Pergamon

European Journal of Cancer Vol. 30A, No. 9, pp. 1259–1263, 1994

0959-8049/94 $7.00+0.00

0959-8049(93)E0022-V

Serum Levels of Tumour Necrosis Factor-α in Patients With B-Cell Chronic Lymphocytic Leukaemia

F. Adami, A. Guarini, M. Pini, F. Siviero, R. Sancetta, M. Massaia, L. Trentin, R. Foà and G. Semenzato

Serum levels of tumour necrosis factor-α (TNF-α) have been evaluated in the peripheral blood of 91 patients with B-cell chronic lymphocytic leukaemia (B-CLL), and have been correlated with the clinical stage (according to Rai's staging system) and relevant haematological and immunological data. Increased values were detected, compared to 36 normal age-matched controls (36 pg/ml ± 5 versus 0.11 pg/ml ± 0.08; $P < 0.05$). An increase of TNF-α serum levels was observed in all stages including stage 0, with a progressive increase in relation to the stage of the disease. A significant relationship between serum TNF-α levels and the number of circulating monocytes ($P < 0.002$) and an inverse correlation with the level of the haemoglobin ($P < 0.001$) was established, as defined by the Pearson's correlation test. In contrast, no correlation was observed between TNF-α serum levels and the other parameters taken into account, including the white blood cell and platelet counts, the absolute number of peripheral blood (PB) lymphocytes, CD5+ B lymphocytes, CD57+ lymphocytes, serum levels of lactic dehydrogenase, total serum immunoglobulins and the serum levels of IgG, IgA and IgM. These data suggest that, in addition to the B-CLL neoplastic cells, the PB monocytes may be involved in the release of TNF-α.

Keywords: Tumour necrosis factor-α, chronic lymphocytic leukaemia, disease progression, clinical parameters, anaemia

Eur J Cancer, Vol. 30A, No. 9, pp. 1259–1263, 1994

INTRODUCTION

TUMOUR NECROSIS factor-α (TNF-α) is a cytokine with a wide spectrum of biological activities including the induction of inflammatory responses, immune modulation and the regulation of tumour cell growth [1–4]. Although major sources of spontaneously produced TNF-α are cells belonging to the monocyte-macrophage lineage, following appropriate stimulation, this molecule can also be released by B and T lymphocytes, and large granular lymphocytes (LGL) [5].

Neoplastic lymphocytes from patients with B-cell chronic lymphocytic leukaemia (B-CLL) and hairy cell leukaemia (HCL) have been shown to express TNF-α-related mRNA, and to release TNF-α spontaneously *in vitro* [6–8]. In both diseases, it has also been shown *in vitro* that TNF-α may promote the proliferation of neoplastic lymphocytes [6–11]. In addition, in neoplastic B lymphocytes the mRNAs of the TNF-α inducible cytokines [interleukin (IL)-1α, IL-1β and IL-6] are expressed both constitutively [12] and after incubation with TNF-α [13]; these cytokines have also been documented to be released *in vivo* [14]. With all these data taken together, it is possible to hypothesise an "autocrine growth factor loop" of the chronic B-cell malignancies, even if the proliferating effect of the IL-6 is quite controversial [15].

Coupled to the observations reported above, the increased serum levels of TNF-α in B-CLL and HCL patients [6, 7] prompted some authors to suggest a role of this cytokine in the regulation of proliferation of the neoplastic clone. While some authors [8, 16, 17] reported a positive effect on proliferative activity, others [6] found an inhibitory function or no effect. Also, in terms of TNF-α receptor (TNF-αR) expression, data in the literature are conflicting [16, 18]. Furthermore, no information is available on whether or not other potential sources of TNF-α, such as cells belonging to the monocyte-macrophage lineage or LGLs, may contribute to the increased amounts of serum TNF-α.

In an effort to clarify the clinical relevance of the increased serum TNF-α levels in B-CLL, and to ascertain if a relationship can be established between this increase and the extent of the disease and/or some of the events naturally occurring in this disorder, such as anaemia and hypogammaglobulinaemia, we

Correspondence to G. Semenzato.
F. Adami, R. Sancetta, L. Trentin and G. Semenzato are at the 1st Medical Clinic and Clinical Immunology Branch, Department of Clinical Medicine, Padua University School of Medicine; A. Guarini, M. Pini and R. Foà are at the Medical Clinic Section, Department of Biomedical Sciences and Human Oncology, Turin University School of Medicine; F. Siviero is at the Department of Clinical Pathology, Cittadella Hospital; M. Massaia is at Hematology Section, Department of Medicine and Experimental Oncology, Turin University School of Medicine; and R. Foà is also at the Immunogenetic and Histocompatibility CNR Center, Turin, Italy.
Revised 18 Nov. 1993; accepted 19 Jan. 1994.

evaluated the serum TNF-α levels in 91 B-CLL patients; the data obtained were correlated with haematological and immunological parameters.

MATERIALS AND METHODS

Source of cells and serum samples

Blood samples were collected from 91 B-CLL patients, 31 males and 60 females (mean age 65 ± 9 years). The diagnosis was based on established criteria, as described previously [19]; in all cases, evidence of a monoclonal CD5+ B-lymphocytosis was obtained. The total leucocyte count and the proportion of circulating monocytes was estimated in a routine haematology laboratory.

Phenotypic evaluations

Phenotypic analysis of peripheral blood lymphocytes (PBL) was performed using fluorescein (FITC)- or phycoerytrin (PE)-conjugated monoclonal antibodies (MAbs), and quantitated using a flow cytometer. MAbs used were CD3 (OKT3) (Ortho Pharmaceutical), CD19 (anti-Leu 12), CD5 (anti-Leu1) and CD57 (anti-Leu7) (Becton-Dickinson, Sunnyvale, California, U.S.A.). Double immunofluorescent cells (i.e. CD19+ CD5+ cells) were detected by CD19-FITC and CD5-PE MAbs. The CD nomenclature was reported according to the Fourth Meeting on Leucocyte Typing [20]. Light chains of surface immunoglobulins were detected with a mouse FITC- (anti-κ) and PE-conjugated (anti-λ) MAb (Simultest, Becton-Dickinson). Predetermined optimum concentrations of primary and secondary antibody were used in all cases. Briefly, 10^7 cells/ml were incubated with the above-quoted FITC- or PE-conjugated antibodies for 30 min in ice, then centrifuged twice in cold phosphate buffered saline (PBS). For indirect fluorescence, a FITC-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin (Ig) (Techno Genetics, Turin, Italy) was added; cells were further incubated for 30 min at 4°C and washed twice. After these incubations, 10000 gated cells were analysed using a FACScan analyser (Becton-Dickinson), and data were processed by using the Consort 30 program. Ten thousand cells bearing the typical lymphocyte scatter were scored.

Patients were graded as follows: group 1 corresponding to Rai stage 0 patients; group 2 corresponding to Rai stages I and II patients, group 3 corresponding to Rai stages III and IV patients. The total tumour mass [21] was evaluated in a selected number of cases. At the time of the analysis, no patient had evidence of infectious disease, and all were untreated or had been off treatment for at least 4 months. Serum samples from 36 healthy volunteers, age- and sex-matched, were used as controls. Serum samples from the patients and from controls were stored at −80°C until titration.

TNF assay

Serum levels of TNF-α were measured using an immunoradiometric assay according to the manufacturer's instructions (Medgenix Group s.a., Fleurus, Belgium). Briefly, 200 μl of each sample to be tested were dispensed in an anti-TNF-α coated tube. Fifty microlitres of the second step reagent (anti-TNF-α^{125}I) were added to each tube. After 16 h of incubation at room temperature, the tubes were decanted and washed twice with 20% Tween 20. The tubes were counted in a γ-counter for 60 s. The standard curve was prepared by plotting the counts per min (cpm) on the ordinate against the standard concentration on the abscissa. TNF-α concentrations were determined by plotting the cpm values using a computer-assisted polynomial function. This immunoradiometric assay measures both free and receptor-bound TNF-α; it is specific for TNF-α and does not cross react with TNF-β, IL-1, IL-2, interferon α, β or γ. The sensitivity of this assay is 5 pg TNF-α/ml.

Statistics

The data were analysed with a SPSS/PC+ program on an Epson personal computer. All data are presented as means ± standard error (S.E.M.). Comparisons between values were carried out running the one-way analysis of variance (with parametric and non-parametric tests: least significant difference and Kruskal–Wallis, respectively), and the Pearson's correlation test. A P value < 0.05 was accepted as significant.

RESULTS

Serum levels of TNF-α

While control sera showed undetectable values of TNF-α (Figure 1), in B-CLL the range of serum TNF-α levels was rather wide, and the mean levels were different in the three considered groups ($F < 0.00005$, at the one-way analysis of variance). Overall, serum levels greater than 10 pg/ml were found in 48 of the 91 cases analysed (53%); 10 out of the 35 (29%) from group 1, 21 out of the 38 (55%) from group 2 and 17 out of the 18 (94%) from group 3. Altogether, undetectable values were recorded in only 30 of the 91 cases (33%), 21 of which were in group 1 (stage 0) patients. Increasing values were observed from group 1 (7 ± 2 pg/ml) to group 2 (39 ± 7 pg/ml) and group 3 (87 ± 13 pg/ml). A statistically significant difference was found to separate the three groups ($P < 0.05$). No significant correlation could be observed between serum levels of TNF-α and the total tumour mass (TTM) (data not shown).

When the TNF-α serum levels were correlated with different

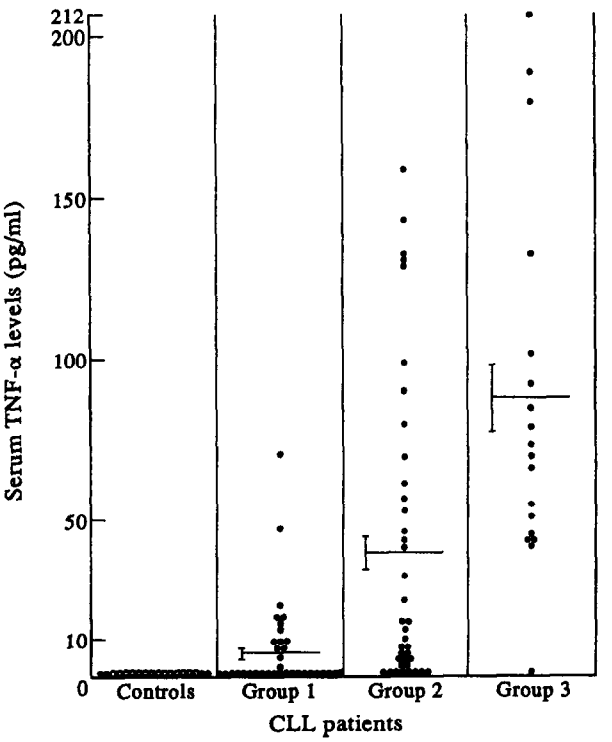

Figure 1. Serum levels of TNF-α (pg/ml) in B-CLL patients (subdivided according to stages) and in healthy controls. Group 1, Rai stage 0; group 2, Rai stages I and II; group 3, Rai stages III and IV. Baseline dots represent undetectable serum TNF-α levels. In the control group, every dot represents two healthy subjects.

haematological and immunological parameters (Table 1), a significant correlation ($r = 0.42$, $P < 0.002$) was found with the absolute number of circulating monocytes. In addition, a significant inverse correlation was established with the level of haemoglobin ($r = -0.43$, $P < 0.001$). No significant correlations were observed between serum TNF-α levels and the absolute number of PB WBC, lymphocytes, CD5+ B-lymphocytes, CD57+ lymphocytes, platelet count and serum levels of IgG, IgA or IgM.

DISCUSSION

This study confirms that in B-CLL patients serum levels of TNF-α are significantly increased with respect to controls, and demonstrates that the values increase with disease progression. A correlation between TNF-α serum levels and the number of PB monocytes and the level of haemoglobin was also established.

In vivo production by neoplastic lymphocytes may account for the higher TNF-α serum levels found in the bulk disease. Both our group [6] and others [22] have reported an increase of *in vitro* TNF-α production by peripheral blood mononuclear cells (PBMC) from early stage B-CLL patients compared to the production by PBMC from patients with advanced stages of disease. These data do not disagree with our hypothesis since high tumour masses may produce increased serum TNF-α levels even in the presence of a low secretive capacity on a per cell basis. Among the other parameters taken into account, the PB CD5+ B lymphocytes, the serum lactic dehydrogenase levels [23], and the degree of the thrombocytopenia do not fully, nor exclusively reflect the tumour burden, the lack of correlation with serum TNF-α levels being, therefore, not surprising.

A direct correlation was found between serum TNF-α levels and the number of PB monocytes, an increase of the latter being a well-known feature in B-CLL [24]; our data indicate that the absolute number of PB monocytes increases from group 1 to group 3 patients. These findings raise the hypothesis of whether cells belonging to the monocyte-macrophage lineage may contribute to the excess of TNF-α found in the sera of B-CLL patients, mostly in those with advanced disease. Flieger and colleagues [25] reported a partially suppressed production of TNF-α by lipopolysaccharide-triggered monocytes of B-CLL patients. However, their *in vitro* conditions are likely to be quite different with respect to the *in vivo* situation.

The strong inverse relationship between serum TNF-α and haemoglobin concentrations further points to the active role of this cytokine in haematopoiesis. In fact, an inhibitory activity of TNF-α on erythroid progenitors [26] and the recovery of haematopoiesis in B-CLL by anti-TNF-α antibodies [27] have been observed *in vitro*. However, anaemia during TNF-α treatment of cancer patients was also reported *in vivo* [28]. In this respect, the high serum TNF-α levels we found in our patients may account for the otherwise unexplained anaemia sometimes observed during the natural history of the disease.

The observed increase of PB CD57+ lymphocytes did not correlate with the serum levels of TNF-α. These data are not consistent with the hypothesis that the CD57+ lymphocytes may be significantly involved in the secretion of this cytokine in B-CLL. Finally, the lack of correlation with the serum levels of immunoglobulins seems to rule out a direct effect of TNF-α on the impairment of immune function of the normal B-cells.

In conclusion, our data confirm the presence of increased TNF-α serum levels in B-CLL patients and show higher values in patients with advanced disease. As the tumour mass is likely to be the main source of the TNF-α, we expected some degree of correlation between serum TNF-α levels and the prognosis. In this regard, 4 patients of our group 3 with high serum TNF-α levels (from 44 pg/ml to 189 pg/ml), died of the disease. However, before a definitive relationship between TNF-α levels and tumour mass can be established, further evaluations with appropriate *in vitro* experiments are needed to assess the putative role of the monocyte-macrophage lineage cells in the release of TNF-α, and the rate of the release of TNF-α by neoplastic lymphocytes. Serum levels of this molecule could then represent a reliable marker of tumour burden and, therefore, an additional prognostic factor in patients with B-CLL.

1. Brenner MK. Annotation. Tumour necrosis factor. *Br J Haematol* 1988, **69**, 149–152.
2. Beutler B, Cerami A. The common mediator of shock, cachexia and tumor necrosis. *Adv Immunol* 1988, **42**, 213–231.
3. Loetscher H, Steinmetz M, Lesslauer W. Tumor necrosis factor: receptors and inhibitors. *Cancer Cells* 1991, **3**, 221–226.
4. Semenzato G. Tumour necrosis factor: a cytokine with multiple biological activities. *Br J Cancer* 1990, **61**, 354–361.
5. Peters PM, Ortaldo JR, Shalaby MR, *et al.* Natural killer-sensitive targets stimulate production of TNF-α but not TNF-β (lymphotoxin) by highly purified human peripheral blood large granular lymphocytes. *J Immunol* 1986, **137**, 2592–2598.
6. Foà R, Massaia M, Cardona S, *et al.* Production of tumor necrosis factor-alpha by B-cell chronic lymphocytic leukemia cells: a possible regulatory role of TNF in the progression of the disease. *Blood* 1990, **76**, 393–400.
7. Foà R, Guarini A, Francia di Celle P, *et al.* Constitutive production of tumor necrosis factor alpha in hairy cell leukemia: possible role in the pathogenesis of the cytopenia(s) and effect of treatment with alpha-interferon. *J Clin Oncol* 1992, **10**, 954–959.
8. Cordingley FT, Bianchi A, Hoffbrand AV, *et al.* Tumour necrosis factor as an autocrine tumour growth factor for chronic B-cell malignancies. *Lancet* 1988, ii, 969–971.
9. Buck C, Digel W, Schöniger W, *et al.* Tumor necrosis factor-α but not lymphotoxin, stimulates growth of tumor cells in hairy cell leukemia. *Leukemia* 1989, **4**, 431–434.
10. Digel W, Stefanic M, Schöniger W, *et al.* Tumor necrosis factor induces proliferation of neoplastic B cells from chronic lymphocytic leukemia. *Blood* 1989, **73**, 1242–1246.
11. Trentin L, Zambello R, Pizzolo G, *et al.* Tumor necrosis factor-α and B-cell growth factor induce leukemic hairy cells to proliferate *in vitro*. *Cancer Det Prev* 1991, **15**, 385–390.
12. Biondi A, Rossi V, Bassan R, *et al.* Constitutive expression of the interleukin-6 gene in chronic lymphocytic leukemia. *Blood* 1989, **73**, 1279–1284.
13. Heslop HE, Bianchi ACM, Cordingley FT, *et al.* Effects of interferon α on autocrine growth factor loops in B lymphoproliferative disorders. *J Exp Med* 1990, **172**, 1729–1734.
14. Aguilar-Santelises M, Loftenius A, Ljung C, *et al.* Serum levels of helper factors (IL-1 alpha, IL-1 beta and IL-6), T-cell products (sCD4 and sCD8), sIL-2R and beta 2-microglobulin in patients with B-CLL and benign B lymphocytosis. *Leuk Res* 1992, **16**, 607–613.
15. Aderka D, Maor Y, Novick D, *et al.* Interleukin-6 inhibits the proliferation of B-chronic lymphocytic leukemia cells that is induced by tumor necrosis factor-α. *Blood* 1993, **81**, 2076–2084.
16. Digel W, Schöniger W, Stefanic M, *et al.* Receptors for TNF on neoplastic B cells from CLL are expressed *in vitro* but not *in vivo*. *Blood* 1990, **76**, 1607–1613.
17. Moberts R, Hoogerbrugge H, van Agthoven T, Löwenberg B, Touw I. Proliferative response of highly purified B-chronic lymphocytic leukemia cells in serum free culture to interleukin-2 and tumor necrosis factor alpha and beta. *Leuk Res* 1989, **13**, 973–980.
18. Trentin L, Zambello R, Agostini C, *et al.* Expression and functional role of TNF receptors on leukemic cells from patients with B-chronic lymphoproliferative disorders. *Blood* 1993, **81**, 752–758.
19. Semenzato G, Foà R, Agostini C, *et al.* High serum levels of soluble interleukin-2 receptors in patients with B chronic lymphocytic leukemia. *Blood* 1987, **70**, 396–400.
20. Knapp W, Dorken B, Rieber P, Schmidt RE, Stein H, von dem Borne AEGKr (eds). *Leucocyte Typing IV*. Oxford, Oxford University Press, 1989.

Table 1. Clinical and haematological parameters of B-CLL patients (mean ± S.E.M.)

	n	sTNF (pg/ml)	Hb (g/l)	Monocytes (× 10⁹/l)	WBC (× 10⁹/l)	Lymphocytes (× 10⁹/l)	CD5+ B-ly (× 10⁹/l)	CD57+ ly (× 10⁹/l)	LDH (U/l)	Platelets (× 10⁹/l)
B-CLL	91	36 ± 5	126 ± 2	923 ± 212	41 774 ± 5 316	34 617 ± 4 824	22 745 ± 5 868	1 106 ± 171	275 ± 24	175 639 ± 9 263
Group 1	35	7 ± 2	137 ± 2	535 ± 53	22 251 ± 3 151	16 261 ± 3 020	5 678 ± 1 234	813 ± 114	174 ± 10	204 150 ± 14 281
Group 2	38	39 ± 7	132 ± 2	807 ± 148	44 397 ± 7 908	36 449 ± 6 679	29 093 ± 11 354	1 358 ± 424	315 ± 341	192 296 ± 13 331
Group 3	18	87 ± 13	98 ± 4	2 517 ± 1 343	70 856 ± 1 667	62 041 ± 15 354	40 955 ± 14 750	1 324 ± 404	322 ± 48	102 785 ± 8 681
Controls	36	0	137 ± 4	469 ± 29	5 236 ± 315	2 140 ± 106	2.5 ± 0.3	264 ± 38	334 ± 15	253 290 ± 12 400

21. Jaksic B, Vitale B. Total Tumour Mass score (TTM): a new parameter in chronic lymphocytic leukaemia. *Br J Haematol* 1981, **49**, 405–413.
22. Hahn T, Kuminsky G, Bassous L, Berrebi A. Tumor necrosis factor production in B cell chronic lymphocytic leukemia. *Leuk Lymph* 1991, **4**, 117–121.
23. Pan L, Beverley PCL, Isaacson PG. Lactate dehydrogenase (LDH) isoenzymes and proliferative activity of lymphoid cells-an immunocytochemical study. *Clin Exp Immunol* 1987, **86**, 240–245.
24. Flieger D, Löms Ziegler-Heitbrock HW. Abnormal blood monocytes in chronic lymphocytic leukemia. *Cancer Res* 1988, **48**, 4812–4816.
25. Flieger D, Emmerich B, Meyer N, Riethmüller G, Löms Ziegler-Heitbrock HW. Deficient production of tumor necrosis factor by peripheral-blood monocytes in chronic lymphocytic leukemia. *Int J Cancer* 1990, **45**, 280–286.
26. Roodman GD, Bird A, Hutzler D, Montgomery W. Tumor necrosis factor-alpha and hematopoietic progenitors: effects of tumor necrosis factor on the growth of erythroid progenitors CFU-E and BFU-E and the hematopoietic cell lines K562, HL60, and HEL cells. *Exp Hematol* 1987, **15**, 928–935.
27. Michalevicz R, Porat R, Vechoropoulos M, *et al.* Restoration of in vitro hematopoiesis in B-chronic lymphocytic leukemia by antibodies to tumor necrosis factor. *Leuk Res* 1991, **15**, 111–120.
28. Blick M, Sherwin SA, Rosenblum M, Gutterman J. Phase I study of recombinant tumor necrosis factor in cancer patients. *Cancer Res* 1987, **47**, 2986–2989.

Acknowledgements—This study was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan), the Consiglio Nazionale delle Ricerche, Special Project on "Applicazioni Cliniche della Ricerca Oncologica" (CNR, Rome), and the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, Rome).

The authors thank Dr D. De Groote of the Medgenix Group s.a., Fleurus, Belgium, for the generous gift of the TNF-α kits and Mr M. Donach for his help in the preparation of the manuscript.

Pergamon

European Journal of Cancer Vol. 30A, No. 9, pp. 1263–1269, 1994

0959–8049/94 $7.00+0.00

0959-8049(94)E0122-K

Efficacy of Combined 5-Fluorouracil and Cisplatinum in Advanced Gastric Carcinomas. A Phase II Trial With Prognostic Factor Analysis

Ph. Rougier, M. Ducreux, M. Mahjoubi, J.P. Pignon, S. Bellefqih, J. Oliveira, C. Bognel, Ph. Lasser, M. Ychou, D. Elias, E. Cvitkovic, J.P. Armand and J.-P. Droz

Combined chemotherapy has demonstrated a degree of efficacy in gastric carcinoma. As 5-fluorouracil (5FU) and cisplatinum are two of the most active drugs, we have tested the efficacy of combined 5FU and cisplatinum in a prospective phase II trial. Cycles were administered every 4 weeks and consisted of 5FU 1000 mg/m²/day 5 days continuous intravenous (i.v.) infusion and cisplatinum 100 mg/m² on day 2. Cycles were repeated according to tolerance and efficacy. 87 patients entered the study, 57 with metastatic or recurrent tumour (M) and 30 with locally advanced gastric cancer (LAGC). The response rate for the 83 evaluable patients was 43% [95% confidence interval (CI) 30–56%]. There were four complete responses (5%), 32 partial responses (39%), 34 cases of stable disease and 13 cases of progressive disease. Responses were more frequent in patients with a good performance status ($P = 0.02$), with their primary located in the cardia ($P = 0.003$), with a non-linitis plastica tumour form ($P = 0.003$) or a tumour containing less than 50% of independent cells ($P = 0.016$). Median survival was 9 months for the total population. It was better in patients with a good performance status ($P = 0.01$), and those who did not have linitis plastica ($P = 0.005$). Toxicity was acceptable, although grade 3–4 neutropenia was reported in 22% of the cycles, mucositis in 14% and 3 patients died of septic complications. The combination of 5FU and cisplatinum is effective in terms of tumour response in advanced gastric cancer and warrants testing with the other active regimens.

Eur J Cancer, Vol. 30A, No. 9, pp. 1263–1269, 1994

INTRODUCTION

CHEMOTHERAPY IN advanced gastric carcinomas is palliative, and has a limited effect [1, 2]. Among the agents with known anti-tumour activity, 5 fluorouracil (5FU) has been extensively used [3], and cisplatinum is one of the most active in terms of response rate (RR) [4, 5]. Many combinations have been tested, and a RR of more than 40% has been reported with a marginal effect on survival [1, 2]. The combination of 5FU, doxorubicin and mitomycin C (FAM) was reported to yield a 42% RR [6], but a randomised trial failed to demonstrate its superiority over 5FU alone [7]. The combination of high-dose methotrexate, 5FU and doxorubicin (FAMTX) initially afforded a 63% RR [8], but this was lower (39%) in a randomised trial of the Gastrointestinal Tract Cooperative Group of the European