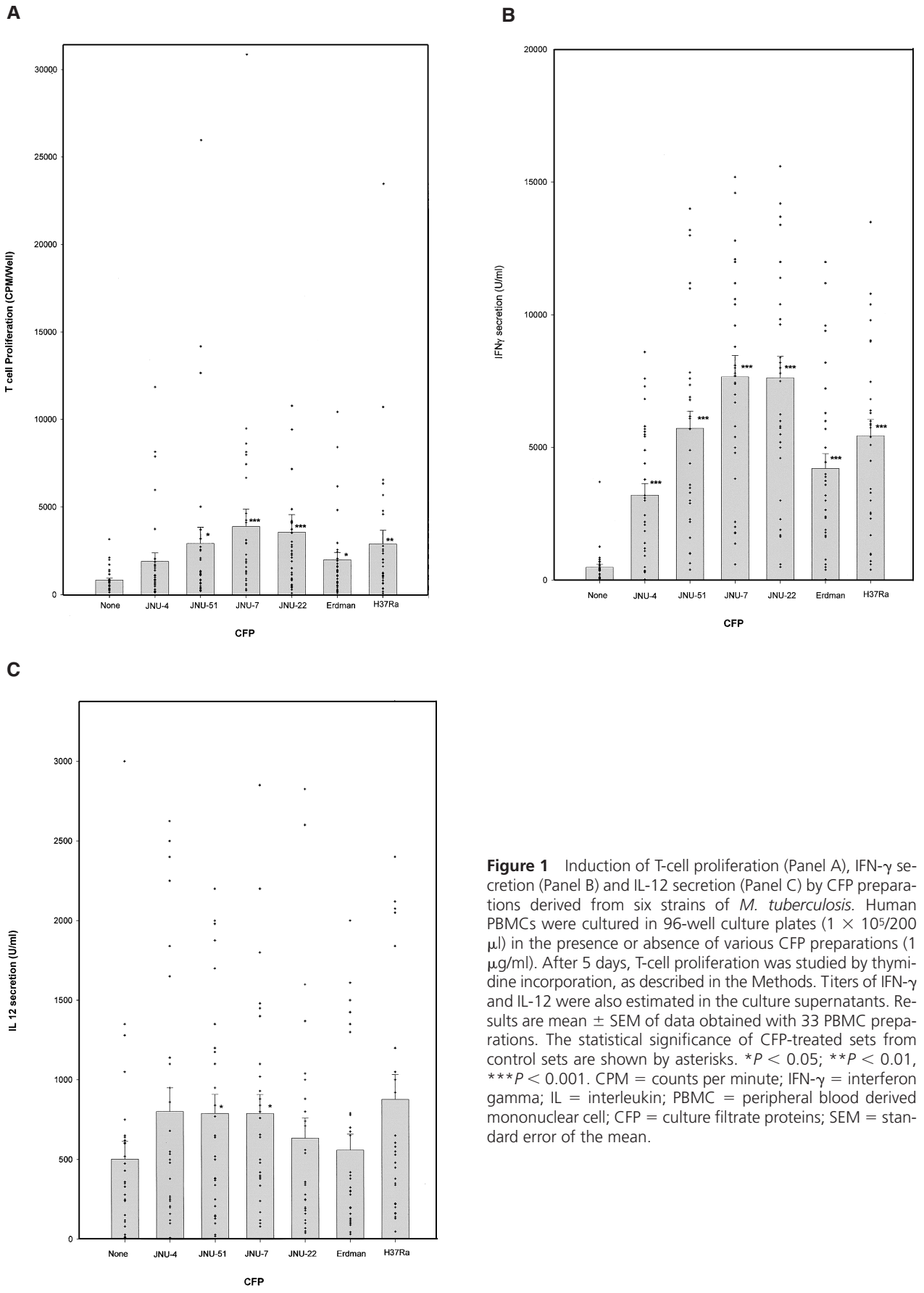

Figure 1 Induction of T-cell proliferation (Panel A), IFN-γ secretion (Panel B) and IL-12 secretion (Panel C) by CFP preparations derived from six strains of *M. tuberculosis*. Human PBMCs were cultured in 96-well culture plates (1×10^5/200 μl) in the presence or absence of various CFP preparations (1 μg/ml). After 5 days, T-cell proliferation was studied by thymidine incorporation, as described in the Methods. Titers of IFN-γ and IL-12 were also estimated in the culture supernatants. Results are mean ± SEM of data obtained with 33 PBMC preparations. The statistical significance of CFP-treated sets from control sets are shown by asterisks. $*P < 0.05$; $**P < 0.01$, $***P < 0.001$. CPM = counts per minute; IFN-γ = interferon gamma; IL = interleukin; PBMC = peripheral blood derived mononuclear cell; CFP = culture filtrate proteins; SEM = standard error of the mean.

between mean response in the different sets was estimated by paired *t*-test.

RESULTS

Response of PBMC preparations from healthy donors to CFP preparations derived from two laboratory strains and four clinical isolates of M. tuberculosis

Experiments were performed to compare the relative efficacies of CFP preparations from different strains of *M. tuberculosis* to induce immune response in PBMCs from healthy donors. Two parameters of T-cell activation, i.e., T-cell proliferation and IFN-γ secretion, and the parameter of release of IL-12 by macrophages, were used for this purpose. CFP preparations were obtained by culturing two standard strains of *M. tuberculosis* (H37Ra and Erdman) and four clinical isolates of *M. tuberculosis* (JNU-4, JNU-7, JNU-22 and JNU-51) derived from sputum samples of Indian patients and adapted to grow in Sauton's medium. PBMC preparations from 33 healthy donors were used in this study, which was carried out over a period of 6 months. These results are summarized in Figure 1 A, B and C. In each figure, individual data points have been shown along with the mean and standard error values.

All CFP preparations except JNU-4, an Indian isolate, induced significant T-cell proliferative activity in PBMC preparations (Figure 1A), which was statistically significant at 5% or higher. A comparison of the different CFP preparations revealed that the CFPs of JNU-7 and JNU-22 Indian isolates were significantly more potent in inducing T-cell proliferation than were those of the H37Ra and Erdman strains ($P < 0.05$ by paired *t*-test).

All CFP preparations were potent inducers of IFN-γ secretion by PBMCs (Figure 1B). The increase in IFN-γ secretion in response to different CFP preparations over control PBMC cultures ranged from 8 to 20-fold ($P < 0.0001$ for all comparisons). A comparison of responses to different CFP preparations indicated that the CFPs derived from JNU-7 and JNU-22 induced maximum IFN-γ, which was significantly greater than the response to CFPs of the H37Ra and Erdman strains ($P < 0.05$).

IL-12 titers were also determined in culture supernatants of PBMCs cultured with or without CFP preparations. The results, summarized in Figure 1C, show that control PBMCs cultured in the absence of CFPs released significant amounts of IL-12. A moderate increase in IL-12 secretion was induced by some CFPs, which was statistically significant only for CFPs from JNU-51 and JNU-7 ($P < 0.05$).

Relative efficacy of CFP preparations to activate PBMCs from healthy donors

As the effect of CFPs on IL-12 secretion by PBMCs was not very significant, the results of two other parameters were further analyzed. The relative efficacies of different CFP preparations to induce T-cell proliferation (TCP) and IFN-γ secretion (IGS), were not similar for all PBMC preparations. In addition, for a given PBMC preparation, the order of efficacy for different CFPs to induce TCP and IGS were not necessarily the same. This can be appreciated from the representative data for TCP and IGS assays for

Table Comparison of T-cell proliferation (TCP) and IFN-γ secretion (IGS) in response to CFP preparations in human PBMC preparations

PBMC sample	Parameter	JNU-4	JNU-51	JNU-7	JNU-22	Erdman	H37Ra	Control
1	TCP*	4.6	15.2	18.1	18.4	6.1	13.8	1.0 (1706)†
	IGS‡	920	2 900	1 772	1 700	1 680	2 550	600
5	TCP	7.0	12.2	7.4	9.3	5.33	9.22	1.0 (1163)
	IGS	7 306	7 828	9 600	7 800	6 000	7 480	420
6	TCP	2.8	2.4	3.9	2.0	2.3	2.7	1.0 (2107)
	IGS	4 400	7 360	12 000	9 840	5 000	9 040	240
12	TCP	1.8	1.8	1.8	1.8	1.6	1.5	1.0 (463)
	IGS	1 100	1 600	10 600	8 400	780	600	360
13	TCP	0.9	1.5	2.3	2.6	1.3	1.5	1.0 (822)
	IGS	3 100	3 000	11 200	12 000	3 000	10 400	400
15	TCP	0.9	1.1	1.5	1.2	2.4	2.0	1.0 (800)
	IGS	5 500	5 700	8 100	5 800	8 200	6 300	120
22	TCP	15.4	16.4	12.3	12.3	10.9	8.5	1.0 (770)
	IGS	5 700	6 900	7 700	8 000	5 700	6 400	680
30	TCP	1.0	1.4	5.6	1.8	1.8	1.2	1.0 (280)
	IGS	5 800	13 200	12 800	15 600	9 400	10 800	100

PBMC preparations were activated with *M. tuberculosis*-derived CFP preparations as described in the Methods.
* T-cell proliferation given as ratios of thymidine counts incorporated in PBMC cultured in the presence and absence of CFPs.
† Absolute values of mean cpm of thymidine incorporation in control cultures.
‡ IFN-γ secretion in U/ml.
CFP = culture filtrate proteins; PBMC = peripheral blood derived mononuclear cell; cpm = counts per minute.

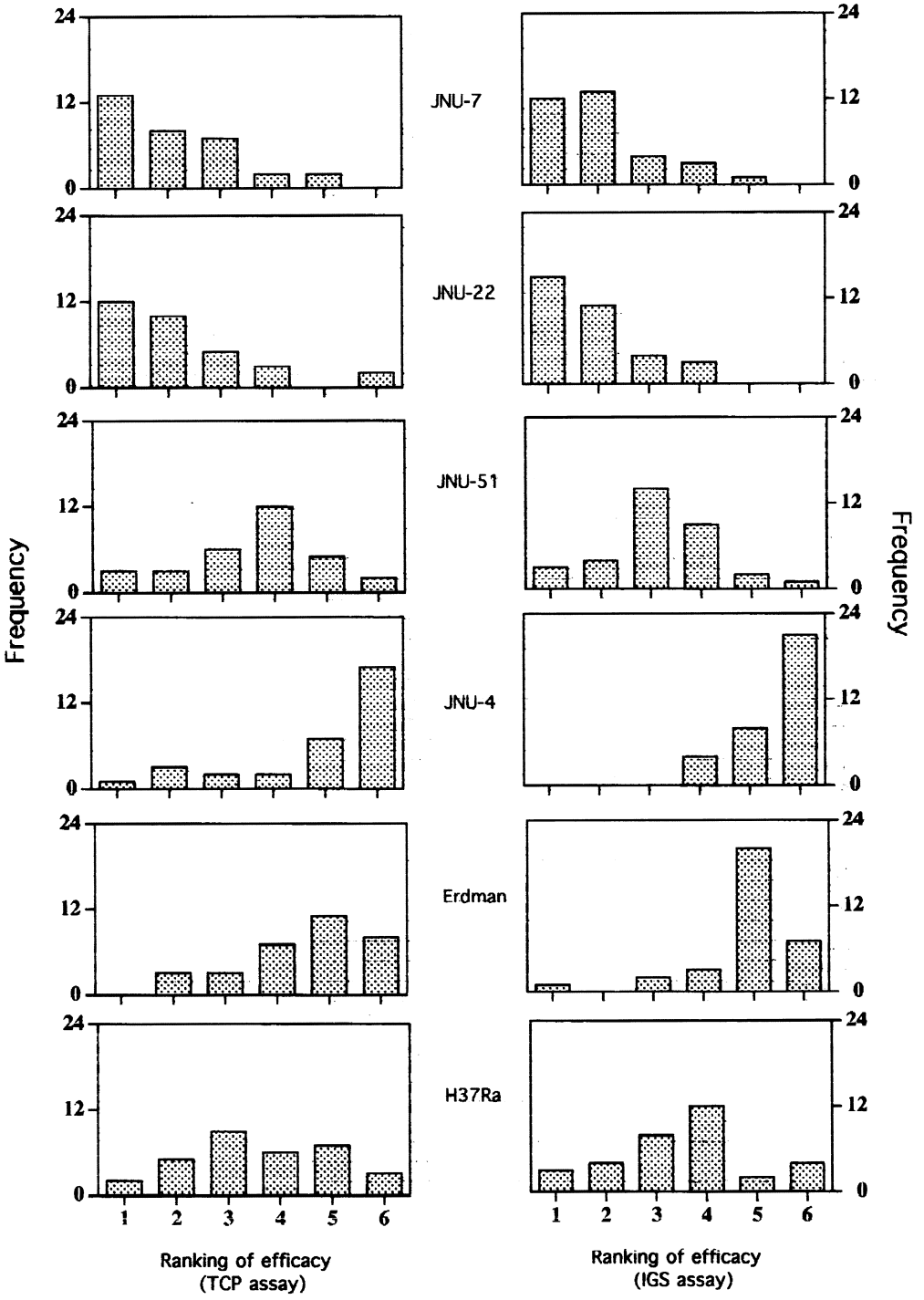

Figure 2 Relative efficacies of CFP preparations for T-cell activation. For each PBMC preparation, responses to the six CFP preparations were ranked from 1 (most effective) to 6 (least effective) in TCP (T-cell proliferation) and IGS (IFN-γ secretion) assays. The frequencies of the various rankings for a given CFP preparation within the set of 33 PBMC preparations are plotted for each CFP in TCP and IGS assays. CFP = culture filtrate proteins; PBMC = peripheral blood derived mononuclear cell.

eight PBMC preparations (Table). Thus the CFP from JNU-22 was most potent in the TCP assay for sample 1, whereas the CFP of JNU-4 was most potent for sample 22. Similarly, in the IGS assay, the CFPs of JNU-51 and JNU-7 were most effective for samples 1 and 2, respectively. Ranking of effectiveness of CFPs in TCP and IGS assays was different for many samples (samples 1, 5, 12, 22 and 30, Table). In addition, in many cases samples showing very good response in IGS assays were poorly activated in TCP assays (samples 12, 13, 15 and 30, Table). Cumulative data on ranking of efficacies of different CFP preparations in TCP and IGS assays are given in Figure 2. In TCP assays, CFPs from JNU-7 ranked first for 13 out of 33 sam-

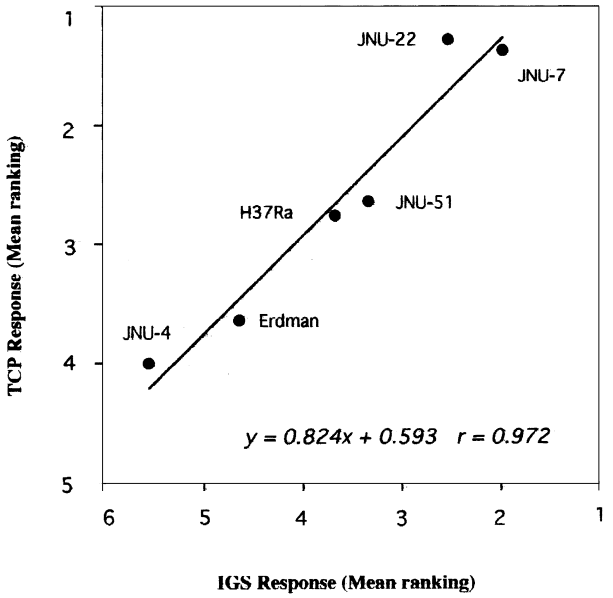

Figure 3 Correlation of efficacies of different CFP preparations in TCP and IGS assays. From Figure 2, mean rankings for all six CFP preparations in TCP and IGS assays were determined and plotted against each other. CFP = culture filtrate proteins; TCP = T-cell proliferation; IGS = IFN-γ secretion.

ples of PBMCs (Figure 2). By contrast, the CFP from Erdman did not rank first for any of the PBMC samples. Similar results were seen for IGS assays. While all CFP preparations induced good IFN-γ response, CFPs from JNU-7 and JNU-22 ranked first or second in 25 of the 33 PBMCs tested, whereas the CFP of JNU-4 did not rank first, second or third in any PBMC preparations.

Due to wide variations in absolute values of counts per minute (cpm, TCP assays) and interferon titers (IGS assays), a significant correlation between these two parameters could not be obtained. If, however, values of mean rankings of different CFP preparations were used, a very good correlation between the two parameters of T-cell activation could be observed ($r = 0.972$, Figure 3).

DISCUSSION

The aim of the current study was to compare the relative efficacies of culture filtrate antigens (CFPs) derived from laboratory strains of *M. tuberculosis* and those from contemporary Indian isolates of *M. tuberculosis*. For this purpose, we studied the in vitro response of PBMCs from normal donors to different CFP preparations. There are two reasons for our use of PBMCs from healthy donors and not from tuberculosis patients. Firstly, a substantial proportion of normal donors from Delhi are expected to be sensitized to mycobacterial antigens. This is because live BCG vaccination at birth is a norm in India, and, in addition, almost two-thirds of the population is estimated to be infected with or exposed to *M. tuberculosis*. Our results clearly support this assumption. Indeed there were only six donors out of a total of 33 examined whose PBMCs uniformly reacted poorly with all CFPs (less than two-fold activation in TCP assays). We have not, however, excluded results from these PBMC samples, as their exclusion did not change the results significantly. Secondly, we also reasoned that healthy donors with exposure to *M. tuberculosis* antigens would be those who might have developed protective immunity to *M. tuberculosis*. Studying their immune response would therefore be more relevant than taking PBMCs from tuberculosis patients, who, by virtue of having established infection, should be considered as lacking effective protective immunity.

The parameters selected for the assessment of the immune response to CFPs were T-cell proliferation and IFN-γ secretion, which denote T-cell activation in response to CFPs. These parameters were selected because their correlation with protective immunity has been established in other studies.[14–16] In addition, we included the parameter of induction of IL-12 secretion, because some recent studies have indicated a crucial role for IL-12 in the generation of protective immunity to tuberculosis.[17,18] Interestingly, we observed a significant spontaneous release of IL-12 in control cultures of PBMCs in the absence of CFPs, the reason for which is not clear. It is possible that the monocytes, the source of IL-12, may be under activation due to some common environmental antigen. It should be noted that the spontaneous secretion of significant amounts of other monocyte derived cytokines, IL-1 and TNF-α (tumor necrosis factor alpha), have also been reported in the literature.[19,20] CFPs induced little if any boosting of IL-12 response in most cases. A statistically significant increase in IL-12 secretion was observed only for CFP preparations from JNU-7 and JNU-51. The T-cell proliferative response and IFN-γ secretory response were, however, markedly boosted by most CFPs. Our results indicated that CFPs from two contemporary Indian isolates of *M. tuberculosis* were significantly more potent in inducing T-cell proliferation and IFN-γ secretion than were the CFP preparations derived from two standard laboratory strains of *M. tuberculosis*. CFPs from one isolate (JNU-4) were generally poorer activators of T cells than those from H37Ra and Erdman. However, there were PBMC preparations which reacted best with JNU-4 CFPs. The better performance of CFPs from contemporary strains of *M. tuberculosis* may be due to the fact that the sensitization of individuals would be to the *M. tuberculosis* strains which are currently infecting the general population and not to the strains that prevailed a century ago. In addition, different individuals may be sensitized to different variants of *M. tuberculosis*, which may explain significant differences in the relative efficacies of different CFP preparations in inducing T-cell activation in different PBMC preparations.

Our results suggest that the search for protective antigens within the pool of CFPs may not be restricted to standard laboratory strains of *M. tuberculosis*, and an analysis of contemporary strains of *M. tuberculosis* for this purpose may provide rich dividends. This study has identified two Indian isolates, JNU-7 and JNU-22, which secrete CFPs with significantly greater potency in inducing T-cell proliferation and IFN-γ secretion in PBMCs from sensitized healthy donors. Further work will be directed towards identifying specific components within CFPs of JNU-7 and JNU-22 which induce these superior responses, and which in turn could be useful in rational vaccine design.

Another important issue raised by this study is that there is a high background reactivity of T cells from healthy individuals towards mycobacterial antigens. While these results are from the Indian population, it is likely that similarly high background activity may also be observed in healthy populations in other countries where tuberculosis infection is endemic. In view of the high background reactivity, testing of a potential anti-tuberculosis vaccine in these populations may prove problematic if T-cell reactivity is taken as an indicator of the protective efficacy of the vaccine.

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RÉSUMÉ

CADRE : Alors que les protéines des filtrats de culture (CFP) de *M. tuberculosis* paraissent être de bons candidats pour un vaccin antituberculeux, seules les CFP provenant de certaines souches classiques de laboratoire de *M. tuberculosis* ont été étudiées dans cette optique.

OBJECTIF : Comparer les efficacités relatives des préparations de CFP provenant de deux souches de laboratoire et quatre isolats cliniques simultanés de *M. tuberculosis* pour l'induction d'une activation des cellules T.

SCHÉMA : Les CFP ont été isolées à partir de six souches de *M. tuberculosis* et utilisées afin d'induire : 1) une prolifération des cellules T, 2) une sécrétion d'IFN-γ, et 3) une sécrétion d'IL-12 par des préparation de cellules mononucléaires dérivées du sang périphérique (PBMC) provenant de 33 donneurs sains.

RÉSULTATS : Les préparations de PBMC ont sécrété spontanément des quantités significatives d'IL-12. Les préparations de CFP provenant de deux isolats cliniques

(JNU-7 et JNU-51) ont stimulé cette réponse de manière significative. Toutes les six préparations de CFP ont entraîné une sécrétion d'IFN-γ par les PBMC, mais à cet égard, celles provenant de deux souches contemporaines de *M. tuberculosis* (JNU-7 et JNU-22) s'avérèrent les plus efficaces. L'effet de CFP provenant de JNU-7 et de JNU-22 est significativement meilleur que celui de souches de laboratoire (H37Ra et Erdman). Des résultats similaires ont été obtenus quand le paramètre était la prolifération des cellules.

CONCLUSION : Ces résultats suggèrent que les CFP provenant d'isolats cliniques sélectionnés de *M. tuberculosis* peuvent dépasser les performances de CFP de souches standard de laboratoire et pour cette raison, pourraient être une meilleure source de candidats potentiels pour un vaccin antituberculeux.

RESUMEN

MARCO DE REFERENCIA : Mientras que el cultivo del filtrado de proteínas (CFP) de *Mycobacterium tuberculosis* parecen ser buenos candidatos de vacunas para la tuberculosis, se han estudiado para este propósito sólo los CFP derivados de ciertas cepas de *M. tuberculosis* de laboratorios conocidos.

OBJETIVO : Comparar la eficacia relativa de las preparaciones de CFP de dos cepas de laboratorios y cuatro de muestras clínicas de *M. tuberculosis* contemporáneas para inducir la activación de las células T.

MÉTODO : Se aislaron CFP de seis cepas de *M. tuberculosis* y se utilizaron para inducir 1) proliferación de las células T, 2) secreción del IFN-γ, y 3) secreción de IL-12 por las preparaciones de las células mononucleares derivadas de la sangre periférica (CMSP) provenientes de 33 dadores sanos.

RESULTADOS : Las preparaciones CMSP secretaron espontáneamente cifras significativas de IL-12 y preparaciones CFP de dos muestras clínicas (JNU-7, JNU-51) estimularon esta respuesta de manera significativa. Las seis preparaciones CFP indujeron la secreción IFN-γ por las CMSP, pero aquellas de las dos cepas contemporáneas de *M. tuberculosis* (JNU-7, JNU-22) fueron más efectivas al respecto. El efecto del CFP de la JNU-7 y de la JNU-22 fue significativamente mejor que aquel de las cepas de laboratorio (H37Ra y Erdman). Se obtuvieron resultados similares cuando el parámetro era la proliferación de las células T.

CONCLUSIÓN : Estos resultados sugieren que los CFP derivados de muestras clínicas seleccionadas de *M. tuberculosis* pueden ser más eficaces que los CFP de cepas de laboratorio estándares y por lo tanto pueden ser una mejor fuente de candidatos potenciales para la vacuna contra la tuberculosis.