LETTERS TO THE EDITOR

Gastric cancer cell lines lack Fas ligand (FasL) expression but kill T cells via a FasL independent pathway

EDITOR,—Bennett et al (Gut 1999;44:156-162) reported that in each of 30 paraffin wax specimens of human gastric adenocarcinomas, FasL mRNA and protein co-localised to neoplastic epithelial cells. TUNEL staining revealed that the high number of tumour infiltrating lymphocytes (TIL) displayed apoptosis features. From these results and from their findings of FasL expression in human colon and oesophageal cancer, the authors propose that FasL might be a mediator of immune privilege in gastrointestinal cancers.

We studied intrinsic FasL expression in gastric cancer cell lines derived from primary (RF-1, SNU-1) or metastatic sites (SNU-16, Kato-III, N-87, RF-48). We did not detect FasL mRNA or protein in any of the six cell lines analysed by RTPCR and by flow cytometry (table 1). We then performed the JAM assay to rule out the presence of a functional FasL expression below the detection limit of our assays. Although we found that gastric cancer cells were able to induce DNA fragmentation in the Fas sensitive T-cell acute lymphocytic leukaemia cell line CEM-C7H2 (fig 1A), blocking FasL on the effector cell site did not reduce the extent of cytotoxicity. This result was confirmed by replacing the target cell line by a subclone stably expressing the viral Fas protein crmA, which inhibits activation of caspases 1 and 8 and thereby mediates resistance to Fas triggering (fig 1B).

Owing to the discrepancy between our results (all cells were FasL negative) and those of Bennett et al (all 30 primary neoplasms were FasL positive), we wondered whether tissue derived factors such as tumour necrosis factor (TNF) might upregulate FasL in vivo, thus explaining the differences observed. In our setting, neither of the cytokines was able to modify FasL expression on gastric cancer cell lines (table 1). In addition, killing of T cell lines was not mediated via secretion of TNF-a as blocking the cytokine using a monoclonal antibody did not influence the result of the JAM assay (fig 1A).

Table 1 Expression of FasL and Fas in gastric cancer cell lines and their sensitivity toward Fas triggering by the CH11 monoclonal antibody

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FasL mRNA</th>
<th>+TNF-a (100 ng/ml)</th>
<th>+IFN-gamma (100 ng/ml)</th>
<th>Fas expression</th>
<th>CH11 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0/1.0</td>
<td>Not done</td>
<td>Not done</td>
<td>1.1</td>
<td>21</td>
</tr>
<tr>
<td>RF-1</td>
<td>Negative</td>
<td>1.3/1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>Kato-III</td>
<td>Negative</td>
<td>0.9/1.2</td>
<td>Not done</td>
<td>Not done</td>
<td>1.4</td>
</tr>
<tr>
<td>SNU-1</td>
<td>Negative</td>
<td>1.1/1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>4.9</td>
</tr>
<tr>
<td>SNU-16</td>
<td>Negative</td>
<td>1.0/1.2</td>
<td>Not done</td>
<td>Not done</td>
<td>1.1</td>
</tr>
<tr>
<td>N-87</td>
<td>Negative</td>
<td>1.0/1.2</td>
<td>Not done</td>
<td>Not done</td>
<td>2.2</td>
</tr>
</tbody>
</table>

RTPCR analysis was done as follows: total RNA from about 10^6 cells was extracted by the acid guanidinium thiocyanate-phenol-chloroform protocol described by Chomczynski and Sacchi; 1 µg RNA together with 250 ng of oligo (dT)15 primer was diluted in 10 µl to a final volume of 14 µl, denatured by heating to 70°C for five minutes and immediately chilled on ice. To each reaction, 6 µl RT mixture containing 4 µl 5× first-strand buffer, 2 pmol each of dATP, dCTP, dGTP and dTTP, and 200 units Moloney-murine leukaemia virus reverse transcriptase, was added (all reagents from Promega, Wisconsin, USA). For cDNA synthesis all samples were incubated at 37°C for 60 minutes. The oligonucleotide primers used were: TTC TTC CCT GTC CAA CCT CTG TGC (sense) and TCA CCA TCC CCA (antisense). 4 PBMC of a healthy individual served as a positive control.

Mean specific killing (%)

Figure 1 CEM-C7H2 T-cell acute lymphocytic leukaemia cells are killed by gastric carcinoma cell lines via a FasL independent pathway. (A) CEM-C7H2 target cells were incubated with 10 µCi/ml [3H]-thymidine for 16-20 hours in the presence of a gastric cancer cell line and a target effector ratio of 1:10. Co-cultivation of cells was performed for 72 hours at 37°C. The reduction in radioactivity was used to calculate the percentages of gastric cells mediated target cell killing. The bars represent mean (SEM) specific killing (%). Statistical analysis of the blocking experiments showed the following: untreated v anti-FasL monoclonal antibody treated effectors (RF-1, p>0.5; RF-48, p>0.5; SNU-1, p>0.2); untreated v anti-tumour necrosis factor (TNF) a treated effectors (RF-1, p>0.07; RF-48, p>0.15; SNU-1, p>0.5). (B) CrmA expressing CEM-C7H2 (C7H2/crm) cells were used as target cells. Experimental conditions were as for (A). Statistical analysis did not reveal any significant reduction in mean specific killing of the crmA expressing C7H2 cells by the gastric cancer cell lines (RF-1, p>0.3; RF-48, p>0.8; SNU-1, p>0.5).

1 Time kinetics (1-3 days’ stimulation) were performed and values are given for day 3. Tumour necrosis factor (TNF) a and interferon (IFN) gamma were purchased from R&D Systems (Minneapolis, Minnesota, USA). Flow cytometric analysis was performed using the NOK-1 monoclonal antibody.

4 Even after 72 hours’ incubation, there was only a very small increase in the percentages of apoptotic cells (e.g. in the SNU-1 cell line the increase was from 3% (control) to 5% (CH11)).
differences between in situ and in vitro results be explained?

Bennet et al mention that CD45+ TIL express FasL mRNA, but they did not analyse Fas expression and sensitivity, features that together characterize activation induced cell death. Although an immunohistochemical examination of slides the authors excluded the possibility of lymphocytes being killed by infiltrating neutrophils potentially attracted by the expression of FasL on the tumour cells, it is possible that lymphocytes succumbed to apoptosis owing to atherocrotic or suicide. This mechanism could well be under the (cytotoxic) control of the tumour as has been discussed for other diseases. Alternatively, lymphocytes could indeed be killed by the tumour cells but by a mechanism independent of the Fas system, a hypothesis supported by our data (fig 1).

Bennet et al did not use the standard Lauren classification system. It has been shown that gastric carcinomas cells of the intestinal and diffuse type (according to Lauren) differ in morphology, growth pattern and risk factors, and also in their expression of molecules involved in apoptosis such as Fas or p53.7 This would be evidence that at least in some tumour models Fas and FasL expression are under transcriptional control of p53.8 Loss-of-function mutations or deletions of p53 have been reported to be involved in gastric carcinogenesis9 and the frequency of these events differs between intestinal and diffuse gastric cancers.10 Also, a correlation between p53 mutation, Fas expression and gastric carcinoma cell differentiation has been demonstrated.11 Furthermore, studies of the impact of differentiation and p53 functional status on FasL expression are therefore mandatory in gastric carcinoma cells.

In sensitivity towards Fas is usually an early step in tumour development, allowing tumour cells to resist the attack of the immune system and to avoid suicide when FasL expression is acquired.12 Furthermore, a sequence of Fas resistance and FasL expression has been demonstrated for hepatocellular carcinoma.13 Secondary loss of the Fas gene, or of its expression during continuous culture of gastric adenocarcinoma cells is unlikely for the following reasons: (I) All cell lines were resistant to Fas and thus loss of FasL expression does not seem to be a prerequisite for their survival, and (ii) to our knowledge, no data are available from other cell (line) systems that tumour cell lines lose FasL expression during long term culture.

In conclusion, we think that Bennett et al’s data suggest that CD45+ lymphocytes die in the immediate proximity of neoplastic cells. Although their data are compatible with Fas induced TIL cell death, our functional data from cell lines suggest that other tumour mediated mechanisms of killing immunocompetent cells might also exist in gastric cancer. Further work clarifying the sequence of Fas/FasL expression and function during the transformation and metatatic processes is needed.

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Reply

Evron—We concur with the view expressed by Tinhofer et al that Fas ligand (Fasl) mediated “counterattack” against antitumour lymphocytes is not the sole mechanism of immune evasion in gastric cancer, or indeed any other form of cancer. Tumours evolve multiple immune evasive strategies. However, there is ample in vitro and in vivo evidence that constitutive expression of FasL enables cancers to promote apoptosis of antitumour immune effector cells.1 For example, FasL has been shown to be associated with apoptosis and loss of tumour infiltrating lymphocytes in human oesophageal cancer1 and depletion of antitumour natural killer cells in a mouse model.2

In stomach cancer, apart from our finding of FasL expression at the mRNA and protein level in vivo in all 30 gastric adenocarcinomas examined,2 Rudi and colleagues also showed Fasl mRNA in all three gastric carcinoma cell lines examined—including one cell line, KATO III, in which Tinhofer et al failed to detect Fasl mRNA. This poses serious questions regarding the sensitivity of the Fasl RT-PCR performed by Tinhofer et al. In fact, appropriate positive controls have not been shown to verify that their negative findings are not merely owing to the insensitivity of their assays for detecting Fasl mRNA and protein in adherent cells.

Successful use of the JAM assay depends on using target cells that exhibit good sensitivity to Fasl mediated apoptosis. Even different cultures of cell lines that are regarded as Fas sensitive, such as Jurkat E6 cells, can vary in their Fas sensitivity for reasons which are unclear, and Fas resistant subclones may be generated. Tinhofer et al need to demonstrate that their cultures of CEM-C7H2 target cells were indeed susceptible to apoptosis via Fas in order to validate their negative results.3

Authentic Fasl mediated killing of Fas sensitive target cells is normally detectable after eight hours of co-culture with FasL expressing effector cells.4 Tinhofer et al performed a prolonged co-culture of 72 hours. It is possible that the cell death detected in target cells at this late stage was from non-specific effects, such as exhaustion of nutrients or growth factors in the presence of proliferating effector cells rather than a specific Fas-mediated apoptosis.4 Tinhofer et al should repeat their JAM assay for a shorter length of time with highly Fas sensitive target cells and include a proved Fasl expressing effector cell line as a positive control.

Tinhofer et al’s findings that gastric carcinomma cell lines are relatively resistant to Fasl mediated apoptosis is consistent with findings for several other types of cancer cell. Fas resistance is a prerequisite for expression of FasL. Colon adenocarcinoma cell lines, for example, are also Fas resistant, enabling most colon adenocarcinoma cell lines to coexpress Fas and Fasl without undergoing Fas mediated apoptosis.1 We agree with Tinhofer et al that the sequence of Fas/Fasl expression and function during gastric carcinogenesis merits further investigation. Their suggestion that these molecules should also be investigated in metastases of gastric cancer is also pertinent as recent evidence suggests that Fasl contributes to the invasion of Fas sensitive organs, such as the liver, by colonic adenocarcinoma cells.2

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Vector manometry and LOS dynamics

**EDITOR,—**We read with interest the recent paper by Kahrilas et al on the effect of hiatus hernia on gastro-oesophageal junction pressure (Gut 1999;44:476–482). These authors used a novel technique that combined vector manometry, fluoroscopy, and endoscopic tagging of anatomical landmarks to map the differences in pressure profile between patients with and without hiatus hernia. Analysis of the vector profiles, taken at end expiration, revealed two distinct high pressure zones in each of the seven patients with hiatus hernia. These were thought to represent an axial separation of the internal and external components of the lower oesophageal sphincter (LOS). When these high pressure zones were repositioned to represent a simulated hernia, these were thought to represent an 'hernia' high pressure zone (3 mm Hg) would be expected. Finally, there can be significant minute to minute variation in lower oesophageal sphincter tone. Therefore, the reproducibility of vector manometry has been described previously by Benelman et al using rapid pull-through vector manometry (8 channel catheter, 0.7 ml/s pull-back speed). They showed that mean LOS pressure varied from 20 to 80 mm Hg in 20 pull-throughs performed in one hour in the same patient. Kahrilas et al did not mention the number of pull-throughs for each patient or the reproducibility of vector profiling. It is therefore difficult to draw accurate conclusions on the size and position of high pressure zones, particularly when the study population is limited to seven patients.

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Mycophenolate mofetil for Crohn’s disease

**EDITOR,—**On the basis of a study reported recently by Neurath et al (Gut 1999;44:625–628), commentators in Gut and the Lancet suggested that mycophenolate mofetil (MMF) should be used in patients with Crohn’s disease who have either not responded to or are intolerant of azathioprine or 6-mercaptopurine. This advice is premature: firstly, because the study was flawed and, secondly, because it examined only management of acute inflammation, not the place of MMF in maintaining remission and in steroid sparing (a fact acknowledged in both commentaries).

The study by Neurath et al compared the effect of MMF 15 mg/kg daily with azathioprine 2.5 mg/kg daily, both with high dose steroids, in the treatment of active chronic Crohn’s disease (six months’ follow up). The main conclusions were that activity, as measured by the Crohn’s disease activity index (CDAI), dropped further at one month in patients given MMF plus steroids than in those given azathioprine plus steroids, and that this was as a result of a faster effect in more severe disease. The major drawbacks of the study were as follows. As pointed out by the authors, neither patients nor investigators were blinded. Four (11%) of 35 patients in the MMF group were lost to follow up compared with none in the azathioprine group: thus, results may have looked different if analysed on an intention to treat basis. The MMF group had higher starting CDAIs: if the levels of CDAI reached at one month were compared between groups, rather than the fall of CDAI, the groups may not have been significantly different. The division of patients into those with moderate and severe activity was retrospective: thus conclusions based on this division should be regarded as hypothesis generating only, especially as differences between the groups do not reach formal statistical significance if adjustments for multiple comparisons are made. Finally, steroid usage in the two groups is not recorded: one can imagine that patients who had a poor early response would lead to more steroids being given and so to a better overall result.

I agree with the authors and commentators that alternatives to azathioprine/6-mercaptopurine are needed. I also agree that the therapeutic effect of MMF in chronic active Crohn’s disease should be assessed in properly performed trials, and pointed out importantly that its effect in maintaining remission and in steroid sparing should be assessed. However, until then, MMF should be considered to have no clear indications for use in Crohn’s disease.

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Reply

**EDITOR,—**Mycophenolate mofetil (MMF) is an immunosuppressive drug that is often used in organ transplantation. It is an ester prodrug of mycophenolic acid that inhibits inosine monophosphate dehydrogenase and potently suppresses lymphocyte proliferation. Furthermore, various clinical trials have shown its efficacy in suppressing autoimmune and chronic inflammatory disorders, such as rheumatoid arthritis, pemphigus vulgaris, and psoriasis. There are several case reports and also our controlled study indicating that MMF can be successfully used in patients with Crohn’s disease. In our study treatment of patients with moderately active Crohn’s disease with MMF/cortisone led to a significant reduction in clinical activity score compared with treatment with azathiopine/cortisone. These data suggested that treatment of chronic active Crohn’s disease with MMF/cortisone would be effective in inducing remission. As corticosteroids were given to patients in addition to
MMF, the data available do not show unequivocally that MMF alone is effective in the maintenance of remission in Crohn’s disease. This question is currently under study in a double blind, randomised controlled trial in Europe and the USA, in which the effects of MMF on maintenance of remission will be analysed.

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The first edition of this handbook was a valuable resource to both junior hospital staff and family doctors for its practical coverage of basic gastroenterology. In the seven years since it was first published, there have been many advances in gastroenterology and these have been included in the new edition, which is a rapid reference book which the authors hope will be of interest to doctors and health professionals in clinics, accident and emergency departments, and in the community. The text is well illustrated and referenced, the style is dogmatic and didactic and, in conjunction with clear algorithms, presents the results of the last. The major disadvantages which the authors constantly remind the reader of, are that uncritical readers may accept these definitions as fixed in stone. This work of course contains much work and renders it in a form readily understandable to clinicians with only vague memories of neuroanatomy. There then follows a section on motility and sensation measurements, again comprehensive but suitably cautious. There are sections on psychological assessments, and a good account of the weaknesses and strengths of various psychological rating scales for non-psychiatric and psychiatric work in gastroenterology but have limited practical experience of the specialty.

R A HARRY


While medical students can confidently hold forth on the mechanisms of the Zollinger-Ellison syndrome, a condition affecting one in a million of the population, they rarely have much to say about functional GI disorders (FGIDs), which can affect up to a quarter of the population at some stage in their life. Part of the reason is that one requires the integration of pathophysiology with psychology, and even sociology. FGIDs also suffer from having no objective measurable abnormalities, so that classifications must of necessity be subjective. The Rome process is a valiant attempt to make this area of study less confused, more consistent, and scientifically respectable. As such, it undoubtedly has had a major impact, and the Rome criteria are now used for the entry into most clinical trials and studies in this area. The senior chairman claims that this process has “done for functional gastrointestinal disorders what the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) has done for psychiatry”. While this may appear grandiose, I think it just might be true.

This book provides an overview of many years’ work, which has contributed much to our understanding of functional gastrointestinal disease (FGID). This is due in no small part to the “Rome” process, which is described in detail in the book. The challenge was to create order out of chaos, by agreeing rigorous criteria for the diagnosis of FGIDs. The major advantage of such a classification is that studies using agreed definitions become comparable and the next study can build on the results of the last. The Rome process is a valiant attempt to make this area of study less confused, more consistent, and scientifically respectable. As such, it undoubtedly has had a major impact, and the Rome criteria are now used for the entry into most clinical trials and studies in this area. The senior chairman claims that this process has “done for functional gastrointestinal disorders what the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) has done for psychiatry”. While this may appear grandiose, I think it just might be true.

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Inflammation, and Sepsis will be held in The 5th World Congress on Trauma, Shock, Inflammation, and Sepsis, 2950 West Cypress Creek Road, Fort Lauderdale, Florida, USA, on 17–19 February 2000. Further information from: Cleveland Clinic Florida, Department of Continuing Education.

Second Annual Gastrointestinal Cancer Update: A Multidisciplinary Approach
The Second Annual Gastrointestinal Cancer Update conference will be held at the Yarrow Hotel and Conference Centre, Park City, Utah, USA, on 15–19 March 2000. Further information from: Rosalie Lammlke. Tel: +1 801 581 8664; fax: +1 801 581 3647; email: rosalie.lammlke@hsc.utah.edu

European Courses on Laparoscopic Surgery
The European Courses on Laparoscopic Surgery will be held at the University Hospital Saint Pierre, Brussels, Belgium, on 4–7 April 2000 and 21–24 November 2000. Further information from: Conference Services S.A., Drève des Tumulii, 18, B-1170 Brussels, Belgium. Tel: +32 2 375 1648; fax: +32 2 375 3299; email: conference.services@skynet.be

Third Scandinavian Course on Inflammatory Bowel Diseases
The Third Scandinavian Course on Inflammatory Bowel Diseases will be held at the Wilanderselén, Örebro Medical Centre, Örebro, Sweden, on 28–30 November 2000. Further information from: Kurksanskiet, Region-sjukhuset, S-701 85 Örebro, Sweden. Tel: +46 19 15 37 05; fax: +46 19 15 37 95.

XVIIIth European Workshop on Gastroenterology and Endotherapy
The XVIIIth European Workshop on Gastroenterology and Endotherapy will be held in Brussels, Belgium, on 26–28 April 2000. Further information from: Administrative Secretariat, Ms Nancy Beauprez, Gastroenterology Department, Academic Hospital, Route de Lennik 808, B-1070 Brussels, Belgium. Tel: +32 2 555 4900; fax: +32 2 555 4901; email: beauprez@ulb.ac.be

Digestive Disease Week
The Digestive Disease Week will be held at the San Diego Convention Centre, San Diego, California, USA, on 21–24 May 2000. Further information from: DDW Administration, 7910 Woodmont Avenue, 7th Floor, Bethesda, Maryland 20814, USA. Tel: +1 301 272 0022; fax: +1 301 654 3978; website: www.ddw.org

International Hepato-Pancreato-Biliary Association 4th World Congress
The International Hepato-Pancreato-Biliary Association 4th World Congress will be held in Brisbane, Australia, from 28 May to 1 June 2000. Further information from: International Hepato-Pancreato-Biliary Association 4th World Congress.