7. ICD-O. International Classification of Diseases for Oncology. In Rilke F, eds. *Istopatologia* WHO, 1983, 5, 1–121.
8. Hutter RVP, Sobin LH. A universal staging system for cancer of the colon and rectum. *Arch Pathol Lab Med* 1986, **110**, 367–368.
9. Cancer Incidence in Five Continents. IARC Publication 42. International Agency for Research on Cancer, Lyon, France, 1982, 207–210, 668–670, 671–674.
10. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959, 22, 719–748.
11. Liang KY, Beaty TH, Cohen BH. Application of odds ratio regression models for assessing familial aggregation from case-control studies. *Am J Epidemiol* 1986, **124**, 678–683.
12. Ponz de Leon M, Sassatelli R, Sacchetti C, Zanghieri C, Scalmati A, Roncucci L. Familial aggregation of tumors in the three-year experience of a population-based colorectal cancer registry. *Cancer Res* 1989, **49**, 4344–4348.
13. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958, **53**, 457–481.
14. Slater GI, Haber RH, Aufses AH, Jr. Changing distribution of carcinoma of the colon and rectum. *Surgery Gynecol Obstetrics* 1984, **158**, 216–218.
15. Enblad P, Adami HO, Bergström R, Glimelius B. Krusemo UB, Pahlman L. Improved survival of patients with cancers of the colon and rectum? *J Natl Cancer Inst* 1988, **80**, 586–591.
16. Levi F, Mezzanotte G, Te VC, La Vecchia C. Cancer survival from the incident cases of registry of Vaud, Switzerland. *Tumori* 1989, 75, 83–89.
17. Cancer in Italy: incidence data from Cancer Registries 1983–87. R. Zanetti, P. Crosignani eds. Lega Italiana per la Lotta contro i Tumori, Torino, 1992.
18. Chu DZJ, Giacco G, Martin RG, Guinee VF. The significance of sinchronous carcinoma and polyps in the colon and rectum. *Cancer* 1986, 57, 445–450.
19. Mecklin JK. Frequency of hereditary colorectal carcinoma. *Gastroenterology* 1987, **93**, 1021–1025.
20. Falterman KW, Hill CB, Markey JC, Fox JW, Cohn I, Jr. Cancer of the colon, rectum and anus: a review of 2313 cases. *Cancer* 1974, **34**, 951–959.
21. Collins RJ, Chan CW. Colorectal carcinoma in Hong Kong Chinese. A pathological survey of 1,117 cases, 1972–1981. *Int J Cancer* 1989, **44**, 410–414.

Acknowledgements—This work has been supported by Grant "60%" of the Ministero dell'Università e della Ricerca Scientifica, Italy; by Grant 1970, 13 May 1986 of the Region Emilia-Romagna, and by a Grant of the Consiglio Nazionale delle Ricerche, Rome, Italy. G. Zanghieri and L. Roncucci were supported by the Lega Italiana per la Lotta contro i Tumori, Sezione di Modena. R. Sassatelli was supported by a Grant of the Associazione Italiana per la Ricerca sul Cancro, Milan, Italy.

Eur J Cancer, Vol. 29A, No. 3, pp. 371–377, 1993.
Printed in Great Britain

0964–1947/93 $6.00 + 0.00

In situ Hybridisation for Cytokine Gene Transcripts in the Solid Tumour Microenvironment

Domenico Vitolo, Anisa Kanbour, Jonas T. Johnson, Ronald B. Herberman and Theresa L. Whiteside

To determine if mononuclear cells (MNC) infiltrating various types of human solid tumours express genes for cytokines, *in situ* hybridisation with ^{35}S-labelled cDNA antisense probes for interleukin 2 (IL2), interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin 1-beta (IL1-β), transforming growth factor beta (TGF-β) and interleukin 2-receptors (IL2R) was performed. Fresh-frozen tissue samples of ovarian carcinomas (n=13), breast carcinomas (n=12), and squamous cell carcinomas of the head and neck (SCCHN, n=7) were evaluated for the presence and localization in the tumour of MNC positive for cytokine genes. In ovarian tumours and those breast carcinomas producing little or no mucin, only rare positive MNC were observed. In contrast, breast carcinomas producing mucin and all SCCHN contained numerous MNC expressing gene transcripts for IL2, IFN-γ, TNF-α, IL2R as well as TGF-β. In tumour-involved lymph nodes of patients with SCCHN, MNC expressing genes for cytokines were found around tumour metastases but not in non-involved areas. These data suggest that tumours expressing immunogenic antigens (e.g. mucin) contain many activated MNC, while other tumours either fail to activate or suppress functions of infiltrating MNC. In SCCHN or tumour-draining lymph nodes, local down-regulation of antitumour responses might be mediated by TGF-β produced by activated tumour-infiltrating MNC.
Eur J Cancer, Vol. 29A, No. 3, pp. 371–377, 1993.

INTRODUCTION

HUMAN MONONUCLEAR cells (MNC) have been most often studied in the peripheral blood, where their phenotypic and functional characteristics can be easily determined both in health and disease. Peripheral blood MNC can be serially monitored to detect disease- or therapy-related changes. However, peripheral blood MNC represent about 2% of those present in the body, and their characterisation does not reflect functional or phenotypic properties of tissue-infiltrating MNC [1, 2]. To study MNC in tissue, histological and immunohistological techniques have been used [3, 4], which provide information about location, distribution, intensity and phenotype of these cells, but not about the state of their activation or function in the local microenvironment. Yet, local interactions between tissue cells

and tissue-infiltrating MNC are of great interest, because they not only appear to be necessary for normal tissue physiology, but, under some circumstances, result in disease [5].

To study tissue-infiltrating MNC functionally, their isolation from freshly harvested tissues and separation, generally by centrifugation on a gradient, from contaminating tissue cells is necessary [6]. Recovery of MNC from tissue depends on a variety of factors, some of which might contribute to unsatisfactory results in the isolation or purification of these cells. One of the most critical factors is enzymatic tissue digestion, which might result in a loss or impairment of MNC functions [7]. Since it could be argued that human MNC recovered from tissue may be altered functionally, an optimal approach is to study these MNC *in situ*, where they accumulate as a result of tissue injury and/or inflammatory or immmunological events, and where they interact with one another or with tissue cells. As recently demonstrated, cellular interactions are mediated by a cytokine network, consisting of soluble factors, cytokines, produced by activated cells and of receptors on target cells found in the tissue microenvironment [8]. Using *in situ* hybridisation it is now possible to reliably determine expression of genes for various cytokines, for cytokine receptors, and/or MNC activation antigens in tissue [9]. Since the tissue microenvironment determines, to a large extent, the types of signals that infiltrating MNC receive, expression of cytokine genes in these MNC provides an estimate of their functional capabilities.

In this study, the ability of MNC found in several types of human solid tumours to express cytokine genes is examined. Tumour-infiltrating lymphocytes freshly isolated from such tumours have been variously reported to be activated, functionally incompetent or enriched in antitumour effector cells [10]. These conflicting reports prompted us to determine functional capabilities of MNC in the tumour microenvironment using *in situ* hybridisation for cytokine gene expression.

MATERIALS AND METHODS

Tissues

Tissue samples were collected from 12 patients with invasive breast cancer, 13 with ovarian adenocarcinoma and 7 with squamous cell carcinoma of the head and neck (SCCHN), all of whom underwent consecutive surgical resections at the hospitals of the University of Pittsburgh Medical Center. All tissues, including tonsils ($n=5$) and reactive lymph nodes ($n=5$) used as controls, were obtained from the Surgical Pathology Laboratories at Presbyterian University Hospital, Magee Womens' Hospital, or Eye and Ear Hospital. Only tissues in excess of those needed for diagnosis were used. The tissues were embedded in OCT compound (Miles Laboratories, Naperville, Illinois) and snap-frozen in liquid N_2 within 45 min following their surgical removal. The samples were stored in OCT at −80°C.

In situ *hybridisation*

Prior to sectioning, tissue blocks were warmed in a cryostat (−20°C). Tissue sections (10 μm) were cut and mounted on gelatin-coated slides. Following air drying, tissue sections were fixed with 4% (v/v) paraformaldehyde (Sigma, St Louis, Missouri) in phosphate-buffered saline (PBS) for 10 min and washed with 70% ethanol in DEP (diethyl-pyrocarbonate) treated H_2O. (All reagents used for *in situ* hybridisation were prepared with DEP-treated H_2O.) Next, the sections were rehydrated in PBS and 50 mmol/l $MgCl_2$ for 15 min and then washed in 200 mmol/l Tris–HCl–glycine buffer, pH 7.4, for 15 min. The specimens were next acetylated with 0.25% acetic anhydride (Sigma) in 0.1 mol/l triethanolamine and 2 × SSC (1 × = 0.15 mol/l NaCl/0.015 mol/l Na citrate) buffer for 15 min. Finally, the slides were washed in 2 × SSC buffer for 10 min before the prehybridisation step. Control slides treated as above were incubated with a solution containing 100 μg/ml DNAse-free ribonuclease A and 10 U/ml ribonuclease T1.

Positive controls for *in situ* hybridisation were included in all experiments and consisted of human peripheral blood mononuclear cells (PBMNC) activated with 1000 Cetus U/ml of recombinant IL2 (rIL2) as described [11]. With PBMNC smears and tissue sections, the presence of 18S ribosomal RNA (rRNA) was considered an indication that cells were synthesising RNA, as described earlier [11, 12]. Negative controls included cytosmears of resting PBMNC or tissue sections hybridised with ^{35}S-labelled p-UC9 plasmid cDNA (see below).

The cDNA probes were isolated from plasmids with the respective restriction enzymes exactly as described previously [13]. The following cDNA fragments were prepared: interleukin 2 (IL2r, 900 bp) and tumour necrosis factor alpha (TNF-α, 800 bp), interferon gamma (IFN-γ, 950 bp), and transforming growth factor beta (TGF-β, 1050 bp) all from Dr M. Palladino, Genentech Inc., So. San Francisco, California; IL2 receptor-β chain p70 (1.05 Kb) from Dr T. Taniguchi, Japan; IL2 receptor-α chain p55 (937 bp) from W. Leonard, NIH; IL1-β (530 bp) from Dr P. Lomedico, Hoffmann La Roche, Nutley, New Jersey; and the 18S rRNA (3000 bp) from C. Milcarek, University of Pittsburgh Medical Center. The p-UC9 plasmid cDNA was cut with *Pst* 1 restriction enzyme to obtain fragments of about 2700 bp for use as a negative control. These cDNA fragments were labelled with [^{35}S]dATP (NEN-DuPont, Boston, Massachusetts) using a kit for random hexanucleotide priming (Promega Corp., Madison, Wisconsin) according to the method described by Feinberg and Vogelstein [14]. The specific activity of the probes used for *in situ* hybridisation assays was at least 1 × 10^8 cpm/μg of labelled fragment.

After rehydration of tissues as described above, tissue sections and cytological specimens were prepared for hybridisation by flooding with 50% formamide (BRL) in 2 × SSC buffer and warming to 70°C. The hybridisation mixture contained 10% dextran sulphate, 2 × SSC, 500 μg of transfer RNA (tRNA) per/ml, 0.2 mg of BSA/ml and 10 mmol/l of DDT (dithiothreitol) in DEP-treated water. Equal volumes of the radiolabelled cDNA probe resuspended in 100% formamide and denaturated by boiling for 10 min and of the hybridisation mixture were placed onto the specimens, covered with parafilm, coverslipped and sealed with rubber cement. The specimens were hybridised overnight in a humidified chamber at 45°C. After hybridisation, the specimens were extensively washed in 50% formamide in 2 × SSC for 30 min at 42°C, and then with 50% formamide in 1 × SSC. The dehydrated slides were dipped in 1:1 mixture of 0.6 mol/l ammonium acetate—NTB-2 Kodak auto-radiographic emulsion melted at 42°C, and exposed in black boxes for 7–10 days. The slides were then developed in Kodak D19 developer for 5 min, rinsed in water for 1 min and treated with Kodak Fixer A for 5 min. The specimens were

Correspondence to T. L. Whiteside.
T. L. Whiteside is at the Pittsburgh Cancer Institute, W1041 Biomedical Science Tower, DeSoto at O'Hara Street, Pittsburgh, Pennsylvania 15213, U.S.A; D. Vitolo, A. Kanbour and R.B. Herberman are at the Department of Pathology; and J. T. Johnson is at the Department of Otolaryngology; University of Pittsburgh, Pittsburgh, Pennsylvania 15213, U.S.A.
Revised 11 Aug. 1992; accepted 21 Sep. 1992.

finally counterstained with haematoxylin, mounted in Permount, and evaluated by light microscopy.

Microscopic examination and cell counts

Serial cryostat sections were used to enumerate tumour-infiltrating MNC positive for expression of different cytokine or IL2R genes. From every tumour biopsy specimen, three series of sections were cut and one section in each series was hybridised with a different ^{35}S-labelled probe. All three sections hybridised with the same probe were examined microscopically, and one was chosen for cell counts. In sections of ovarian and breast tumours, which contained well-defined MNC infiltrates, labelled cells were counted per 200 MNC in representative areas selected after careful microscopic evaluation of the MNC distribution. Cells considered positive for expression of cytokine genes contained at least 25 grains of radioactivity/cell. In most cases, the counts were performed independently by two people and averaged. In the case of lymphoid tissues (tonsils or lymph nodes used as controls) or tumour-involved lymph nodes from patients with SCCHN, positive cells per five representative high power fields (HPF; at 400 × magnification) were counted using an ocular grid, and the results expressed as the number of positive cells per one HPF. In cryostat sections of tumour tissues, it was generally possible to distinguish between MNC infiltrates in the stroma and the tumour cells.

The haematoxylin and eosin (H&E)-stained microscopic sections of tumours were evaluated for the histological type and grade of tumour and for degree of tumour infiltration with MNC by two pathologists. Tumour-infiltrating MNC were counted on H&E-stained serial sections in five HPF (×400), averaged, and expressed per one HPF. The grading scale was as follows: 10–30 cells = +; 30–50 cells = ++; ≥ 50 cells = +++. In all cases of breast carcinoma, the presence of intraluminal or intracellular mucin was determined using mucicarmine-stained sections. The presence and amount of mucin and its focal or diffuse distribution were noted. The amount of mucin present was semi-quantified by evaluation of the intensity of the crimson dye.

RESULTS

In situ hybridisation for expression of cytokine and IL2R genes was used on serial cryostat sections of human tumour tissues to determine if infiltrating MNC were activated, as judged by their ability to upregulate gene transcripts for these products in the tumour microenvironment. Different types of human solid tumours used in this study included those prominently infiltrated with MNC as well as those containing scarce MNC infiltrates, as determined histologically. In SCCHN, cytokine gene expression was examined in infiltrating MNC in the primary tumours and in lymphoid cells surrounding tumour metastases into the cervical lymph nodes. The results indicate that considerable differences exist between various tumour types as well as within each histological tumour type in a degree of infiltration with MNC and in the ability of tumour-infiltrating MNC to express cytokine or IL2R transcripts *in situ*.

As shown in Table 1, the invasive ductal breast carcinomas examined were divided into two groups based on the tumour ability to secrete mucin and on the degree of MNC infiltration in the tumour stroma. The tumours which produced large amounts of intraluminal or intracellular mucin, as determined by the mucicarmine stain, also contained large numbers of infiltrating MNC (see Fig. 1c). Among these MNC, from 18 to 30% contained gene transcripts for different cytokines and IL2R (Fig. 1a). In contrast, breast tumours which produced no or little mucins were poorly infiltrated with MNC, and only rare MNC expressing cytokine genes (IL2 and TNF-α) were present. No MNC expressing mRNA for IL2R-α or -β were detected in these tumours (Fig. 1b). These results suggest that the ability of breast tumours to produce mucins may be associated with a more vigorous inflammatory or immune response, as compared to that seen in breast carcinomas which make little or no mucins.

In contrast to breast carcinomas, only rare MNC expressing mRNA for cytokines were detected in the ovarian adenocarcinomas examined (Table 2). Even in those ovarian tumours which were well infiltrated, only few cells positive for IL2 (up to 5%) and IL2R-α (up to 15%) were observed (Fig. 2). No cells positive for IFN-γ mRNA were detected in any of these tumours. All tumours contained a small proportion of MNC (up to 5%) expressing transcripts for TGF-β (Table 2).

The three primary SCCHN examined all contained grade ++ (30–50 MNC/HPF) infiltrates into the tumour stroma, and activated MNC positive for cytokine and IL2R gene transcripts were present in these infiltrates (Table 3). The data in Table 3

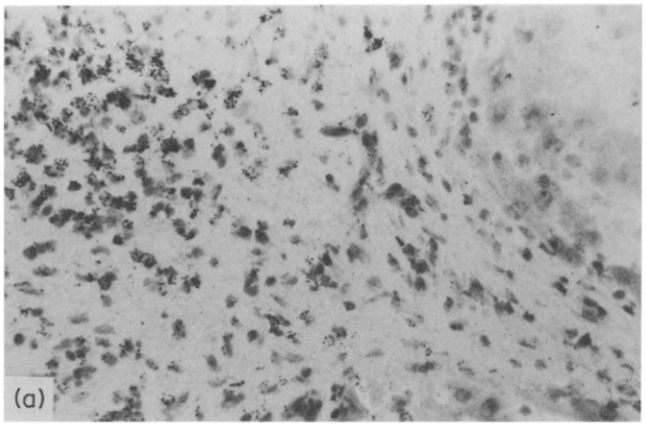
*Table 1. Expression of gene transcripts for cytokines and IL2R by tumour-infiltrating MNC in human invasive breast carcinomas**

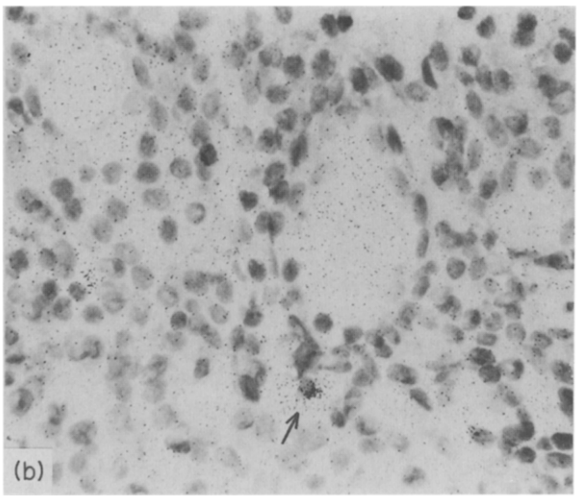
Histology	Infiltrate†	[^{35}S]cDNA probe					
		IL2R-α	IL2R-β	IL2	IFN-γ	TNF-α	TGF-β_1
		% positive cells					
Ductal invasive‡ (n=8)	+/++	0	0	0.9 ± 1	0	1.2 ± 1.6	0
Ductal invasive, mucin-producing (n=4)	+++	23 ± 3	15 ± 4	4 ± 4	21 ± 7	23 ± 2	0

*The data obtained by *in situ* hybridisation are expressed as mean percentages ± SEM of positive cells per 200 MNC counted.

†Numbers of infiltrating MNC were counted on H&E-stained serial sections as described in the Materials and Methods.

‡Among these eight tumours, four contained cells producing some detectable mucin by staining with mucicarmine.





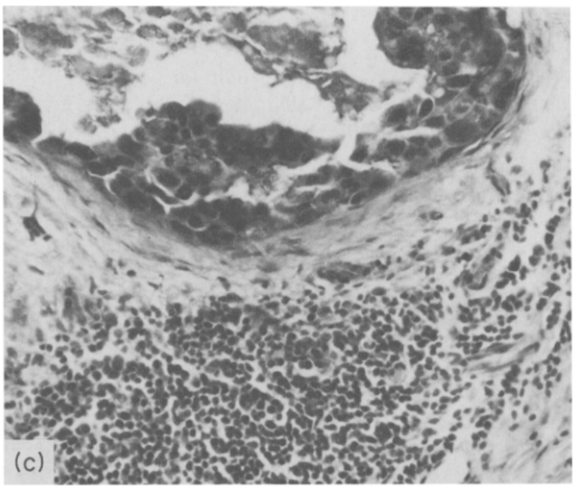


Fig. 1. ***In situ*** **hybridisation with the ^{35}S-labelled cDNA probe for TNF-α gene transcript on cryostat sections of a mucin-producing breast carcinoma well-infiltrated by MNC (in a, magnification × 250) and of a poorly infiltrated breast carcinoma (in b, magnification × 400) which does not produce mucin. Note that only one positive cell is present in (b) (see arrow) vs. numerous cells positive for TNF-α mRNA in (a). (c), A paraffin section of the same mucin-producing breast carcinoma as that shown in (a) but stained here with mucicarmine. Note the presence of intra- and extracellular mucins and of a considerable periductal MNC infiltrate. Magnification × 250.**

*Table 2. Expression of gene transcripts for cytokines or IL2R by tumour-infiltrating MNC in human ovarian carcinomas**

		[^{35}S]cDNA probe					
Infiltrate†	*n*	IL2R-α	IL2R-β	IL2	IFN-γ	TNF-α	TGF-β_1
		% positive cells					
+/++	10	0	0.5 ± 0.2	0.6 ± 0.2	0	0.4 ± 0.2	2.5 ± 2
+++	3	9 ± 4	0	4 ± 1	0	0.5 ± 0.1	1.0 ± 0.2

*The data from *in situ* hybridisation experiments are expressed as mean percentages ± SEM of positive cells per 200 MNC counted.
†Numbers of infiltrating MNC were determined as described in the Materials and Methods.

are presented as numbers per HPF, not percentages, of positive cells, because it was not possible to accurately enumerate MNC in cryostat sections of the tumour or tumour-involved lymph nodes. As shown in Fig. 3, the tumour stroma contained many cells positive for IFN-γ gene transcripts. In tumour-involved lymph nodes obtained from 4 patients with SCCHN, many cells in an immediate proximity to tumour metastases were activated and contained transcripts for cytokines IL2, IFN-γ, TNF-α or IL1-β as well as for IL2R-α or -β (Table 3, Fig. 4a and b). In addition, numerous lymph node lymphocytes located next to tumour metastases contained mRNA encoding TGF-β (Fig. 5). On the other hand, in the lymph node areas not containing visible tumour metastases, few cells expressing cytokine or IL2R genes were present (Fig. 4). Thus, it appeared that MNC located closest to the tumour, both in the SCCHN stroma and around metastases in the lymph nodes, were activated, while only few activated MNC were found in the tumour-uninvolved areas of lymph nodes.

As controls for tumour-involved lymph nodes, normal reactive lymph nodes and tonsils were examined for expression of cytokine and IL2R genes. These lymphoid tissues were found to contain few positive cells (e.g. per HPF: 0 for IL2R-α, 0–1 for IL2R-β, 0–5 for IL2, 0 for IFN-γ and 0–5 for TGF-β). In

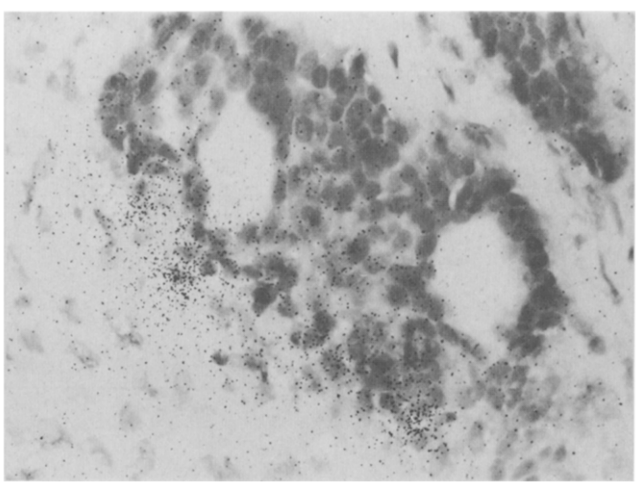


Fig. 2. ***In situ*** **hybridisation with the ^{35}S-labelled cDNA probe for IL2 gene transcripts on a cryostat section of human ovarian carcinoma. Note several MNC positive for IL2 gene transcripts. Magnification × 400.**

*Table 3. Expression of gene transcripts for cytokines or IL2R by tumour-infiltrating MNC in tumour tissue or tumour-involved lymph nodes in squamous cell carcinomas of the head and neck**

Tissue	Location†	[^{35}S]cDNA probe						
		IL2R-α	IL2R-β	IL2	IFN-γ	TNF-α	IL1-β	TGF-β_1
		Number of positive cells/HPF						
Tumour (n=3)	Stroma	2–5	0–2	0–2	0–2	0–5	2	2–5
Tumour-involved	Immediately adjacent to tumour cells	5–10	0–1	5–10	5–10	2–10	10	5–10
Lymph nodes (n=4)	Tumour-free areas	0–1	0	1–2	1–2	0–2	1–2	1–2

*The data from *in situ* hybridisation experiments are presented as ranges of the mean numbers of positive cells per high power field (400 ×, HPF) determined as described in the Materials and Methods.
†Positive cells were counted in the tumour stroma or, on lymph node sections, immediately adjacent to tumour cells and also in lymphoid tissue not involved by the tumour.

Fig. 6, a section of human tonsil hybridised to the IL2 probe, shows the presence of a few MNC positive for IL2 transcripts.

DISCUSSION

Human solid tumours are frequently infiltrated by MNC [10, 15], and suggestions have been made that the presence of MNC infiltrates reflects the host immune response to the tumour [16]. These infiltrates contain variable proportions of lymphocytes (which are largely CD3$^+$T cells) and macrophages, and they vary considerably in both cell numbers and composition in different human solid tumours [10, 17]. Infiltrates of MNC are localised mainly to the stroma and peritumorally, although both T-lymphocytes and macrophages may be sometimes found in the tumour parenchyma [10, 17]. Both the degree of tumour infiltration and the infiltrate composition have been positively correlated with a better prognosis or survival in some tumours [18, 19]. However, no consistent data in support of prognostic or survival significance of MNC infiltrates are available so far.

In view of the possible role of tumour-infiltrating MNC in tumour growth or metastasis, attention has been focused on functional and phenotypic characterisation of these cells [6, 10, 20]. In many recent studies, fresh tumour-infiltrating lymphocytes (TIL) have been shown to be partly or completely deficient

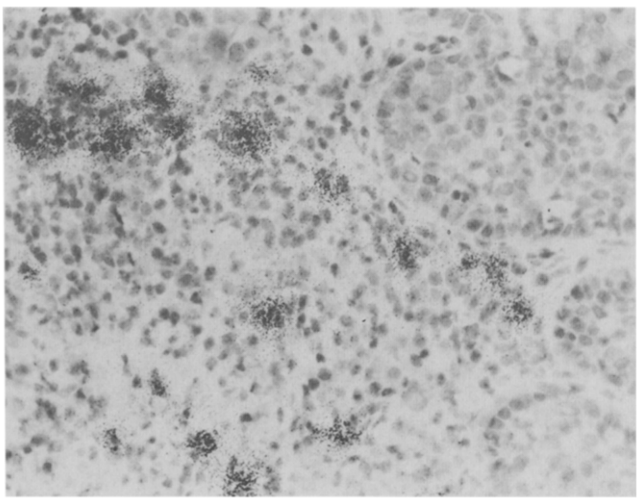


Fig. 3. *In situ* hybridisation with the ^{35}S-labelled cDNA probe for IFN-γ gene transcripts on a cryostat section of human SCCHN. Note numerous positive cells located in the stroma next to tumour cells. Magnification × 400.

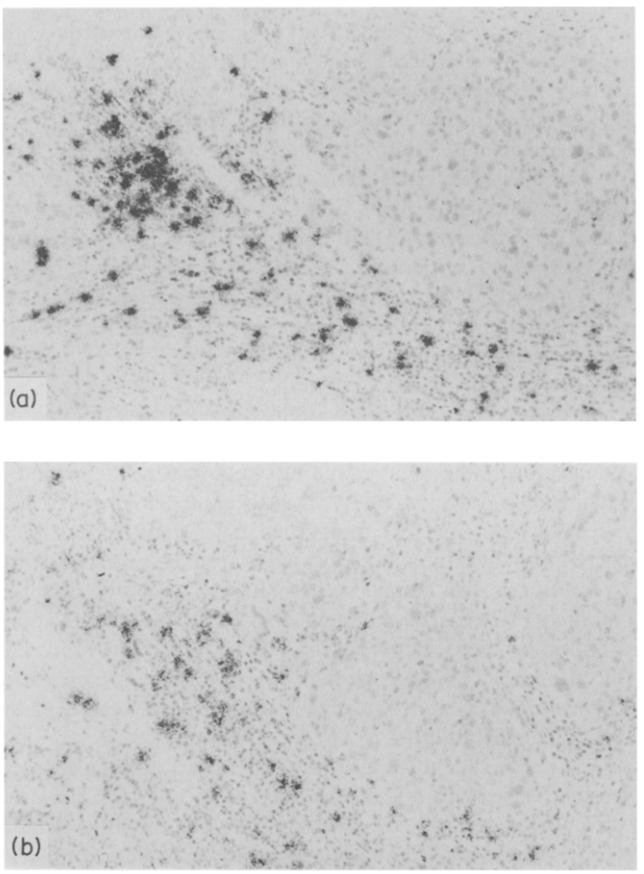


Fig. 4. *In situ* hybridisation with the ^{35}S-labelled cDNa probe for TNF-α gene transcripts (in a) and for IL2R p75 gene transcripts (in b) on serial cryostat sections of a tumour-involved lymph node in SCCHN. Positive cells are in lymphoid tissue surrounding tumour metastases. Magnification × 100.

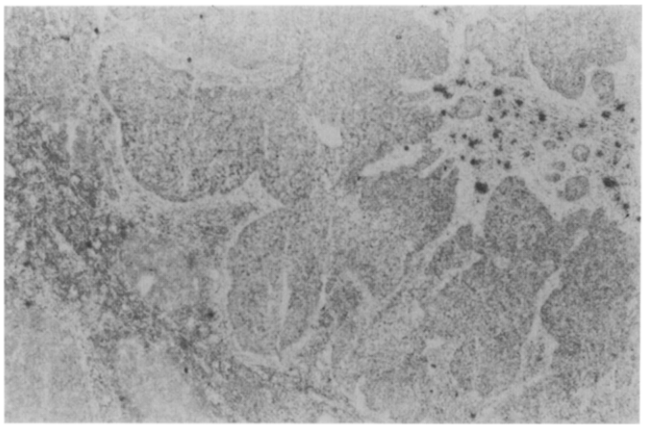


Fig. 5. *In situ* hybridisation with the ^{35}S-labelled cDNA probe for the TGF-β gene transcripts on a cryostat section of tumour-involved lymph node in SCCHN. Most positive cells are located next to the tumour (right) and only few positive cells are present in lymphoid tissue more distant from the tumour (left). Magnification × 10.

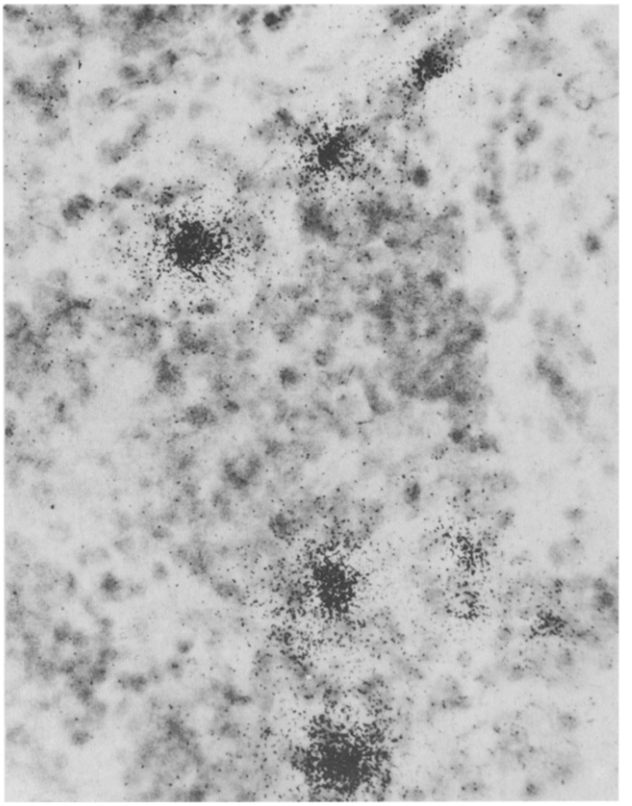


Fig. 6. *In situ* hybridisation with the ^{35}S-labelled cDNA probe for the IL2 gene transcripts on a cryostat section of human tonsil. Several strongly positive cells can be seen in lymphoid tissue. Magnification × 600.

in proliferative or cytotoxic functions after isolation from tumour tissue, although *in vitro* culture in IL2 or in IL2 combined with other cytokines restored these functions [10, 21]. In human SCCHN, fresh TIL produced less cytokines *in vitro* than autologous PBL, and lymph node lymphocytes (LNL) obtained from paired tumour-involved or non-involved lymph nodes were unable to produce IL1-β or TNF-α and showed depressed IFN-γ production [9]. These observations were consistent with the possibility that local immunosuppression in the tumour microenvironment is responsible for poor *in vitro* responsiveness of TIL to activation stimuli [7, 10]. However, phenotypic characterisation of freshly isolated TIL by two-colour flow cytometry showed that considerable proportions of $CD3^+$T lymphocytes obtained from different solid tumours were activated, i.e. expressed CD25 (IL2R p55) and HLA-DR antigens [10]. As it was possible that isolation of tumour-infiltrating MNC from tumour tissue might result in impaired function, we used *in situ* hybridisation for cytokine or IL2R gene expression to examine the activation state of TIL or LNL in three different types of human solid tumours.

Our results indicated that various tumour types differed in the ability to modulate cytokine gene expression in infiltrating MNC. To a certain extent, this ability was related to the numbers of infiltrating MNC, in that MNC expressing cytokine or IL2R gene transcripts were more numerous in well-infiltrated than in poorly infiltrated tumours. This observation is consistent with the hypothesis that tumour cells in certain, but not all solid tumours, can mediate recruitment of MNC and induce activation of infiltrating MNC. On the basis of earlier reports by Finn and her colleagues, it appears that most mucin-producing pancreatic or breast tumours induce strong immune responses and contain large MNC infiltrates ([22] and see Fig. 1a). Certain tumour-derived mucins have been shown to be good immunogens *in vitro*, and our results with mucin-producing breast carcinomas are in agreement with this finding [23]. On the other hand, the paucity of MNC infiltrates or activated MNC expressing cytokine genes in ovarian carcinomas, which produce mucins, requires an explanation. Biochemically and immunologically, mucins isolated from breast or pancreatic tumours are similar, in that they contain short amino acid sequences which can induce *in vitro* generation of cytolytic T lymphocytes [23]. In contrast, ovarian carcinomas, expressing different type of mucins are unable to induce generation of $CD3^+CD8^+$T cells specific for tumour mucin [22]. Thus, experimental evidence obtained by Finn and co-workers suggests that breast but not ovarian carcinoma mucins are immunogenic [23]. However, factors other than mucin expression by tumour cells might be involved in modulating immune responses in these tumours.

In SCCHN, recent experiments in our laboratory showed that tumour cells could induce activation of MNC *in vitro*. Human lymphoid cells incubated with a SCCHN cell line were induced to upregulate IL2R, express genes for different cytokines, produce IFN-γ or TNF-α, and to proliferate [24]. *In vivo*, using local adoptive transfers of human MNC to SCCHN growing in nude mice [25], similar activation of the MNC localised in the tumour stroma was observed [24]. In this study, we observed that in the tumour stroma as well as in tumour-involved lymph nodes of patients with SCCHN, activated MNC positive for cytokine or IL2R gene transcripts were localised in a close proximity to tumour cells or to tumour metastases, respectively. These observations, together with our earlier findings that SCCHN are generally well infiltrated with MNC [20, 21] and that TIL as well as cervical lymph node lymphocytes from patients with SCCHN are a good source of anti-tumour effector cells [26], indicate that this tumour can induce a strong immune response.

In situ hybridisation, which allows for detection and localisation of activated MNC in the tumour microenvironment, also provided evidence that cells containing mRNA for TGF-β were frequently seen in SCCHN, and were present in small numbers

in ovarian carcinomas but not in breast carcinomas. The localisation of MNC containing TGF-β message in SCCHN or tumour-involved lymph nodes deserves attention. These MNC were found in the immediate proximity of tumour cells and were infrequent in tumour-uninvolved areas of lymph nodes. Thus, it appears that MNC activated by tumour-derived signals might express genes for TGF-β in addition to genes for other cytokines or IL2R. In certain solid tumours, TGF-β-producing MNC may be responsible for local regulation of antitumour responses and, perhaps, for local immunosuppression observed in *in vitro* experiments with freshly isolated TIL [10].

Our results indicate that considerable differences existed among the three types of solid tumours studied in cytokine gene expression by TIL. It is likely that these differences are related to the ability of a tumour to activate MNC present in the local microenvironment, on the one hand, and/or to down-regulate their functions by delivering immunosuppressive signals on the other. Interactions between TIL and tumour cells in the tumour microenvironment are probably mediated by cytokines, and, thus, *in situ* studies of cytokine gene expression in either TIL or tumour cells, or preferably both, provide important information about the cytokine circuits involved in immunoregulation of tumour-infiltrating MNC functions in different types of human solid tumours.

1. Westermann J, Pabst R. Lymphocyte subsets in the blood: A diagnostic window on the lymphoid system? *Immunol Today* 1990, **11**, 406–410.
2. Whiteside TL, Herberman RB. Extravasation of antitumor effector cells. A review. *Invasion Metastasis* 1992, **12**, 128–146.
3. Underwood JC. Lymphoreticular-cell infiltration in human tumors: prognostic and histological implications. A review. *Br J Cancer* 1974, **30**, 538–548.
4. Chen K, Demetris AJ, Van Thiel DH, Whiteside TL. Double immunoenzyme staining method for analysis of tissue and blood lymphocyte subsets with monoclonal antibodies. *Lab Invest* 1987, **56**, 114–119.
5. Potarroyo M. Leukocyte adhesion in host defence and tissue injury. *Clin Immunol Immunopathol* 1991, **60**, 333–348.
6. Whiteside TL, Miescher S, Hurlimann J, Von Fliedner V. Separation, phenotyping and limiting-dilution analysis of T-lymphocytes infiltrating human solid tumors. *Int J Cancer* 1986, **37**, 803–811.
7. Miescher S, Stoeck M, Quiao L, Barras C, Barrelet L, Von Fliedner V. Preferential clonogenic deficit of CD8-positive T lymphocytes infiltrating human solid tumor. *Cancer Res* 1988, **48**, 6992–6998.
8. Mantovani A, Dejana E. Cytokines as communication signals between leukocytes and endothelial cells. *Immunol Today* 1990, **10**, 370–375.
9. Vitolo D, Letessier EM, Johnson JT, Whiteside TL. Immunologic effector cells in head and neck cancer. *J Natl Cancer Inst Monograph #13: Biology of and Novel Therapeutic Approaches for Epithelial Cancers of the Aerodigestive Tract.* 1992, **13**, 203–208.
10. Whiteside TL, Jost LM, Herberman RB. Tumor-infiltrating lymphocytes: potential and limitations to their use for cancer therapy. *Critical Reviews in Oncology/Hematology*, Amsterdam, Elsevier Science Publishers. 1992, **12**, 25–47.
11. Vitolo D, Zerbe T, Kanbour A, Dahl C, Herberman RB, Whiteside TL. Expression of mRNA for cytokines in tumor-infiltrating mononuclear cells in ovarian adenocarcinoma and invasive breast cancer. *Int J Cancer* 1992, **51**, 573–580.
12. Vitolo D, Vujanovic N, Rabinowich H, Schlesinger M, Herberman RB, Whiteside TL. Rapid interleukin-2 induced adherence of human natural killer (NK) cells II. Expression of mRNA for cytokines and IL2 receptors in adherent NK cells. 1992, Submitted.
13. Maniatis T, Fritsch EF, Sambrook J. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor, New York; Cold Spring Harbor Laboratory, 1982.
14. Feinberg AP, Vogelstein B. Addendum: A technique of radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1984, **137**, 266–267.
15. Ioachim HL. The stroma reaction of tumors: An expression of immuno surveillance. *J Natl Cancer Inst* 1976, **57**, 465–475.
16. Haskill S. Some historical perspectives on the relationship between survival and mononuclear cell infiltration. In Haskill S, ed. *Tumor Immunity and Prognosis: The Role of Mononuclear Cell Infiltration.* New York, Marcel Dekker, 1982, 1–10.
17. Vose BM, Moore M. Human tumor-infiltrating lymphocytes: A marker of host response. *Semin Hematol* 1985, **22**, 27–40.
18. Svennevig JL, Lunde OC, Holter J, Bjorgsvik D. Lymphoid infiltration and prognosis in colorectal carcinoma. *Br J Cancer* 1984, **49**, 375–377.
19. Wolf GT, Hudson JL, Peterson KA, Miller HL, McClatchey KD. Lymphocyte subpopulations infiltrating squamous carcinomas of the head and neck: Correlations with extent of tumor and prognosis. *Otolaryngol Head Neck Surg* 1986, **95**, 142–152.
20. Snyderman CH, Heo DS, Chen K, Whiteside TL, Johnson JT. T cell markers in tumor infiltrating lymphocytes of head and neck cancer. *Head Neck Surg* 1989, **11**, 331–336.
21. Heo DS, Whiteside TL, Johnson JT, Chen K, Barnes EL, Herberman RB. Long-term interleukin 2-dependent growth and cytotoxic activity of tumor-infiltrating lymphocytes (TIL) from human squamous cell carcinomas of the head and neck. *Cancer Res* 1987, **47**, 6353–6362.
22. Barnd DL, Lamm S, Metzgar RS, Finn OJ. Specific major histocompatibility complex unrestricted recognition of tumor associated mucins by human cytotoxic T cells. *Proc Natl Acad Sci USA* 1989, **26**, 7159–7163.
23. Jerome KR, Barnd DL, Boyer CM, *et al.* Breast adenocarcinoma reactive cytotoxic T lymphocytes recognition epitope present on the protein core of the epithelial mucin molecules. In: Lotze MT, Finn OJ, eds. *Cellular Immunity and the Immunotherapy of Cancer.* UCLA Symposium on Molecular and Cellular Biology, New Series, New York, Wiley-Liss, Inc., 1991.
24. Rabinowich H, Vitolo D, Altarac S, Herberman RB, Whiteside TL. The role of cytokines in the adoptive immunotherapy of an experimental model of human head and neck cancer by human IL2-activated natural killer cells. *J Immunol* 1992, **149**, 340–349.
25. Sacchi M, Vitolo D, Sedlmayr P, *et al.* Induction of tumor regression in experimental model of human head and neck cancer by human A-LAK cells and IL2. *Int J Cancer* 1991, **47**, 784–791.
26. Letessier E, Heo DS, Okarma T, Johnson JT, Herberman RB, Whiteside TL. Enrichment in tumor-reactive $CD8^+$T lymphocytes by positive selection from the blood and lymph nodes in patients with head and neck cancer. *Cancer Res* 1991, **51**, 3891–3899.