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TITLE: Diagnostic value of Interleukin 12 p40 in tuberculous pleural effusions.
SHORT TITLE: Interleukin 12 p40 and pleural effusions.

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ABSTRACT

BACKGROUND: The diagnosis of tuberculous pleural effusion (TBPE) is frequently problematic. Several markers of TBPE in pleural fluid have been evaluated, with different results.

METHODS: Pleural effusions from 96 patients were classified on the basis of definitive diagnosis as tuberculous (39), neoplastic (42) or parapneumonic (15). Adenosine deaminase (ADA), ADA isoform 2 (ADA-2), interferon γ (IFN-γ), $CD3^+DR^+$ T lymphocytes ($CD3^+$ DR^+ T), and interleukin 12 p40 (IL-12 p40) were determined in all 96 effusions. The efficiency of IL-12 p40 for diagnosis of tuberculous pleural effusions (TBPEs) was evaluated in comparison with those of the other parameters; by comparing the areas under their receiver operating characteristics (ROCs).

RESULTS: With the threshold value of 550 pg/mL for IL-12 p40, had a sensitivity of 92.3% (36/39); specificity of 70.2% (17 false positives). The misclassification rate of IL-12 p40 was significantly greater than those of ADA-2 and ADA. Among TBPEs, ADA correlated significantly with ADA-2; and IFN-γ with ADA and IL-12 p40.

CONCLUSIONS: Although tuberculous pleural effusions show values of IL-12 p40 significantly higher than neoplastic and parapneumonic fluids, it is less efficient than ADA, ADA-2 and IFN-γ. Its routine determination is accordingly not justified.

KEY WORDS: Pleural effusion. Tuberculosis. Interleukin.

INTRODUCTION

The diagnosis of tuberculous pleural effusion (TBPE), one of the most frequent causes of pleural effusion [1], is not infrequently problematic due to the unspecificity of the diagnostic used tests [2-5]. Some determinations from pleural fluid (mycobacterial DNA detected by means of polymerase chain reactions (PCR) [6-12], interferon γ (IFN-γ) [12-17], lysozyme [15, 18, 19] and adenosine deaminase (ADA) [2, 12, 15, 19-24]) can be useful in these diagnostic processes.

It has become clear in recent years that as part of the immune response to infection by *Mycobacterium tuberculosis*, interleukin 12 increases macrophage activation by upregulating the production of IFN-γ by natural killer (NK) cells [25, 26]. This raises the possibility that IL-12 or its subunits may also be of diagnostic value. Here we report the results of a study in which the efficiency of IL-12 p40 in discriminating between TBPE and pleural effusions of neoplastic or parapneumonic origin was evaluated in comparison with the previously characterized parameters.

MATERIAL AND METHODS

We prospectively studied, during a 24-month period, 146 patients who were admitted to the Pulmonology Service of our centre with what proved tuberculous (TBPEs; 24 men, 15 women), neoplastic (NPPEs; 22 men, 20 women -Table 1) or parapneumonic pleural effusions (PPPEs; 12 men, 3 women) according to previous established criteria [15]. The Review Board on Human Studies at our institution approved the protocol. Informed consent was obtained from all patients.

Sample collection and analyses

Pleural fluid samples were taken by thoracocentesis at admission before institution of any treatment. Total cell counts were determined with a Siemens ADVIA 2120 haematology system. IL-12 p40 was determined in 96 effusions (39 tuberculous, 42 neoplastic and 15 parapneumonic) using a sandwich ELISA from AMS Biotechnology (pg/mL). Adenosine deaminase (ADA), expressed in U/L, was determined by the Giusti method [27]; isoform ADA-2, was determined by inhibition with EHNA [28]; Interferon γ (IFN-γ) was determined using an ELISA kit (Intertest-γ) from Genzyme (pg/mL). and $CD3^+ DR^+$ T counts by the method previously described [29, 30] (cells/μL).

Statistical analysis

Kolmogorov-Smirnov tests were used to check distributional normality; non-normal distributions were subjected to log transforms. Groups were compared using a post-hoc multiple comparison test (Bonferroni). The relative abilities of the various diagnostic parameters for pairwise discrimination between TBPEs and non-TBPEs were

evaluated by comparing the areas under their receiver operating characteristics (ROCs) [31]. The diagnostic performance of the various parameters when used with the diagnostic thresholds afforded by the ROC analysis (see Results) was evaluated in terms of sensitivity, specificity and positive and negative likelihood ratios (PLR, NLR). The PLR indicates how much more frequent positive test results occur in TBPEs, calculated as the proportion of positive test result in patients with TBPEs (sensitivity) divided by the proportion in non-TBPEs (1-specificity). The NLR indicates how much to decrease the probability of disease if the test is negative ((1-sensitivity) / specificity).

All statistical analyses were performed using MEDCALC®.

RESULTS

The distributions of the biochemical parameters in each group of pleural effusions are summarized in Table 2. For all these parameters, the median of the tuberculous group differed significantly from those of the neoplastic and parapneumonic groups in all cases, except comparison with PPPEs with regard to $CD3^+$ DR^+ T. The diagnostic thresholds afforded by the ROC analysis for IL-12 p40, ADA, ADA-2, IFN-γ and $CD3^+$ DR^+ T were 550 pg/mL, 54.3 U/L, 44.5 U/L, 169 pg/mL and 80.3 cells/mm^3 respectively. However, the ROCs of the diagnostic parameters (Fig. 1) show that IL-12 separated TBPEs from NPPEs and PPPEs less well than ADA, ADA-2 and IFN-γ. The area under the IL-12 p40 ROC, 0.837, was significantly smaller than the areas of these other parameters. The area under the IL-12 p40 ROC does not differ significantly from the area under the $CD3^+$ DR^+ T ROC. The area under the ADA-2 ROC, 0.996, was significantly higher than the areas of the other parameters.

Fig. 2 shows the ADA and ADA-2 levels in each group of effusions. All TBPEs except one had values higher than the established diagnostic thresholds: 54.3 U/L for ADA, 44.5 U/L for ADA-2. Sub-threshold levels of ADA and ADA-2 were found in 97.6% of NPPEs, while among PPPEs 80% had sub-threshold levels of ADA and 100% sub-threshold levels of ADA-2. Fig. 3 shows IL-12 and IFN-γ levels in each group of effusions. IL-12 p40 was above the threshold (550 pg/mL) in 92.3% of TBPEs, 35.7% of NPPEs, and 13.3% of PPPEs. IFN-γ was above the threshold (169 pg/mL) in 82.1% of TBPEs, 7.1% of NPPEs, and 6.7%% of PPPEs. $CD3^+$ DR^+ T was above the threshold (80.3 cells/mm^3) in 82.1% of TBPEs, 31% of NPPEs, and 46.6% of PPPEs.

Table **3** lists the numbers of misclassifications by each parameter and group. From worst to best, $CD3^+$ DR^+ T misclassified 28.1% of effusions, IL-12 20.8%, IFN-γ

11.5%, ADA 5.2%, and ADA-2 2.1%. The misclassification rates of ADA and ADA-2 did not differ significantly. The misclassification rate of ADA-2 was significantly lower than those of IFN-γ, IL-12 p40 and $CD3^+$ DR^+ T. The misclassification rate of IL-12 p40 did not differ significantly from that of $CD3^+$ DR^+ T and IFN-γ, but was significantly greater than those of ADA and ADA-2.

Table 4 lists other performance parameters emphasizing that IL-12 p40 had a sensitivity of 92.3%, a specificity of 70.2%, a PLR of 3.10 and an NLR of 0.11. Between the studied parameters, we did not found significant differences concerning to sensitivity. The specificity of IL-12 p40 was significantly lower than ADA, ADA-2 and IFN-γ, while ADA-2 showed no significant differences with respect to ADA and IFN-γ. Table 5 lists correlations among the various parameters in the TBPE group.

DISCUSSION

The results of this study appear to confirm that IL-12 p40 levels in the TBPEs are significantly higher than in the NPPEs and the PPPEs, but it is less efficient than the established markers ADA, ADA-2 and IFN-γ.

IL-12 is a cytokine composed of two polypeptide subunits, p40 and p35, that are encoded by distinct genes and are linked covalently by disulphide bridges in the active heterodimer, p70. IL-12 stimulates the production of IFN-γ by NK cells, and thereby indirectly promotes the activation of macrophages. It is therefore believed to play an important role in the immune response to intracellular pathogens such as *M. tuberculosis*. Previous studies have in fact found, like this study, that IL-12 p40 levels are significantly higher in TBPEs than in NPPEs and other pleural effusions [32-36], and have reported areas under the IL-12 p40 ROC similar to the 0.837 observed in this study [33]; but this value is significantly less than the areas under the ROCs of IFN-γ, ADA and ADA-2.

Our results confirm that most TBPEs are identifiable by their high IL-12 p40 levels (sensitivity 92.3%), but their specificity is poor, 70.2%. In a previous study using a diagnostic threshold of 560 pg/mL, we found a similar sensitivity but a higher specificity, 85.4% [34], the difference probably being mainly due to this previous study having included transudates, which only rarely have IL-12 p40 levels above the threshold. We believe that the specificity obtained excluding transudates from the study is the more relevant result for clinical practice, because the aetiological diagnosis of transudates is generally unproblematic; it is to distinguish among TBPEs, NPPEs and PPPEs that tests are needed. The overall misclassification rate of IL-12 p40 in the

present study was 20.8%, a figure that is significantly larger than the misclassification rates of ADA (5.2%), and ADA-2 (2.1%).

Other authors reported IL-12 p40 to have a specificity of 96.9% and a PLR of 17.45 but less sensitivity, 54.5% [35]. The immediate cause of the wide discrepancy between these values and ours is their having used a diagnostic threshold more than twice as high as ours, 1296 pg/mL. That this was the optimal threshold for their sample is in turn probably attributable mainly to that sample having included no PPPEs and three times as many NPPEs as TBPEs, an aetiological distribution very different from ours; as well the small size of their sample (43 effusions), and a different method to determine IL-12 p40.

The yield of the rest of parameters is similar to previous studies [37]. Although the overall misclassification rate of ADA-2, 2.1%, did not differ significantly from that of ADA, 5.2%, it did have a significantly larger area under its ROC.

IFN-γ was positively correlated with IL-12 p40, in keeping with current accounts of the role of IL-12 in inducing IFN-γ production during infection by *M. tuberculosis*. As expected, ADA-2 was closely correlated with ADA among TBPEs.

In conclusion, the observed elevation of IL-12 p40 levels in TBPEs is coherent with IL-12 playing a role in the immune response to infection by *M. tuberculosis*. The correlation between IL-12 p40 and IFN-γ in these effusions is in line with current knowledge about the relationship between both of them. However, IL-12 p40 is less efficient in this role than ADA, ADA-2 or IFN-γ, and its routine determination is accordingly not recommended.

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TABLES

Table 1. Types or locations of tumours associated with the 42 neoplastic pleural effusions in this study.

Bronchogenic carcinoma	17
Breast	10
Lymphoma	4
Thyroid	2
Thymus	2
Prostate gland	2
Melanoma	1
Ewing's sarcoma	1
Oesophagus	1
Uncertain	2

Table 2. Descriptive statistics of diagnostic parameters considered, for each type of pleural effusion.

	TBPE		**NPPEs**		**PPPEs**	
Males (%)	62.6		52.4		80	
Mean age (years±SD)	39.7±20		63.7±12.8		57.9±21.4	
n	39		42		15	
	Median	CI95	Median	CI95	Median	CI95
IL-12 p40 (pg/mL)	2873.0	1219-5224	351.8	99.5-697.1	155.8	22.6-462.3
ADA (U/L)	125.7	104.3-136.1	23.6	19.3-27.9	27	13.8-87.2
ADA-2 (U/L)	107.7	92.3-118.8	19.1	14.7-23.6	19.5	12.6-28.8
IFN-γ (pg/mL)	1952	645-3549	22.4	8.8-52.3	16	8.3-52.3
$CD3^+$ DR^+ T ($cells/mm^3$)	153.1	106.5-265.5	31.9	16.6-69.5	99.6	20.5-297.3

TBPE, tuberculous pleural effusion; NPPEs, neoplastic pleural effusions; PPPEs, parapneumonic effusions. CI95: 95% confidence interval.
SD: Standard Deviation. n: number of patients.
For all parameters, values in the tuberculous group differ significantly from values in the other two groups ($p < 0.0005$ in all cases except comparison of $CD3^+$ DR^+ T cell count in tuberculous and parapneumonic effusions, for which $p = 0.1110$).

Table 3. Numbers of misclassified effusions of each group, for each diagnostic parameter studied.

	IL-12 p40	**ADA**	**ADA-2**	**IFN-γ**	**$CD3^+$ DR^+ T**
TBPE	3/39 (7.6%)	1/39 (2.6%)	1/39 (2.6%)	7/39 (17.9%)	7/39 (17.9%)
NPPEs	15/42 (35.7%)	1/42 (2.4%)	1/42 (2.4%)	3/42 (7.1%)	13/42 (31%)
PPPEs	2/15 (13.3)	3/15 (20%)	0/15 (0%)	1/15 (6.7%)	7/15 (46.7%)
Total	20/96 (20.8%)	5/96 (5.2%)	2/96 (2.1%)	11/96 (11.5%)	27/96 (28.1%)
p^*	< 0.0005	0.4443		0.0225	< 0.0005
$p^\#$		< 0.0035	< 0.0005	N.S.	N.S.

TBPE, tuberculous pleural effusion; NPPEs, neoplastic pleural effusions; PPPEs, parapneumonic effusions. N.S.: not significant. * p values for comparisons with ADA-2. $^\#$ p values for comparisons with IL-12.

Table 4. Performance measures for diagnosis of TBPEs by each diagnostic parameter with the stated thresholds.

	Threshold	**Sensitivity**	**Specificity**	**PLR**	**NLR**
IL-12 p40	> 550 pg/mL	92.3%	70.2%	3.10	0.11
ADA	> 54.3 U/L	97.4%	93.0%	13.9	0.03
ADA-2	> 44.5 U/L	97.4%	98.2%	55.5	0.03
IFN-γ	> 169 pg/mL	82.1%	93.0%	11.7	0.19
$CD3^+$ DR^+ T	> 80.3 cel/mm^3	82.1%	64.3%	2.3	0.28

PLR, positive likelihood ratio; NLR, negative likelihood ratio.

Table 5. Coefficients of correlation between the parameters studied in the TBPE group, with *p* values in parentheses.

	ADA	**ADA-2**	**IFN-γ**	**$CD3^+$ DR^+ T**
IL-12 p40	0.105 (0.516)	0.025 (0.878)	0.468 (0.004)	0.146 (0.369)
ADA		0.931 (0.000)	0.319 (0.050)	0.009 (0.956)
ADA-2			0.181 (0.265)	- 0.080 (0.623)
IFN-γ				0.281 (0.083)

LEGENDS OF THE FIGURES

Figure 1. Receiver operating characteristics (ROCs) of the parameters studied, for diagnosis of tuberculous pleural effusion versus neoplastic or parapneumonic pleural effusion.

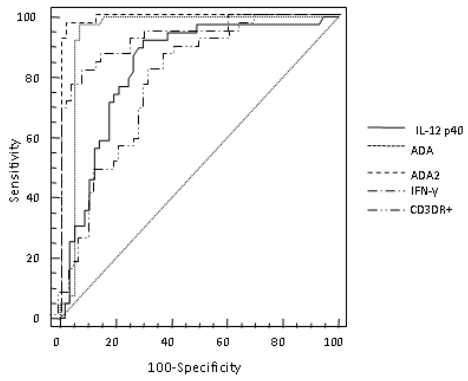

Figure 1. Receiver operating characteristics (ROCs) of the parameters studied, for diagnosis of tuberculous pleural effusion versus neoplastic or parapneumonic pleural effusion.

Figure 2. ADA and ADA-2 levels (U/L) in the pleural fluid of the three groups of patients studied (cut-off point of 54.3 and 44.5 respectively).

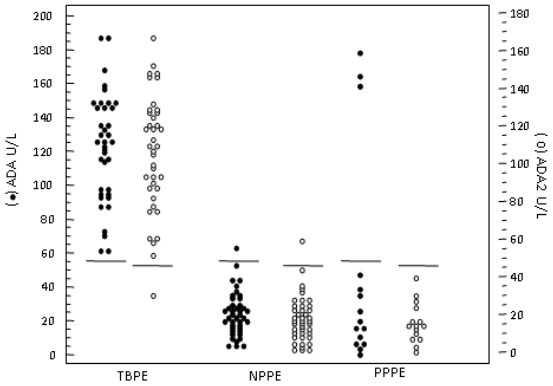

Figure 2. ADA and ADA-2 levels (U/L) in the pleural fluid of the three groups of patients studied (cut-off point of 54.3 and 44.5 respectively).

Figure 3. IFN-γ and IL-12 p40 levels (pg/mL) in the pleural fluid of the three groups of patients studied (cut-off point of 550 and 169 respectively).

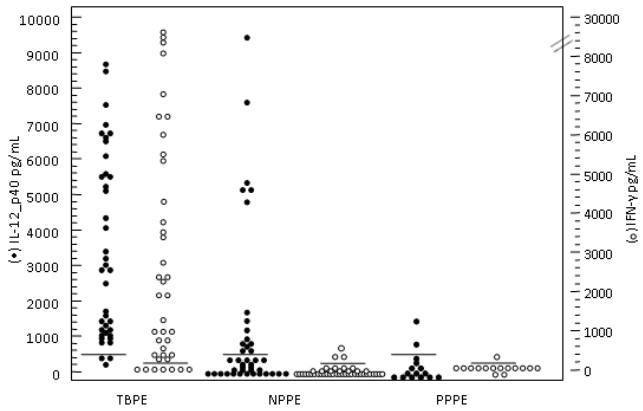

Figure 3. IFN-γ and IL-12 p40 levels (pg/mL) in the pleural fluid of the three groups of patients studied (cut-off point of 550 and 169 respectively).