

# T cell receptor- $\zeta$ and granzyme B expression in mononuclear cell infiltrates in normal colon mucosa and colon carcinoma

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## Abstract

**Background**—Whereas the presence of a lymphoid infiltrate has been associated with a favourable prognosis in colorectal carcinoma, the proliferative and cytotoxic responses of freshly isolated tumour infiltrating lymphocytes are frequently impaired. In mice, tumour induced immune suppression has been associated with a decreased expression of the  $\zeta$ -chain of the T cell receptor (TCR)-CD3 complex, and loss of mRNA for granzyme B.

**Aim**—To compare the expression of TCR- $\zeta$  and granzyme B in lymphocytes infiltrating normal colonic mucosa and Dukes's A and D colorectal carcinomas.

**Specimens**—Paraffin wax embedded normal (n=10) and malignant colonic mucosa (seven Dukes's A, nine Dukes's D).

**Method**—Immunohistochemistry.

**Results**—The numbers of TCR- $\zeta^+$  lymphocytes decreased from normal mucosa to Dukes's D carcinomas. In contrast, granzyme B $^+$  lymphocytes were more frequent in Dukes's A carcinomas than in normal mucosa, but disappeared from advanced stage tumours. Granzyme B expressing cells were mainly CD3 $^-$  (natural killer/lymphokine activated killer cells) in normal mucosa, but CD3 $^+$  in tumours, indicating the presence of activated cytotoxic T lymphocytes. In vitro culture of tumour infiltrating lymphocytes rapidly restored the expression of both molecules.

**Conclusion**—The frequency of TCR- $\zeta^+$  and granzyme B $^+$  lymphocytes is decreased in advanced stage colorectal carcinomas. The restoration of expression during in vitro stimulation suggests the presence of tumour derived suppressive factors in situ.

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hypothesis.<sup>3</sup> However, although tumour infiltrating lymphocytes can effectively kill autologous tumour cells in vitro stimulation and expansion,<sup>4,5</sup> proliferative and cytotoxic responses of freshly isolated tumour infiltrating lymphocytes are frequently impaired.<sup>3,6</sup>

In tumour bearing mice it has been demonstrated that a loss of two important signal transduction molecules in T lymphocytes, the T cell receptor (TCR)- $\zeta$  chain and p56<sup>lck</sup>, could be the basis of this functional deficit.<sup>7</sup> The TCR- $\zeta$  chain is a disulphide linked homodimer associated with the CD3 complex. Its cytoplasmic domain is involved in signal transduction and subsequent activation of T cells.<sup>8,9</sup> Recently, two groups independently reported that the expression of TCR- $\zeta$  in peripheral blood lymphocytes and in tumour infiltrating lymphocytes from patients with renal cell carcinoma and colorectal carcinoma is decreased compared with peripheral blood lymphocytes from healthy subjects.<sup>10,11</sup> Subsequent investigations by Matsuda *et al* demonstrated that lymphocytes infiltrating normal colon mucosa derived from colorectal cancer patients also have decreased expression of TCR- $\zeta$  compared with peripheral blood lymphocytes.<sup>12</sup> They showed that the intensity of TCR- $\zeta$  staining on isolated lymphocytes was inversely related to their distance from the tumour, with mucosal lymphocytes in the proximity of the tumour expressing less TCR- $\zeta$  than lymphocytes obtained from mucosa at a greater distance from the tumour. They hypothesised that this reduction in TCR- $\zeta$  expression might be caused by soluble factors, produced by the tumour cells.<sup>12</sup>

Granzyme containing lymphocytes have been demonstrated at sites of acute inflammation.<sup>13</sup> Granzymes have a pivotal role in exocytosis mediated cytotoxic processes.<sup>14</sup> These serine proteases are constituents of the so called 'cytotoxic granules' in cytotoxic T lymphocytes and natural killer cells.<sup>15</sup> Granzyme B is the most abundant granzyme in cytotoxic T lymphocytes. As in vitro stimulation with interleukin 2 (IL2) or phorbol myristate acetate (PMA) in combination with anti-CD3 rapidly induces messenger RNA for granzymes in peripheral blood T lymphocytes,<sup>16</sup> the expression of granzymes can be considered as a sign of T cell activation. In tumour bearing mice, the impaired cytotoxic capacity of CD8 $^+$  lymphocytes has been associated with a delay and a decrease in the expression of mRNA granzyme for granzyme B.<sup>17</sup>

In this study we analysed the expression of TCR- $\zeta$  and granzyme B in situ in T

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In several tumours, including colorectal carcinoma, the presence of a lymphoid infiltrate has been associated with a favourable prognosis.<sup>1,2</sup> It is assumed that these tumour infiltrating lymphocytes reflect the host's immune response against the tumour cells. The increased precursor frequencies of tumour specific cytotoxic T lymphocytes in some tumours in comparison with peripheral blood mononuclear cells (PBMC) strengthens this

lymphocytes infiltrating early and late stage colorectal carcinomas (Dukes's A and D) using immunohistochemical methods. For comparison, normal colon mucosa specimens from non-cancer patients were examined. In Dukes's D colon carcinomas the density of the CD3<sup>+</sup> lymphocyte infiltration was decreased in comparison with Dukes's A carcinoma and normal mucosa, whereas granzyme B expressing T lymphocytes were most frequent in early stage tumours, but decreased in late stage tumours. This is probably due to tumour derived suppressive influences as both TCR- $\zeta$  and granzyme B expression were restored upon *in vitro* stimulation of the tumour infiltrating lymphocytes.

## Methods

### Specimens

Formalin fixed, paraffin wax embedded material was obtained from primary colorectal carcinomas (seven Dukes's A, nine Dukes's D). Formalin fixed, paraffin wax embedded specimens of normal colonic mucosa were obtained from surgical specimens after correction of an anus praeter (n=4), or from biopsy specimens that were taken because of a suspicion of Hirschsprung's disease (n=6).

### Antibodies

The anti-TCR- $\zeta$  monoclonal antibody (mAb) TIA-2 (IgG1; Coulter Immunology, Hialeah, USA) recognises an intracytoplasmatic epitope on the  $\zeta$ -chain of the TCR complex and in natural killer cells.<sup>18</sup> The murine GB7 mAb (IgG2a) recognises human granzyme B in formalin fixed paraffin wax embedded tissues.<sup>19</sup> AntiCD8 (CD8/144, IgG1<sup>20</sup>) was a kind gift of Dr D Y Mason, Oxford. Rabbit polyclonal anti-CD3 recognises the  $\epsilon$ -chain of the CD3 complex and was purchased from Dakopatts (Glostrup, Denmark).

### Immunohistochemistry

Four  $\mu$ m thick tissue sections were mounted on poly-L-lysine coated glass slides and air dried. Endogenous peroxidase activity was blocked by incubation in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol for 30 minutes. To optimise the immunoreactivity of the epitopes, antigens were 'retrieved' as described previously.<sup>21</sup> Briefly, tissue sections were immersed in sodium citrate buffer (0.1 M, pH 6.0) and boiled for 10 minutes followed by a 20 minute period of cooling down. After intensive washing in tap water followed by phosphate buffer saline (PBS), staining was performed using the biotin-streptavidin technique (ABC). Staining was developed with diaminobenzidine (Sigma Chemical, St Louis, MO) and the tissue sections were counterstained with haematoxylin.

### Double staining with GB7

Double staining was performed for granzyme B in combination with CD3 or with CD8,

according to the procedure as described by Merchenthaler.<sup>22</sup> Briefly, after a pre-incubation in normal saline solution, both antibodies were incubated simultaneously in the appropriate dilution. After washing in saline sodium citrate buffer (SSC) and PBS, detection of granzyme B was performed using biotinylated goat antimouse-IgG2a followed by a horseradish peroxidase labelled streptavidin. Staining was developed with diaminobenzidine/Ni/H<sub>2</sub>O<sub>2</sub> (1.5 ml NiSO<sub>2</sub>(NH<sub>2</sub>)<sub>2</sub>SO<sub>4</sub> \*6H<sub>2</sub>O 4% in 50 ml TRIS buffer 0.05 M) resulting in a grey-black staining (DAB/Ni, Sigma Chemical, St Louis, MO). Remaining peroxidase activity was inhibited by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> methanol for 15 minutes. After mild fixation using 4% paraformaldehyde (two minutes) anti-CD8 and anti-CD3 staining procedures were performed using the ABC technique and developed with DAB resulting in the normal brown colour. If necessary, GB7 staining was increased by silver enhancement.<sup>22</sup>

### Lymphocyte culturing

Healthy donor peripheral blood lymphocytes were isolated by Ficoll Hypaque gradient (Pharmacia, Uppsala, Sweden) using standard procedures. Tumour infiltrating lymphocytes were obtained from colorectal carcinoma cell suspensions that were prepared from primary colon carcinomas as described previously<sup>23</sup> and cryopreserved in liquid nitrogen. Before use, tumour cell suspensions were rapidly thawed and gradually diluted in Hank's balanced salt solution containing 0.02% DNase. After washing in Hank's balanced salt solution, peripheral blood lymphocytes and tumour infiltrating lymphocytes were cultured at  $1 \times 10^6$  cells per ml in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, 2 mM glutamin, and 50  $\mu$ M  $\beta$ -mercapto-ethanol. The lymphocytes were polyclonally activated using a combination of mAbs to CD3 and CD28<sup>24</sup> with 10 IU/ml IL2 (Cetus, Emeryville, CA, USA).

Cytospin preparations were prepared using  $4 \times 10^4$  cells per cytospin, air dried, and fixed in 4% buffered formalin for 10 minutes. When the cytospin preparations were not stained immediately, they were dehydrated, air dried, and stored at room temperature until further use.

### Evaluation

Quantification of immunolabelled cells was done by counting positively stained lymphoid cells in 10 high power fields (400 $\times$ ) within the tumour area. The fields were randomly chosen and had to contain at least 75% tumour cell surface. For granzyme B, the proportion of double stained cells was assessed by counting at least 100 granzyme B<sup>+</sup> cells and scoring whether they were double or single stained. When less than 100 granzyme B<sup>+</sup> cells were present, all granzyme B<sup>+</sup> cells in the tumour area were counted. Statistical analysis of the data was performed by the Mann-Whitney U

TABLE I Median (range) of the number of lymphocytes infiltrating 10 high power fields (400×) of normal colon mucosa and Dukes's A and D carcinoma

	Normal mucosa (n=10)	Dukes's A (n=7)	Dukes's D (n=9)	Significance level*		
				A v N	D v N	D v A
CD3 <sup>+</sup>	421 (171-1477)	511 (341-810)	235 (214-484)	0.49	0.08	0.01
CD8 <sup>+</sup>	191 (35-463)	119 (33-415)	60 (11-246)	0.77	0.02	0.11
TCR- $\zeta$ <sup>+</sup> †	113 (59-1073)†	78 (7-316)†	28 (3-105)†	0.12	0.01	0.12
Granzyme B <sup>+</sup>	9 (2-46)	34 (6-68)	5 (0-60)	0.03	0.31	0.01
CD3 <sup>+</sup> /granzyme B <sup>+</sup>	3 (1-7)	30 (4-55)	3 (0-41)	0.01	0.49	0.09

\*p Values were calculated by Mann-Whitney U test for the differences between Dukes's A v normal mucosa (A v N), Dukes's D v normal mucosa (D v N), and Dukes's D v Dukes's A (D v A). †Normal mucosa: n=6; Dukes's A: n=5; Dukes's D: n=7.

test. Two tailed p values lower than 0.05 were considered significant.

## Results

### CD3<sup>+</sup> and CD8<sup>+</sup> lymphocytes

Table I summarises the composition of the mononuclear infiltrates in Dukes's A and D colon carcinoma in comparison with normal colon mucosa from non-cancer patients. In normal and malignant tissues, a large variation was observed between different donors in the numbers of lymphocytes expressing each of the markers. In normal mucosa, a median number of 421 CD3<sup>+</sup> lymphocytes was counted in 10 high power fields (HPF, 400×). The numbers of CD3<sup>+</sup> lymphocytes in Dukes's A carcinomas were slightly higher than in normal mucosa (median 511). In contrast, Dukes's D carcinomas contained less CD3<sup>+</sup> cells per 10 HPF than normal mucosa and Dukes's A tumours. The difference with Dukes's A tumours was significant (Dukes's D v Dukes's A: p=0.01; Table I). In comparison with normal mucosa, the numbers of CD8<sup>+</sup> cells were somewhat lower in Dukes's A tumours (Dukes's A: median 119; normal mucosa: median 191; Table I). In Dukes's D carcinomas both the absolute numbers of CD8<sup>+</sup> cells (median number 60; Dukes's D v normal: p=0.02; Table I) and their percentages compared with CD3<sup>+</sup> cells were lower than in normal mucosa: 20-60% of the CD3<sup>+</sup> lymphocytes in normal mucosa were CD8<sup>+</sup>

(median 35%), whereas in Dukes's D tumours the median percentage of CD8<sup>+</sup> lymphocytes was 12% (p=0.04; Fig 1A).

### TCR- $\zeta$ <sup>+</sup> lymphocytes

The expression of TCR- $\zeta$  was analysed in the same mononuclear infiltrates. As shown in Table I, both in normal mucosa and carcinoma specimens the numbers of TCR- $\zeta$  expressing cells were always substantially lower than the number of CD3<sup>+</sup> lymphocytes. It was discounted that the difference between the numbers of CD3<sup>+</sup> and TCR- $\zeta$ <sup>+</sup> lymphocytes were due to the immunohistochemical procedure because T lymphocytes surrounding lymphoid follicles present in the mucosa showed equal numbers of TCR- $\zeta$ <sup>+</sup> and CD3<sup>+</sup> lymphocytes (Fig 2A and B). Despite the interindividual variability, the median numbers of TCR- $\zeta$ <sup>+</sup> lymphocytes were significantly lower in Dukes's D carcinomas (n=7, median 28) than in normal mucosa (n=6, median 113; Dukes's D v normal: p=0.01, Dukes's A+D v normal p=0.01; Table I). Calculated as a proportion of the number of CD3<sup>+</sup> cells, the percentage of TCR- $\zeta$ <sup>+</sup> lymphocytes was lower in tumour tissue than in normal mucosa (medians: 17% v 50%), but due to the high interindividual variability this difference was not statistically significant (Fig 1B). Figure 2C and D shows consecutive tissue sections of a Dukes's D colon carcinoma stained for CD3 (C) and TCR- $\zeta$  (D).

### Granzyme B expression

The mononuclear infiltrates were also examined for the expression of granzyme B, as shown in Table I. In normal mucosa as well as in Dukes's D carcinoma, very low numbers of granzyme B<sup>+</sup> cells were found (Table I). In general, they were located in the lamina propria, and had the morphology of large granular lymphocytes (Fig 2E). The median number of granzyme B expressing cells in Dukes's A carcinomas (median 34) was significantly higher compared with normal mucosa (median 9) and Dukes's D carcinomas (median 3) (Dukes's A v normal: p=0.03, Dukes's A v Dukes's D: p=0.01; Table I).

Double staining for granzyme B and CD3 and for granzyme B and CD8 showed that most of the granzyme B<sup>+</sup> cells in Dukes's A and D carcinomas also expressed CD3 and CD8, indicating that these cells were activated cytotoxic T lymphocytes (CD3<sup>+</sup> granzyme B<sup>+</sup>).

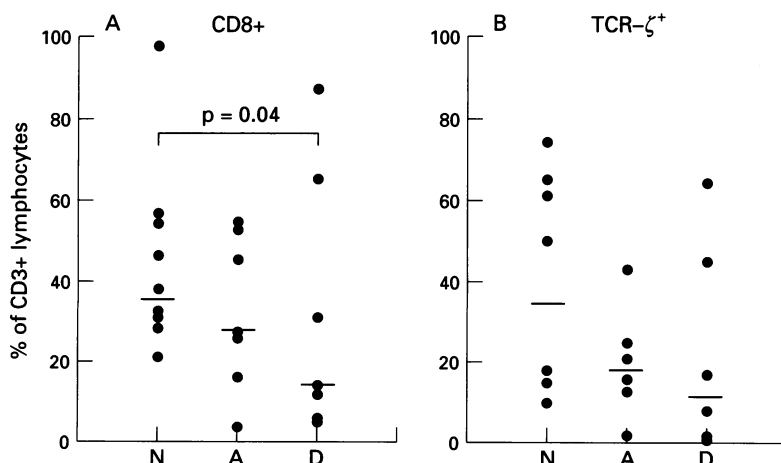
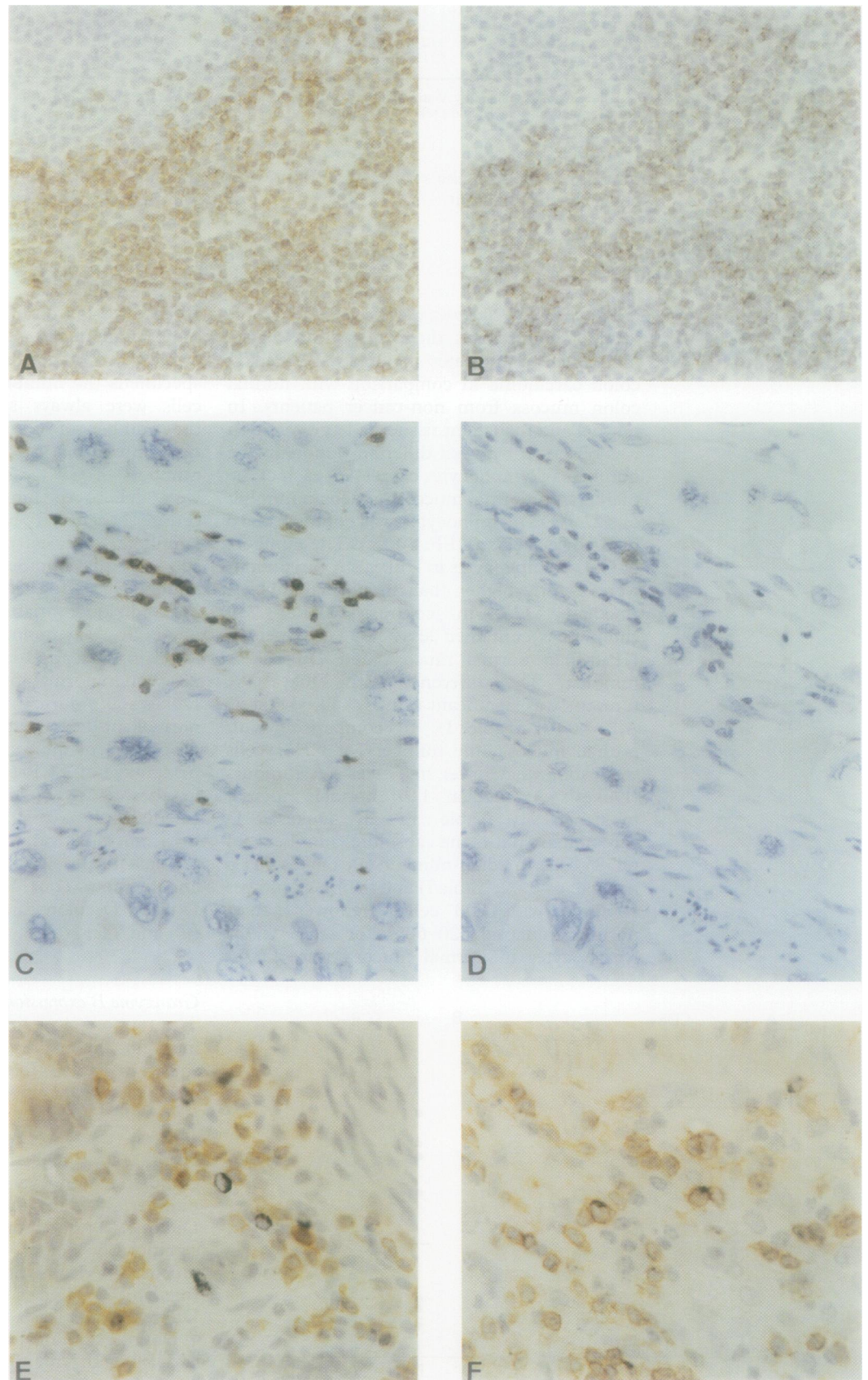


Figure 1: Percentages of CD8<sup>+</sup> and TCR- $\zeta$ <sup>+</sup> lymphocytes compared with the number of CD3<sup>+</sup> lymphocytes in normal mucosa (N), Dukes's A (A), and Dukes's D (D) carcinoma. Bars represent median values within each group. Statistical differences were calculated using the Mann-Whitney U test.

median 68%; CD8<sup>+</sup> granzyme B<sup>+</sup>: median 65%; Table II). In contrast, in normal colon mucosa most of the granzyme B<sup>+</sup> cells were negative for CD3 and CD8, which indicates a high amount of natural killer cells present (CD3<sup>+</sup> granzyme B<sup>+</sup>: median 32%; CD8<sup>+</sup> granzyme B<sup>+</sup>: median 25%; Table II). Figure 2 E and F shows examples of double staining



**Figure 2:** TCR- $\zeta$  and granzyme B expression in normal colonic mucosa and colorectal carcinomas. Mucosa associated lymphoid follicle stained with CD3 (A) and TCR- $\zeta$  (B). CD3 (C) and TCR- $\zeta$  (D) expression in sequential tissue sections of a Duke's D carcinoma. Double staining for CD3 and granzyme B in normal colonic mucosa (E) and Duke's A carcinoma (F).

TABLE II Phenotype of granzyme B expressing lymphocytes in normal colonic mucosa and colorectal carcinomas

Double positive cells as % of granzyme B <sup>+</sup> cells	Normal mucosa (median % (range))	Tumour (median % (range))	p Value
CD3 <sup>+</sup> /granzyme B <sup>+</sup>	32 (6-58) (n=10)	68 (12-82) (n=13)	0.002
CD8 <sup>+</sup> /granzyme B <sup>+</sup>	25 (0-48) (n=6)	65 (36-90) (n=5)	0.02

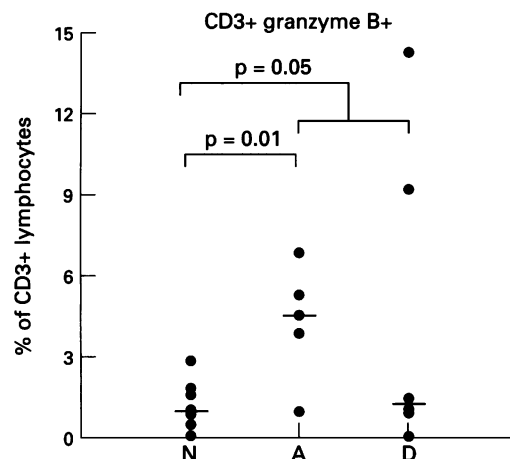


Figure 3: Proportions of T lymphocytes expressing granzyme B in normal colonic mucosa (N), Dukes's A (A) and Dukes's D (D) carcinoma. Bars represent median values within each group. Statistical differences were calculated using the Mann-Whitney U test.

for CD3 and granzyme B in normal and tumour tissue. Figure 3 shows that in Dukes's A tumours the proportion of CD3<sup>+</sup> lymphocytes containing granzyme B was significantly higher than in normal colonic mucosa (Dukes's A v normal:  $p=0.01$ ; Fig 3).

#### In vitro culture of tumour infiltrating cells

To analyse whether the low TCR- $\zeta$  expression in tumour infiltrating lymphocytes was a stable trait, we investigated whether it could be restored upon stimulation in vitro with a

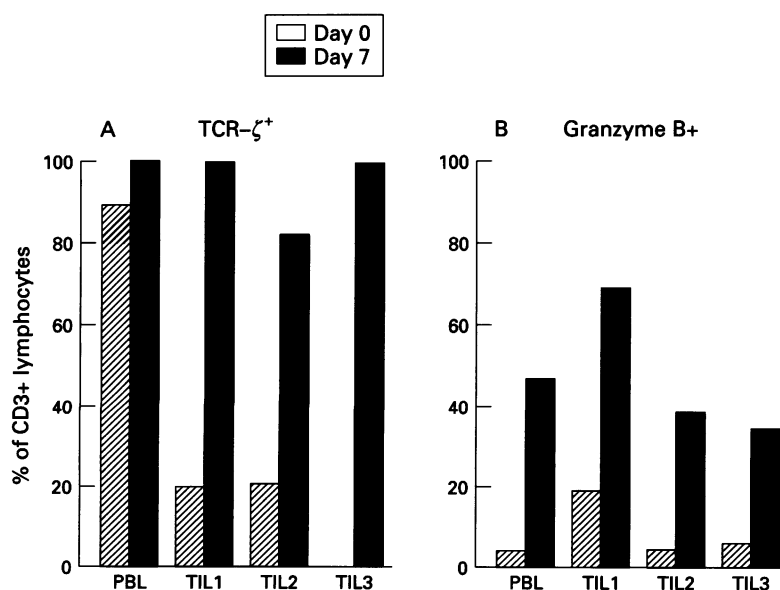


Figure 4: Increase in the percentage of TCR- $\zeta$ <sup>+</sup> (A) and granzyme B<sup>+</sup> (B) T lymphocytes in peripheral blood lymphocytes and tumour infiltrating lymphocyte cultures after a seven day culture period with a combination of mAbs to CD3 and CD28.

combination of stimulatory mAbs to CD3 and CD28. TCR- $\zeta$  expression was not affected by the enzymatical dissociation procedure (data not shown). For comparison, peripheral blood lymphocytes from a healthy donor were stimulated in the same way. Figure 4A shows that whereas in peripheral blood lymphocytes approximately all CD3<sup>+</sup> lymphocytes expressed TCR- $\zeta$ <sup>+</sup>, the proportion of CD3<sup>+</sup> tumour infiltrating lymphocytes expressing TCR- $\zeta$ <sup>+</sup> was below 25%. Upon stimulation with anti-CD3 and anti CD28, the proportion of TCR- $\zeta$ <sup>+</sup> cells significantly increased in all tumour infiltrating lymphocyte cultures, and reached a level comparable to cultured peripheral blood lymphocytes. In addition, granzyme B expression was induced in 30%-70% of CD3<sup>+</sup> tumour infiltrating lymphocytes (Fig 4B). Seven days after initiation of the culture, virtually all granzyme B<sup>+</sup> cells were T lymphocytes as demonstrated by CD3 reactivity (data not shown).

#### Discussion

Upon stimulation of the TCR complex, phosphorylation of the TCR- $\zeta$  chain is an essential step in the intracellular signalling pathway leading to T lymphocyte activation.<sup>8</sup> Recently it was observed that T cells from tumour bearing mice lacked the CD3- $\zeta$  chain and exhibited impaired cytotoxic function and a decreased ability to mediate an antitumour response, which was associated with a decrease in the expression of granzyme B mRNA.<sup>7, 17</sup> In humans, tumour infiltrating lymphocytes from renal cell carcinoma and colorectal carcinoma showed a pronounced decrease in the expression of TCR- $\zeta$ .<sup>10-12</sup> In this study, we observed that the numbers of TCR- $\zeta$ <sup>+</sup> lymphocytes in human Dukes's A and D colorectal carcinomas were lower than in normal mucosa of non-cancer patients. In addition, activated granzyme B<sup>+</sup> cytotoxic T lymphocytes were less frequently present in Dukes's D than in Dukes's A colorectal carcinomas.

Whereas the numbers of CD3<sup>+</sup> infiltrating cells in Dukes's A tumours were approximately equal to their numbers in normal colonic mucosa, CD3<sup>+</sup> lymphocytes were decreased in number in Dukes's D in comparison with Dukes's A carcinomas. CD8<sup>+</sup> lymphocytes formed a minority of the CD3<sup>+</sup> lymphocytes and their contribution to the infiltrates in tumour tissue decreased with the tumour stage. This contradicts the findings of Umpleby *et al*<sup>25</sup> but confirms those of Norazmi *et al*<sup>26</sup> and Østenstadt *et al*<sup>27</sup> who reported a predominance of CD4<sup>+</sup> lymphocytes in colonic tumours.

Compared with normal mucosa, the numbers of TCR- $\zeta$  expressing lymphocytes in tumours were decreased in Dukes's A and D colorectal carcinomas, being lowest in Dukes's D tumours. This finding adds to the observation by Matsuda *et al* that the intensity of TCR- $\zeta$  staining on the cell membranes of peripheral blood lymphocytes of colorectal carcinoma patients decreased with tumour stage.<sup>12</sup> Moreover, as the number of TCR- $\zeta$ <sup>+</sup> lymphocytes

decreased already in Dukes's A carcinomas, our results suggest that TCR- $\zeta$  downregulation in tumour infiltrating lymphocytes is a comparatively early event in the interaction between a tumour and the immune system, which may hamper the adequate activation of antitumour immune effector cells.

On the other hand we observed an increase in the numbers of granzyme B expressing lymphocytes in early stage colorectal tumours compared with normal colonic mucosa. The induction of mRNA for perforin and granzymes in T lymphocytes requires that, additional to T cell receptor triggering, a co-stimulatory signal is provided.<sup>16, 28</sup> Thus, the presence of granzyme B<sup>+</sup> lymphocytes suggests that some immune stimulation still takes place in Dukes's A carcinomas. In Dukes's D carcinomas, however, granzyme B expressing cells had disappeared from the tumour tissue. Earlier also Nakanishi *et al*<sup>29</sup> have reported that perforin expressing lymphocytes may be present in Dukes's A tumours, but are decreased with advancing tumour stage. These data from human colorectal carcinoma are in line with the findings in mouse models.<sup>7, 17</sup> Ochoa *et al* reported that T lymphocytes from late (>26 days) tumour bearing mice had an impaired cytotoxic function combined with a decreased expression of the tumour necrosis factor  $\alpha$  and granzyme B genes<sup>17</sup> and a loss of TCR- $\zeta$  expression in T lymphocytes.<sup>7</sup>

The TCR- $\zeta$  chain is required for correct assembly, transport, and surface expression of the TCR/CD3 complex.<sup>30</sup> In addition, the TCR- $\zeta$  chain connects the antigen binding TCR chains with the intracellular signalling machinery of the T cell.<sup>8</sup> A (tumour induced) defect interfering with the signal transducing capacity of the TCR- $\zeta$  chain would severely hamper the function of antitumour immune effector cells by uncoupling antigen recognition and effector functions. As it has been demonstrated that colorectal carcinoma cell lines can produce factors that inhibit T lymphocyte function,<sup>31, 32</sup> it might be envisaged that tumour cell derived factors cause progressive decrease in TCR- $\zeta$  expression, ultimately leading to a decrease in granzyme B expression. Moreover, the rapid increase in the expression of both TCR- $\zeta$  and granzyme B with tumour infiltrating lymphocytes are stimulated in vitro supports the view that they are suboptimally activated, or even suppressed in situ. Further support for tumour induced immune suppression stems from our finding that TCR- $\zeta$  expression in healthy donor peripheral blood lymphocytes is decreased by incubation in supernatants of colon cancer cell lines (Mulder *et al*, unpublished results).

Notably, also in normal colonic mucosa, we observed low numbers of TCR- $\zeta$  expressing lymphocytes compared with the numbers of CD3<sup>+</sup> cells. This lower expression of TCR- $\zeta$  was confined to the mucosa. Lymphocytes surrounding lymphoid follicles present in the mucosa always expressed TCR- $\zeta$  to the same extent as CD3. It is not clear what causes the differences between the numbers of TCR- $\zeta$ <sup>+</sup> and CD3<sup>+</sup> lymphocytes in normal colonic

mucosa. It could be that it results from local immunoregulatory influences in the gut, preventing overactivation of the immune cells in response to the constant antigenic pressure. It is tempting to speculate that immunosuppressive properties of colorectal carcinoma cells are malignant derivatives of the immunoregulatory function of normal intestinal epithelial cells.<sup>33</sup>

In conclusion, this study shows an increase in the numbers of granzyme B expressing T lymphocytes in Dukes's A carcinomas in comparison with normal mucosa suggesting an initial antitumour immune response. However, both the numbers of T lymphocytes with a good TCR- $\zeta$  expression and the numbers of activated cytotoxic T lymphocytes decreased in late stage carcinomas. Tumour derived factors are probably involved because the expression of both TCR- $\zeta$  and granzyme B by tumour infiltrating lymphocytes was rapidly restored during in vitro culture. Whether these findings reflect the existence of distinct suppressive mechanisms, and whether the downregulation of TCR- $\zeta$  and granzyme B expression are related at the (sub)cellular level should be the subject of further investigations.

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