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ORIGINAL ARTICLE

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Disregulation in TH1 and TH2 subsets of CD4+ T cells in peripheral blood of colorectal cancer patients and involvement in cancer establishment and progression

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Abstract Recent theories have established that, during an ongoing immune response, the lymphokines produced by TH1 and TH2 subsets of $CD4^+$ T cells are critical to the effectiveness of that response. In vivo and in vitro studies have demonstrated that the type of environmental cytokines plays a determinant role in directing the development of naive T cells into TH1 or TH2 effector cells. Disregulated expansion of one or other subset may contribute to the development of certain diseases. To establish whether a similar situation might exist in the cells of the peripheral blood (PBMC) of colorectal cancer patients, we have performed immunological studies on a group of patients and a group of healthy subjects. We examined the interleukin-2 (IL-2), interferon γ (IFNγ), IL-4, IL-6 and tumour necrosis factor α levels in serum; the production of IL-4 and IL-2, with and without activating agents, by PBMC, tumour-draining lymph node lymphocytes and tumour cells; and the proliferative response of PBMC to IL-2, IL-4 and anti-CD3 monoclonal antibody (anti-CD3), which were variously combined. The data of the present study lead us to hypothesize that, because of suppressive effects probably due to environmental IL-4, in the peripheral blood of patients there seems to be a disregulation in the functionality of TH1 and TH2 subsets of $CD4^+$ T cells, with an expansion in TH2 and a malfunction in TH1 cells. Moreover it seems that this disregulation increases with as the disease progresses through the stages, suggesting that it can be directly implicated in the mechanisms that allow the tumour to locate and progress in the host.

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Introduction

The theory shared by recent studies is that the protective value of the immune response is highly dependent on the type of cytokines produced by T cells [19, 43, 46]. According to their cytokine profile, these immune responses have been classified into types I and II [6, 7], respectively regulated by TH1 and TH2 cell subsets of the $CD4^+$ T helper (TH) cellular population. Although both subsets secrete interleukin-3 (IL-3), granulocyte-macrophage-colony-stimulating factor and tumor necrosis factor α (TNFα), only TH1 cells produce IL-2, IFNγ and TNFβ [11], whereas TH2 secrete IL-4, IL-5, IL-6 and IL-10 [47]. The cytokines produced by TH1 and TH2 cell subsets are very important for the functionality and immune response of cytotoxic T lymphocytes (CTL), because they can regulate the differentiation of these cells.

Various studies [10, 17, 25, 52] have described an essential role for IL-2 in the generation of the CTL precursor in both the type I and type II immune response [56]. In the absence of the T helper cellular population, which produces IL-2, Ag-specific CTL can be generated only from the precursors of the type I immune response because of their ability to produce IL-2 in an autocrine fashion [3]. IL-6 may be important for the generation of CTL but this role appears to be caused by IL-2-dependent mechanisms [29]. The TH2 and TH1 cells seem to derive from a common precursor that may be either a TH0 or a naive cell [1, 36, 38], which produces both TH1 and TH2 cytokines. Generally the polarization of the immune response into TH1 or TH2 cellular types is not absolute and the ratio of these cells varies according to the physiological demand and clinical condition [28]. In vivo and in vitro studies in mice have demonstrated that cytokines play a very important role in directing the development of naive T cells into TH1- or TH2-type effector cells [12, 33, 44, 49].

Table 1 Clinical and histopathological data of patients with colorectal cancer

Characteristic	Number of patients in stages: I		II		III		IV	
Rectum/colon sigmoideum[a]		2				2		1
Transverse colon		1		2		1		1
Descending colon		1		5		1		1
Ascending colon		1		10		4		3
Sigmoid		4		7		2		5
Rectum		9		9		2		3
pTNM	T2N0M0	14	T3N0M0	33	T3N1M0	5	T3N1M1	4
	T1N0M0	4			T3N2M0	3	T3N3M1	2
					T3N3M0	1	T4N1M1	2
					T4N3M0	1	T4N3M1	5
					T4N1M0	2	T1N1M1	1
Number of patients		18		33		12		14

[a] Location of colorectal cancer

IL-4 was shown to be essential for the development of TH2 cells [9, 22] and to inhibit the differentiation of precursors into TH1 cells [45, 51]. Moreover, some pathological conditions may arise from an abnormal balance between the production of TH1 and TH2 cytokines [34]. It seems that the relative proportion of each cell type depends on the exogenous cytokines present during the activation event [16, 13, 31, 48, 53]. In fact, the cytokines present during the primary stimulation were found to have an important influence on the type of TH cell differentiation [12, 13, 31, 44]. In this paper we describe our results from the immunological evaluation of a group of colorectal cancer patients and a group of healthy subjects, which we performed in order to determine whether a similar situation exists in the cells of the peripheral blood of cancer patients.

Materials and methods

Patients and healthy subjects

A group of 77 patients (44 men and 33 women, aged from 44 to 85 years), who were diagnosed for the first time as having colorectal cancer and had to undergo colectomy, were studied. Clinical diagnosis was confirmed histopathologically and patients were subtyped by pTNM classification according to the diagnostic criteria of the American Joint Committee on Cancer and the Committee of the International Union Against Cancer. After this classification the patients were divided into four groups that corresponded to the four stages shown in Table 1. None of the patients received radiation or chemotherapy before surgery. Tumours varied from 2.5 cm to 9.0 cm in diameter.

A group of 70 healthy subjects (40 men and 30 women, aged from 43 to 78 years: laboratory staff, blood donors, subjects undergoing surgery for dermic cysts) served as the control group. In both the patient (N) and healthy subject (N_c) groups there was the same distribution of both age (N = 77, 66.1 ± 10.5 years compared to N_c = 70, 64.5 ± 9.1 years, P = 0.1) and sex (P = 0.132, odds ratio = 1.000). Venous blood (donated by both patients and healthy subjects) was collected by venepunture into syringes containing preservative-free heparin (Liquemin-Roche), 1 h prior to anaesthesia and surgery.

Detection of IL-2, IFNγ, IL-4, IL-6, and TNFα

Sera from patients and the control group, collected within 1 h of withdrawal, and the cell-free supernatant obtained in vitro were centrifuged at 250 *g* and stored frozen in aliquots at −80 °C until use. Enzyme-linked immunosorbant assays (ELISA) were employed. The method has been described in detail elsewhere [5]. The sensitivities of these assays were as follows: TNFα <1.5 pg/ml (T Cell Diagnostics, Cambridge, USA); IL-2 <5 pg/ml, IL-4 <1 pg/ml (Endogen, Cambridge, USA), IL-6 <2 pg/ml (Medgenix, Belgium), IFNγ <100 pg/ml (Genzyme, Cambridge, USA).

PBMC, tumor-draining lymph node lymphocytes and tumour cell preparation

Peripheral blood mononuclear cells (PBMC) were separated by centrifugation over a Ficoll/Hypaque gradient (20 min, 1000 *g*), and washed with RPMI-1640 medium (Gibco). Isolated cells were cultured at a concentration of 1×10^6 cells/ml in RPMI-1640 complete medium (supplemented with 10% heat-inactivated human serum or fetal calf serum, 0.2 nM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin; Sigma). Lymph node lymphocytes (LNL) and tumour cells were initially rinsed in modified RPMI-1640 medium containing 10% human serum and then finely minced with surgical iris scissors. The mechanically disaggregated specimens were then filtered to separate mononuclear and tumoral cells from surrounding tissue fragments and centrifuged for cell number determination.

Cytokine production from PBMC, LNL and tumour cells

Supernatants were obtained from PBMC, LNL and tumour cell culture in RPMI-1640 complete medium. The cells [with and without 3 µg/ml phytohaemagglutinin (PHA)] were incubated at concentrations of 1×10^6 cells/ml at 37 °C in a humidified atmosphere of 5% CO_2. After 24 h and 72 h of culture without change of medium, 120 µl supernatant was removed from each well, centrifuged at 250 *g* and stored frozen in aliquots at −80 °C until use.

Proliferation assays

Cellular cultures were performed in triplicate in a 96-well microtitre plate (Falcon Plastics). Each well contained 2×10^5 mononuclear cells in 200 µl complete medium, and cytokines were added: 1000 U/ml IL-2 (Proleukin, Cetus Corp., Emeryville, USA), 1000 U/ml IL-4 (Genzyme, Boston) and 2.5 µg/ml anti-CD3 (Orthoclone OKT3 Cilag), which were variously combined. The plate was incubated at 37 °C in a 5% CO_2 incubator for 7 days and 0.8 µCi [^{3}H]thymidine was added to each well 18 h prior to harvesting. The incorporation was counted in triplicate and expressed as cpm.

Statistical analysis

The results were expressed as means ± standard deviations and the differences between groups were assessed by the Mann-Whitney U test, the two-tailed Student's t-test and the χ^2-test as appropriate. Correlations between immunological parameters were analysed using Spearman's rank test or the Pearson correlation test as appropriate. The level of significance was set at $P < 0.05$. The immunological evaluation in relation to the progression of the illness was carried out by comparing the groups of patients at various stages.

Table 2 Cytokine serum levels in the group of patients and in the control group. This table shows the cytokine serum levels for the patients and the control group. Values are expressed as means ± SD; the numbers of patients, stages and controls are shown in parentheses. *IL* interleukin, *IFN* interferon, *TNF* tumour necrosis factor

Cytokines	Cytokine level (pg/ml)					
	Controls	All patients	Stage I	Stage II	Stage III	Stage IV
IL-2*	(27) 24.2 ± 10.6	(54) 10.4 ± 9.7 $P < 0.0001$	(15) 20.1 ± 9.2 NS	(19) 9.1 ± 7.8 $P < 0.0001$	(9) 6.2 ± 6.4 $P < 0.0001$	(11) 2.9 ± 3.4 $P < 0.0001$
IFN-γ**	(41) 111.5 ± 178	(63) 189.5 ± 244 NS	(12) 144.1 ± 194.5 NS	(32) 144.5 ± 200.6 (NS	(9) 331.8 ± 386.8 NS	(10) 243.7 ± 248.4 NS
IL-4*	(33) 16.4 ± 6.2	(24) 330.4 ± 283.5 $P < 0.0001$	(8) 52.7 ± 36.4 $P < 0.0001$	(3) 105 ± 111 $P < 0.0001$	(6) 411.5 ± 111.2 $P < 0.0001$	(7) 675 ± 137.1 $P < 0.0001$
IL-6**	(70) 16.2 ± 91.6	(70) 118.8 ± 278.2 $P < 0.0001$	(16) 23.2 ± 63.6 NS	(31) 24.1 ± 69.3 $P = 0.015$	(11) 136.6 ± 141.9 $P < 0.0001$	(12) 474.6 ± 372.2 $P < 0.0001$
TNFα**	(33) 0	(51) 4.9 ± 20 NS	(10) 0 NS	(25) 0 NS	(10) 23.5 ± 41.8 $P = 0.001$	(6) 2.6 ± 6.3 $P = 0.02$

*, ** The statistical evaluation of the patients, both in total and in the different disease stages, was carried out by comparing them with the control group. This was done by using the most appropriate statistical test depending on the populaton distribution: * by Student's *t*-test; ** by the Mann-Whitney *U*-test

Results

Evaluation of IL-2, IFNγ, IL-4, IL-6, TNFα in the serum

In patients (Table 2) the serum levels of IL-4 and IL-6 were significantly higher than in the controls. By contrast, the IL-2 serum level was lower. IFNγ and TNFα levels were not different from those of the control group. By comparing the serum levels of each group of patients in the various stages with those of the control group it is possible to note an increase in the levels of IL-4, IL-6 and TNFα. However, the IL-2 level decreased as the disease progressed. This environmental model of peripheral blood suggests that the TH cells functionally present are of the TH2 type and they increase with the progression of the disease.

IL-4 and IL-2 production from PBMC, LNL and tumour cells.

After stimulation with the mitogen PHA, the production of IL-4 from PBMC of patients was significantly higher than in the control group (Table 3) and positively correlated with

Table 3 Production of IL-2 and IL-4 from peripheral blood mononuclear cells (*PBMC*) and lymph node lymphocytes (*LNL*). Values are expressed as means ± SD; numbers of patients, stages and controls are shown in parentheses. In this table the production of IL-2 and IL-4 coming from PBMC and LNL (without and with phytohaemagglutinin, *PHA*, 3 µg/ml, 24 h, 37 °C) in the group of patients and the control group is shown. As regards the production of cytokines from PBMC, the statistical evaluation of the patients, both in total and in the various disease stages, was carried out by comparing them with the control group. This was done by using the most appropriate statistical test depending on the distribution of the population. As regards the lymph node cells, it was not possible to perform a comparison with the healthy subjects owing to the lack of lymph nodes from this group

Cytokine and its source	Mitogen stimulation	Cytokine production (pg/ml)					
		Controls	All patients	Stage I	Stage II	Stage III	Stage IV
PBMC							
IL-2*	–PHA	(20) 19.7 ± 19.5	(19) 1.1 ± 3.2 $P < 0.0001$	(3) 3.3 ± 5.8 NS	(7) 1.5 ± 3.9 $P = 0.010$	(5) 0.16 ± 0.3 $P = 0.026$	(4) 0 $P = 0.01$
IL-2*	+PHA	(20) 330.2 ± 210.9	(19) 47 ± 71.6 $P < 0.0001$	(3) 134.8 ± 104.4 NS	(7) 78.3 ± 69.6 $P = 0.0008$	(5) 1.9 ± 4.4 $P = 0.0007$	(4) 6.1 ± 10.6 $P = 0.001$
IL-4**	–PHA	(7) 4.3 ± 4.1	(18) 6.2 ± 3.3 NS	(2) 1.05 ± 0.9 NS	(2) 6.2 ± 6.3 NS	(6) 3.96 ± 0.04 NS	(8) 8.5 ± 2.1 $P = 0.042$
IL-4**	+PHA	(7) 11.5 ± 5.2	(18) 31.5 ± 16.1 $P = 0.0043$	(2) 9.6 ± 1.2 NS	(2) 7.4 ± 5.6 NS	(6) 28.2 ± 8 $P = 0.0009$	(8) 45.7 ± 4.5 $P < 0.0001$
LNL							
IL-4	–PHA		(8) 1.6 ± 0.3	ND	(6) 1.7 ± 0.3	(2) 1.3 ± 0.03	ND
IL-4	+PHA		(9) 6.8 ± 5.7	ND	(6) 9.0 ± 5.9	(2) 2.3 ± 0.3	(1) 2.8
IL-2	–PHA		(8) 3.2 ± 3.9	ND	(6) 2.6 ± 4.4	(1) 5.15	(1) 5
IL-2	+PHA		(11) 323.9 ± 525.7	ND	(8) 391.6 ± 612	(2) 159.8 ± 77	(1) 110.5

*, ** Statistical analysis by: * Student's *t*-test; by the Mann-Whitney *U*-test

Table 4 Statistical correlations in the group of patients. When present, PHA was 3 μg/ml; IL-2, IL-4 1000 U/ml; anti-CD3 2.5 μg/ml. Cytokine levels were measured in pg/ml, PBMC proliferation in cpm

	Serum level of cytokines					Cytokine production by PBMC				Proliferative response of PBMC to:				
						–PHA		+PHA						
Parameter	IL-2 (*a*)	IFNγ (*b*)	IL-4 (*c*)	IL-6 (*d*)	TNFα (*e*)	IL-2	IL-4 (*f*)	IL-2 (*g*)	IL-4 (*h*)	IL-2 (*i*)	IL-2 +anti-CD3 (*l*)	anti-CD3 (*m*)	IL-4 (*n*)	IL-2+IL-4 +anti-CD3 (*o*)
Stage (*A*)	[–]	NS	[+]	[+]	[+]	NS	[+]	[–]	[+]	[–]	[+]	[+]	NS	NS
Serum levels of:														
IL-2 (*B*)		[–]	[–]	NS	NS	NS	NS	NS	NS	NS	[–]	NS	NS	NS
IFNγ (*C*)	[–]		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
IL-4 (*D*)	[–]	NS		[+]	NS	NS	[+]	[–]	[+]	[–]	[+]	[+]	NS	NS
IL-6 (*E*)	NS	NS	[+]		NS	NS	NS	[–]	NS	NS	NS	NS	[+]	NS
TNFα	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS

The statistical correlations were carried out using the Spearman's Rank and the Pearson correlation tests.
Correlations with stage (Spearman's rank test)
Serum level of cytokines: *Aa* (N = 54, r = –0.62, P <0.0001); *Ac* (N = 24, r = 0.89, P <0.0001); *Ad* (N = 70, r = 0.43, P = 0.0004); *Ae* (N = 51, r = 0.35, P = 0.014)
Production of cytokines: *Af* (N = 18, r = 0.63, P = 0.012); *Ag* (N = 19, r = –0.58, P = 0.0095); *Ah* (N = 18, r = 0.92, P = 0.0001)
PBMC proliferative response: *Ai* (N = 36, r = –0.51, P = 0.0024); *Al* (N = 36, r = 0.45, P = 0.0071); *Am* (N = 36, r = 0.92, P = 0.009)
Correlation with serum levels of cytokines:
Serum level of cytokines: *Bb* = *Ca* [N = 48, r = –0.45, P (Pearson) = 0.001]; *Bc* = *Da* [N = 21, r = –0.81, P (Pearson) = 0.0001]; *Dd* = *Ec* (N = 24, r = 0.42, P (spearman) = 0.040]
Production of IL-2 and IL-4 from PBMC: *Df* [N = 7, r = 0.85, P (Pearson) = 0.014]; *Dg* [N = 7, r = –0.76, P (Pearson) = 0.04]; *Dh* (N = 7, r = 0.97, P (Pearson) = 0.0002]; *Eg* [N = 19, r = –0.47, P (Spearman) = 0.040]
PBMC proliferative response: *Bl* [N = 26, r = –0.50, P (Pearson) = 0.0091]; *Di* [N = 15, r = –0.58, P (Pearson) = 0.002]; *Dl* [N = 15, r = 0.62, P (Pearson) = 0.014]; *Dm* [N = 15, r = 0.99, P (Pearson) = 0.011]; *En* [N = 27, r = 0.48, P (Spearman) = 0.017]
Moreover, correlations between the production of IL-4 and the proliferative response of PBMC to various stimuli were noticed (Pearson correlation test)
fl (N = 9, r = 6.9, P = 0.038) *fo* (N = 9, r = 0.76, P = 0.0179; *hl* (N = 9, r = 0.70, P = 0.034); *ho* (N = 9, r = 0.68, P = 0.042). On the other hand, significant correlations between the production of IL-2 and IL-4 from PBMC with and without PHA were not noticed

the disease stage (Table 4). By contrast, the production of IL-4 without PHA was not different between patients and the control group (Table 3) even though it increased with disease stage (Table 4). Preliminary results show that the $CD4^+$ cells of patients, after PBMC separation with Dynabeads M-450 (for use with Detachabead; Dynabeads, Dynal, Oslo, Norway), also produced higher IL-4 levels than did the control group ($N = 5$, $N_c = 9$; t-test $p = 0.008$) in the supernatant collected after 72 h of culture. In addition, the production of IL-2 from PBMC of patients (with and without PHA) was significantly lower than in the control group (Table 3) and negatively correlated with the disease stage (Table 4). These statistics suggest that in the PBMC of the patients there is an increase in the TH subpopulation that produces IL-4 (TH2) and that this subpopulation increases with disease progression. Our observations agree with the results showing that patients′ LNL in culture produce IL-4 (Table 3) and the highest production of IL-4 was at stage II, which preceded lymph node infiltration. Thus, it may be possible that when the tumour cells infiltrate the lymph node, the generation of TH cells in the lymph node environment is shifted towards a TH2 type of immune response. The fact that the functionality of the TH cells is directed towards the TH2 subpopulation is probably due to the cell suppression effect which, when the IL-4 is present in the environment, suppresses the production of TH1-type cells. In fact, in 1 patient with colon adenoma (data not shown) IL-4 production with PHA from LNL was lower than for patients at stage II (2.36 versus 9.0±5.9 pg/ml). In patients the production of IL-2 from LNL with PHA seems to decrease with the progression of the illness. This decrease also goes to show the possible phenotypical and functional shifting of the TH subpopulation towards the TH2 type as the disease progresses. In order to support what is stated above, the results of the experiments on the production of IL-4 by the tumour cell culture are reported. These results highlight that the IL-4 is ever present in all the supernatants (without PHA) collected after 24 h of culture (N = 6; 4.5±3.2 pg/ml) and increases in the 72-h supernatant (N = 3; 253.8±256.9 pg/ml). In the latter it is also possible to note the increase from stage I (N = 1, 91.3 pg/ml) to stage IV (N = 1, 550 pg/ml).Whereas, must be noted that the IL-2 production is always absent in the 72-h supernatant (without PHA) from tumour cells (N = 7, IL-2 < 5 pg/ml).

PBMC proliferative response

Mean values and the statistical evaluation of proliferative response in patients and the control group of healthy subjects are shown in Table 5. It can be seen that the proliferative responses to IL-2, and IL-2+anti-CD3 are not statistically different from those of the healthy control group. However, when we added IL-4 to the stimulation with IL-2+anti-CD3 there was a decrease in the proliferative response in the group of patients in respect to the control group. Moreover, when we observed the proliferative response to IL-2 in the groups of patients at the various stages a decrease in the response to IL-2 with the progres-

Table 5 Proliferative responses of PBMC in patients and in controls. Results show the PBMC proliferative response to IL-2 (1000 U/ml), IL-4 (1000 U/ml) anti-CD3 (2.5 μg/ml), which were variously combined. The values are expressed as means ± SD

PBMC	Proliferative response ([^{3}H]dThd incorporation, cpm)					
	Controls (N_c = 23)	All patients (N = 36)	Stage I (N = 6)	Stage II (N = 12)	Stage III (N = 5)	Stage IV (N = 13)
Without activation**	326.2 ± 265.3	768.2 ± 125.8 NS	625 ± 195.6 P = 0.022	729.4 ± 897.9 NS	318 ± 206.2 NS	1 043.4 ± 1 853.4 NS
+IL-2*	43 530.1 ± 22 369.8	54 088.9 ± 36 962.6 NS	93 067.2 ± 39 896.3 (*a*) P = 0.004	59 614.6 ± 38 185.8 NS	44 018.6 ± 32 370 NS	34 871.4 ± 20 079.6 (*b*) NS
+IL-2+anti-CD3*	19 653.0 ± 15 753.4	88 510.1 ± 38 480.6 NS	78 861.5 ± 21 140.7 (*c*) NS	74 577.7 ± 37 672.6 NS	50 824.4 ± 9 938.9 P = 0.0001	120 319 ± 29 500 (*d*) NS
+IL-2+anti-CD3+IL-4*	141 031.0 ± 14 487.4	102 808.0 ± 30 864.2 P = 0.005	106 937 ± 43 793.3 NS	104 012 ± 34 421.7 P = 0.028	76 258.8 ± 13 956.3 P < 0.0001	110 902 ± 21 862.5 P = 0.0041
+IL-2+IL-4*	38 965.5 ± 8 393.5	32 749.9 ± 14 402.9 NS	46 268.5 ± 11 914.1 NS	30 916.7 ± 9 204.5 NS	28 037.4 ± 11 316.9 NS	29 329.6 ± 16 929.7 NS
+IL-4+anti-CD3*	64 026.0 ± 13 455.2	13 128.6 ± 8 767.0 P < 0.0001	21 950.7 ± 6 608.5 P = 0.015	9 855.2 ± 5 416.3 P = 0.015	19 644.3 ± 18 082.8 P = 0.0020	11 206.3 ± 4 558.3 P < 0.0001
+IL-4	635.2 ± 316.0	1 248.0 ± 1 659.4 NS	245.2 ± 124.1 P = 0.049	892.1 ± 1 064.5 NS	2 384.2 ± 2 298.5 NS	1 375.8 ± 1 853.7 NS
+anti-CD3**	539.1 ± 979.1	8 271.7 ± 9 087.1 P = 0.015	184 ± 31.1 NS	282 ± 66.5 NS	3 647.4 ± 2 069 P = 0.033	19 327 ± 4 467.6 P = 0.0043

*, ** The statistical evaluation was carried out using: * Student's *t*-test; ** the Mann-Whitney *U*-test, depending on the population distribution. The statistical evaluation of the values of the patients, both in total and in various stages of disease, was carried out by comparing them with the control group. Differences between patients in stages I and IV: *a* versus *b*, P = 0.0005; *c* versus *d*, P = 0.0068; *a* versus *c*, P = 0.46; *d* versus *b*, P < 0.0001

sion of the illness could be seen (Tables 4, 5). Such an effect seems to be neutralized by the addition of the anti-CD3 to the IL-2 in that such a stimulus determines an increase in the proliferative response as the disease progresses (Tables 4, 5). Thus, it would seem that there are faults at the level of the mechanisms of the proliferative responses of patients′ PBMC to the IL-2, which increase with the stage progression, and that these faults are eliminated by the costimulator effects of the signals produced in vitro with the addition of anti-CD3. The increase in the proliferative response with the addition of anti-CD3 backs-up our hypothesis of the expansion of the TH2 cells with as the disease advances in that other researchers have noticed that a good proliferative response of PBMC to IL-2+anti-CD3 demonstrates an enrichment in the TH2 cell subpopulation in respect to the TH1 cells [37, 54, 55]. The decrease in the proliferative response to IL-2+anti-CD3 after the addition of IL-4, in the patients relative to the control group (recreating the situation in vivo), might suggest a suppressive action on IL-2 by IL-4.

Statistical correlations

In Table 4 the correlations between the immunological parameters of patients are shown. It is of great interest to note that the progression of the disease was positively correlated with the IL-4 serum level. By contrast, the correlation with IL-2 level was negative. In patients the production of IL-4 from PBMC (with and without PHA) also significantly increased with disease stage and was correlated with the level of IL-4 in the serum. Instead the production of IL-2 from PBMC (with PHA) decreased as the disease advanced. Moreover, in patients the proliferative response of PBMC to IL-2 was negatively associated with disease progression, suggesting that the IL-2 mechanism does not work well because it is damaged in some way; however, the costimulator signals produced by anti-CD3 reversed this situation, in that an increase of PBMC proliferative response with stage was noted when we added anti-CD3 to IL-2. Moreover this increase, which is well-known to be indicative of a TH2 expansion, was positively correlated with serum level and production of IL-4 by PBMC, confirming the TH2 expansion hypothesis. In addition, it must be noted that the serum level of IL-6 was negatively correlated with IL-2 production by PBMC after PHA stimulation. Moreover the IL-6 serum level increased with disease progression, but IL-2 decreased. Other researchers have shown that to allow the generation of reactive CTL effector cells the mechanisms of IL-2 and IL-6 should be positively correlated [29, 50].

Discussion

The data of the present study lead us to hypothesize that, probably through IL-4 mechanisms, in the cells of peripheral blood of colorectal cancer patients there is a disregulation in the functionality of TH1 and TH2 cells with an expansion in TH2 and a malfunction in TH1.

This hypothesis is supported by the following results. In patients the serum levels of IL-4 and IL-6 were significantly higher than in the control group, whereas the level of IL-2 was lower and IFNγ was not different (Table 2). It must be noted that, as the disease progressed, the level of IL-4 increased in the serum; by contrast, IL-2 decreased (Tables 2, 4). We also found that, in patients, the IL-4 production from PBMC was higher than in the control group and significantly increased with as the disease stage advanced, being positively correlated to the level of IL-4 in the serum. Non-T cells, such as basophils and mast cells, could provide IL-4 but, after PBMC separation, the $CD4^+$ cells of patients also produced a higher level of IL-4 than did those of the control group. In addition IL-2 production from PBMC was lower than in the control group and decreased with disease progression.

Our hypothesis is also supported by the experiments on proliferative responses of PBMC to IL-2, IL-4 and anti-CD3, which showed interesting statistical correlations (Table 4). In patients the proliferative response to IL-2 also decreased with the disease stage, denoting damage to its mechanisms. However, the addition of anti-CD3 to IL-2 reversed this situation, resulting in a significant increase in PBMC proliferation as the disease advanced. It must also be noted that the proliferative response to IL-2+anti-CD3 was positively correlated with the serum level and with the production of IL-4 from PBMC (with and without PHA) and, consequently, with TH2 cells. Moreover, the results of other researchers [37, 54, 55] underline the conclusion that IL-2+anti-CD3 stimulation favours the *in vitro* proliferation of TH2 cells.

Thus, taken together, these data suggest that, in patients, the presence of IL-4 during the ongoing immune response to tumour suppressed the response of TH1 cells and favoured the expansion of TH2. Our results may indicate that, in these patients, the TH2 type of immune response can be induced by three principal causes: (a) IL-4 production from tumour cells, which we found in all samples examined (whereas IL-2 is always absent) and can affect the type of immune response at either the local or systemic level, or both together, by suppressive effects on IL-2 mechanisms; (b) the absence of TH1 cell costimulator signals, which induces an unresponsive anergy state in these cells and a transition from TH1 to a TH2-like signalling phenotype; (c) the presence of the cytokine IL-4 in the lymph node environment which, for the activity of suppressor cells, may be sufficient to induce changes in the type of lymphocyte trafficking between the lymph nodes and peripheral blood, turning the response towards TH2-type cells. The existence of the mechanisms by which IL-4 down-regulates IL-2 production is confirmed by the results of other researchers [23, 35, 41, 42, 48], which have shown that, in several infectious disease models, IL-4 inhibits the generation of protective cell-mediated immunity and that the environmental IL-4 is able to down-regulate IL-2 production by the inhibition of IL-2 gene transcription.

Our hypothesis about the mechanisms determining this situation in the cells of peripheral blood of patients is also confirmed by our results, which concern the immunological evaluation in stages. In fact, it is possible to note that, compared to the control group, at stage I the patient serum only showed an increase in IL-4 while the IL-2, IL-6, IFNγ and TNFα levels were still within the normal range (Table 2). Instead, after the progression of the disease from stage I to II, compared to controls the patient's serum levels of IL-4 and IL-6 increased, whereas that of IL-2 decreased (Table 2) and as did its production from PBMC (Table 3). In addition, the IL-4 and IL-6 serum levels were increasing while IL-2 was decreasing, which continued until stage IV (Table 2). Our observations are also confirmed by results from PBMC proliferative responses. The stimulation by IL-2 and IL-2+anti-CD3 in patients did not show significant differences from the control group (Table 5). Therefore, when we added IL-4 to IL-2+anti-CD3 (recreating the situation in vivo) there was a decrease of the PBMC proliferative responses of patients compared to the control group (Table 5). Thus it is possible to suppose that anti-CD3 in vitro signals induced costimulatory effects on the PBMC response because the cells that are functionally increasing are of the TH2 type; however, the suppressive effects due to IL-4 damage the activation pathway of these cells with an anti-CD3 stimulus. This is confirmed by the results of the PBMC proliferative response to anti-CD3 and IL-4+anti-CD3 (Table 5). In fact, it is possible to note that in patients, compared to the control group, the proliferative response to anti-CD3 was significantly higher, but when we added IL-4 to anti-CD3 this response was significantly lower. Moreover our results show that disease progression was negatively associated with serum level and IL-2 production from the patients′ PBMC; instead, there was a positive correlation with IL-6 serum level and PBMC production after PHA stimulus (data not shown: $N = 21$, $r = 0.88$, Spearman's rank test $P = 0.0001$). IL-2-producing TH1 cells may be required for the generation and function of mature CTL [10, 17]. IL-6 has been shown in vitro to be a CTL differentiation factor [50], but it may not be sufficient by itself to drive the generation of reactive CTL effector cells in vivo, because for this it also needs IL-2 [29]. Consequently, the result of an IL-2 deficiency may be the suppression of the generation of reactive CTL effector cells, which allows the tumour to locate and progress in the host. In fact, an IL-4-dependent mechanism by which CD8 cytolytic responses can be turned into non-cytolytic ones [14] was found, which probably allows a pathogen to escape elimination. This mechanism has been implicated in the pathology of leprosy [40] leishmania major [20] and AIDS [26]. It must be underlined that, as in the last pathology, in our patients we previously found [5] an increase in soluble IL-2 receptor and a decrease in $CD4^+$ PBMC expression connected with disease progression. Moreover, other papers show that T cells isolated from cancers are defective in their response to IL-2 [2] and to autologous tumour [57] and have a reduced level of CTL [24].

It therefore seems that, in colorectal cancer patients, the initial tumour establishment is enabled because the immune system of the patients is functionally directed toward a TH2 type of immune response, which nevertheless is not able to

oppose and destroy the first cells contaminated by tumour mechanisms. The inability of the TH2 cells to destroy the initial cancer cells seems to be due to the IL-2 deficiency, which does not allow the generation of specific reactive antitumour CTL [4, 14, 18]. Consequently, to allow the generation of a specific and productive immune response which destroys the tumour, it is very important to elucidate how these mechanisms should be manipulated in vivo to shift the cytokine balance in patients.

These events may not only take place in colorectal cancer patients; it is possible that they are involved in the basic mechanisms of tumour pathology, because in other types of cancers [27] their effects may also be noticed, since the positive resolution of the disease was obtained by therapy inducing TH1 cytokines. Moreover, immunotherapy led to an improvement in the resolution of the disease, following the very frequent administration of IFNα. Data from other studies suggest that this may because IFNα but not IFNγ is able to oppose the inhibition of IL-4 mechanisms on TH1 cells and redirect the immune response from type II to type I [8].

In conclusion, further research is necessary but current studies have underlined the fact that IFNγ and TGFβ favour TH1 cell development [15, 32, 49]. Interestingly, the latest research also reports that IL-12 induces TH1 differentiation and development in CD4+ T cells undergoing primary activation [21, 43]. Moreover, TH1 cells are very sensitive to glucocorticoids [39] and inducers of cAMP [30], which can aid an appropriate choice of pharmacological therapy. Thus, it is of great interest to study the possible applications of these agents and new agents in order to improve the evaluation and treatment in colorectal cancer patients, and to begin to find the right way to solve this increasingly urgent problem.

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