

Immunoglobulin E antibodies from pancreatic cancer patients mediate antibody-dependent cell-mediated cytotoxicity against pancreatic cancer cells

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Summary

In addition to allergy and parasitic infections, immunoglobulin E (IgE) has been shown recently to possess anti-viral and anti-cancer effects. We investigated serum levels of IgE, its low-affinity receptor, soluble CD23 (sCD23) in patients with pancreatic cancer and the effect of IgE against pancreatic cancer cells. Twelve patients were evaluated for pancreatic cancer by imaging and confirmed by biopsy. Fifteen healthy volunteers served as controls. Serum Igs (IgG, IgM, IgA, IgE) and sCD23 levels were determined (enzyme-linked immunosorbent assay, nephelometry) and the presence of cancer-specific IgE was assessed (fluorescence microscopy, Western blot). IgE anti-cancer activity was determined by antibody-dependent cell-mediated cytotoxicity (ADCC). Serum levels of IgE and sCD23 were elevated significantly in patients with pancreatic cancer *versus* controls, whereas no differences were observed in other Ig isotypes (IgG, IgM, IgA). Flow cytometry and immunofluorescence microscopy demonstrated similar presence of IgG and IgE pancreatic cancer Igs. However, Western blot analysis indicated differences in IgG and IgE antigen-specific antibodies; IgE antibody recognized a 50 kD protein. ADCC studies demonstrated that serum and purified IgE-mediated cytotoxicity against pancreatic cancer cells, effects which were reversed with anti-IgE neutralizing antibody and IgE depletion (immunoaffinity); greater cytotoxicity was observed in patient serum when compared with healthy controls. These data suggest that IgE and sCD23 may serve as useful biomarkers for patients with pancreatic cancer and may be important in the immune response to this disease in that IgE-directed therapy may help to direct treatment.

Keywords: ADCC, adenocarcinoma, CD23, cytotoxicity, IgE, IgE receptor, immunoglobulins, pancreatic cancer

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Introduction

Pancreatic cancer continues to be one of the leading causes of cancer-related death in the United States [1] despite surgical treatment and numerous recent therapeutic interventions, such as gemcitabine [2] and topoisomerase inhibitors [3]. As predisposing host factors remain unclear [4], early diagnosis of this disease is extremely difficult, with the diagnosis usually made through imaging techniques with subsequent histopathological confirmation from biopsy specimens. In most cases, patients present in late stages of the disease and are thus no longer amenable to surgery. Therefore, it is imperative to find better diagnostic evaluations of this disease in addition to better therapeutic interventions in

order to make an impact on the lethal sentence associated with the diagnosis of this disease.

The aetiology and biology of pancreatic cancer is probably multi-factorial [5]. Viral infection has been postulated as a precedence or contributor to pancreatic cancer pathogenesis [6–11]. Others have suggested recently that allergic status correlated with cancer prognosis and survival; that patients with allergic diseases had a decreased risk for pancreatic and other cancers [12–15]. In this regard we have demonstrated previously that immunoglobulin (Ig)E anti-viral antibodies obtained from non-allergic patients may provide a beneficial role in human immunodeficiency virus (HIV) and other viral diseases [16–18]; the presence and persistence of IgE anti-viral antibodies may contribute to

IgE immunosurveillance and effect pathogenesis. In those studies we have shown that IgE can directly inhibit viral production and destroy virus-infected cells, probably by antibody-dependent cell-mediated cytotoxicity (ADCC) [16]. With regard to the role of IgE in malignancy, recent studies by Karagiannis *et al.* have investigated the role of IgE anti-cancer antibodies in ovarian carcinoma [19,20]. In those studies IgE antibodies were able to destroy ovarian cancer cells by ADCC. In addition, tumour-bearing nude mice showed prolonged survival with administration of anti-tumour IgE [19]. Recent studies by Wrensch and colleagues have reported that glioblastoma patients with elevated IgE had 9 months longer survival than those with normal or borderline IgE levels [21]. Here we investigated the role of IgE in pancreatic cancer and show that IgE is regulated uniquely with respect to other Ig isotypes and can destroy pancreatic cancer cells through ADCC mechanisms. These results provide a novel utility for IgE with respect to potential anti-cancer immunotherapy.

Materials and methods

Patient specimens

Peripheral blood (5 ml total) was obtained from 12 pancreatic cancer patients treated at State University of New York Downstate and Kings County (KCH), Medical Center's GI and Surgery Clinics and its affiliates. Serum obtained from 15 healthy volunteers served as controls. Patients and controls did not have any history of recent infection, atopy or parasitic infections. Patient consent for specimens and internal review board approval was obtained. Blood was collected into red-top monoject tubes (Sherwood Medical, St Louis, MO, USA) and allowed to clot for 30 min, after which tubes were spun at 800 *g* and serum collected. Sera were stored at -80°C until analysis.

Serum Igs (IgG, IgM, IgA, IgE) and soluble CD23

Serum Ig (IgG, IgM, IgA) levels were detected by nephelometry (MININEPHTM, The Binding Site, San Diego, CA, USA) according to the manufacturer's instructions. Total serum IgE and soluble CD23 (sCD23) levels were detected by enzyme-linked immunosorbent assay (ELISA) (IgE-BioQuant, San Diego, CA, USA; sCD23-BioSource, Camarillo, CA, USA) which was performed according to standard procedure. IgG, IgM and IgA are expressed as g/l; IgE and sCD23 levels are expressed as IU/ml and U/ml, respectively, [mean $\pm$ standard error (s.e.)] with significance between groups set at $P \leq 0.05$ (Student's *t*-test).

Cell culture

Pancreatic cancer cell lines [pancreatic adenocarcinoma (PANC-1), human pancreatic adenocarcinoma (HPAC) and

MiaPaCa-2] were obtained from American Tissue and Cell Culture (ATCC) and cultured in complete media according to ATCC guidelines in T-75 cm² flasks in a 37°C incubator w/5% CO₂. PANC-1 and HPAC cells were grown in Dulbecco's modified Eagle's medium (DMEM), 15% fetal calf serum (FCS), 5% penicillin/streptomycin and 0.5 mM sodium pyruvate. MiaPaCa-2 cells were grown in DMEM with Ham F-12, 5% FCS, 5% penicillin/streptomycin, 0.002 mg/ml insulin, 0.005 mg/ml transferrin, 40 ng/ml hydrocortisone and 10 ng/ml epidermal growth factor.

Immunofluorescence

Flow cytometry. Pancreatic cancer cell lines (HPAC and PANC-1) were incubated with patient or control serum for 30 min. Cells were centrifuged at 800 *g* for 10 min and washed three times with phosphate-buffered saline (PBS). Cells were then incubated with rabbit anti-human IgE or IgG antibody (Bethyl Laboratories, Montgomery, TX, USA) for 10 min, followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Bethyl) for 10 min. Cells were centrifuged and washed with PBS between each incubation. Flow cytometric analysis was performed on a Coulter Epics XL/MCL Flow Cytometer using System II software (Coulter) and CytoComp (Coulter), as described previously [17]. Specific fluorescence was reported as the percentage of cells with relative fluorescence intensity scored above background.

Fluorescence microscopy. Pancreatic cancer cell lines HPAC, PANC-1 and MiaPaCa-2 ($3\text{--}4 \times 10^5$ cells/ml) in appropriate media were incubated in 24-well plates at 37°C, 5% CO₂, overnight. Media was removed and cells were fixed with 500 μl of 10% buffered formalin for 15 min. Formalin was removed and cells were incubated with either pancreatic cancer patient serum, normal serum (1%) or no serum in 300 μl final volume (PBS) with 1% bovine serum albumin (BSA) overnight at 4°C. Cells were then washed three times with PBS (10 min incubation per wash) and incubated with either rabbit anti-human IgE or IgG antibody (1%) (Bethyl) in 300 μl of 1% BSA for 1 h at room temperature. Cells were washed three times with PBS and incubated with FITC-conjugated anti-rabbit antibody (0.2%) (Bethyl) for 1 h at room temperature. Cells were washed three times, resuspended in 1 ml of PBS and visualized with fluorescence microscopy. Cells from representative wells were scraped, transferred to microslides and visualized under confocal microscopy.

Western blot analysis of antigen-specific IgE

Pancreatic cancer cell lines HPAC, PANC-1 and MiaPaCa-2 were grown to confluence and lysed as described previously [22]. Briefly, 1×10^7 cells were lysed with 1.0 ml of ice-cold radio immunoprecipitation assay buffer [150 mM NaCl,

10 mM Tris-HCl pH 7.2, 0.1% sodium dodecyl sulphate (SDS), 1.0% Triton X-100, 1% deoxycholate, 5 mM ethylenediamine tetraacetic acid containing 1% protease inhibitor cocktail (Sigma P8340)]. Cell lysates were pipetted into a 1.5-ml microcentrifuge tube on ice, agitated for 15 min and centrifuged for 15 min at 14 000 g at 4°C. The supernatants were collected immediately into 200 µl aliquots and stored at -80°C. Prior to storage, protein quantification of the cell lysates were measured by Bradford Assay.

Cell lysates were run on 15% acrylamide gels by SDS-polyacrylamide gel electrophoresis at differing concentrations (2–25 µl) and developed with Coomassie Blue. Subsequent analysis used 10 µl of lysates, which yielded best results in preliminary studies. Lysates were transferred onto nitrocellulose and stained with Ponceau Red stain to visualize the lanes. Each lane was cut out as strips and destained with PBS. Each strip containing pancreatic cancer cell lysate was incubated individually in plastic trays with gentle rotation on an orbital shaker. Strips were first incubated with wash buffer [5% powdered milk/1× Tris-buffered saline/0.1% Tween20] for 1 h at room temperature. Each strip was then incubated overnight with either pancreatic cancer patient serum, normal serum (1%) or no serum in 2 ml wash buffer. Strips were washed three times with PBS and incubated with rabbit anti-human IgE or IgG antibody (2%) (Bethyl) in 2 ml wash buffer. Strips were washed three times in PBS and incubated with horseradish peroxidase labelled-goat anti-rabbit antibody (0.1%) (Bethyl) in 2 ml wash buffer for 1.5 h. Strips were washed three times and developed with 2 ml 3,3-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma, St Louis, MO, USA).

Serum IgE removal

Depletion and recovery of IgE (immunoaffinity). Serum obtained from patients and controls was depleted of IgE as described previously [16]. IgE was recovered from immunoaffinity columns with 0.5 volumes 0.05 M glycine (Sigma), pH 3.0 for 3 min and eluted IgE was immediately neutralized with 0.31 volumes of 1 M Tris-NaOH, pH 11.0 (final adjusted pH 7.0–7.4). IgE depletion and recovery was determined by ELISA (BioQuant, San Diego, CA, USA), recovered IgE contained no detectable IgM, IgG or IgA (nephelometry). Unfractionated, IgE-depleted serum and eluted (purified) IgE were stored at -70°C until utilized.

IgE neutralization. Serum obtained from patients with pancreatic cancer were incubated with increasing amounts (1–10%) of anti-human IgE or IgG (as a comparison) (Bethyl) for 30 min at room temperature prior to culture in cytotoxicity assays.

Antibody-dependent cell-mediated cytotoxicity

Effector cells. Peripheral blood mononuclear cells (PBMC) served as effector cells. Ten ml of blood was collected from a

healthy volunteer and diluted 1:1 with PBS. Diluted blood was layered 1:1 on Ficoll-Hypaque solution (Pharmacia, Uppsala, Sweden) and centrifuged at 800 g for 30 min at room temperature. The buffy coat layer, consisting of PBMC, was transferred into fresh tubes, washed twice and resuspended in PBS. PBMCs were resuspended in PBS. Cell viability was > 99% (trypan blue exclusion dye).

Target cells. HPAC (5×10^3) cells were aliquoted into each well of a 96-well plate and served as the target cells. PBMCs (2.5×10^4) were aliquoted into each well and served as the effector cells. Preliminary studies demonstrated a 5:1 effector to target (E:T) cell ratio to be the most effective for cytotoxicity studies. E:T mixtures in phenol red dye free RPMI-1640 (HyClone, Logan, UT, USA) complete medium (1 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5% heat-inactivated fetal bovine serum, GIBCO-BRL, Grand Island, NY, USA) were incubated at 37°C, 5% CO₂ overnight with either pancreatic cancer patient serum or normal serum (100 µl final volume).

Cytotoxicity assay. Assays were carried out using a CytoTox 96 non-radioactive cytotoxicity assay (Promega, Woodshole, MA, USA), in accordance with the manufacturer's directions. This assay measures lactate dehydrogenase (LDH) release by target cells, after conversion of a tetrazolium salt into a formazan red product. Serum (1–5%) was added to 96-well tissue culture plates (Fisher Scientific, Springfield, NJ, USA) containing 5:1 E:T cell mixtures (100 µl final volume) and incubated at 37°C, 5% CO₂, for 18–20 h. In some experiments, IgE neutralized, IgE-depleted serum or purified IgE was added to cytotoxicity assays. To report maximum lysis, lysing solution (9% v/v Triton-X 100) (10 µl) was added to representative culture wells. After incubation supernatant (50 µl) was transferred to a fresh 96-well plate. Chromogenic substrate solution (50 µl) was added to each well and the plates incubated in the dark at room temperature for 30 min, after which stop solution (1 M NaAc) (50 µl) was added to each well to stop the reaction. The plates were read using a 96-well plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 490 nm. When cells were cultured without serum, cell viability, as judged by trypan blue dye exclusion, and cell recovery was > 99%. It was established in preliminary studies that when E:T cells were used in ratios of 10:1 or greater, or when serum concentrations were 10% or greater, LDH release could not be measured accurately as readings obtained with the spectrophotometer were above the limits of the instrument at the requisite wavelength set forth by the manufacturer of the assay kit used. Further, when aliquots of LDH containing medium were diluted, linear curves were not obtained. Therefore, cells were used at 5:1, and serum was used at 1%. Data shown represent the mean of at least three experiments ± s.e., and are expressed as percentage of cytotoxicity. Background controls were corrected for and consisted of target cell spontaneous (HPACs in media), target cell

maximum (HPACs in media with 10 µl lysis solution) and effector cell spontaneous (PBMCs in media). The target cell maximum was corrected for by a volume control (media with 10 µl lysis solution). Each experiment was corrected for by a serum control (serum in media). These controls were incubated on the same 96-well plate as experimental assays. Percentage (%) cytotoxicity was calculated according to the general equation below:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Target cell spontaneous} - \text{Effector cell spontaneous}}{\text{Target cell maximum} - \text{Target cell spontaneous}} \times 100$$

Results

Pancreatic cancer patient demographics included an average age of 65 years, with a female predominance of 3 : 2. The cancer stage was IIa or greater (T3NxMx), with the location of the adenocarcinoma located at the head of the pancreas. The majority of co-morbid conditions included hypertension, diabetes and end-stage renal disease (Table 1). Serum levels of IgG, IgM and IgA were similar in both pancreatic cancer and control groups (Fig. 1) (IgG: 11.2 ± 1.2 versus 13.2 ± 2.1 , IgM: 2.39 ± 0.8 versus 1.66 ± 0.3 , IgA: 2.72 ± 0.7 versus 1.92 ± 0.4 ; $P > 0.05$ for all comparisons). In contrast, serum levels of IgE were elevated significantly in patients with pancreatic cancer (148 ± 45 IU/ml) compared with controls (30 ± 6.3 IU/ml) ($P = 0.022$). Serum levels of sCD23 were also elevated significantly in patients with pancreatic cancer (2.82 ± 0.91 U/ml) compared with controls (1.42 ± 0.22 U/ml) ($P = 0.006$) (Fig. 2); serum levels of IgE and sCD23 were increased significantly (fivefold and twofold respectively) in patients with pancreatic cancer compared with controls.

Flow cytometry analysis of IgE and IgG anti-pancreatic cancer antibodies to cell surface antigens were similar between patients and controls (IgE: $12.91 \pm 0.02\%$ versus $11.31 \pm 0.01\%$, IgG: $30.98 \pm 0.15\%$ versus $44.81 \pm 0.28\%$, $P > 0.05$) (Fig. 3). To establish further the presence of Ig

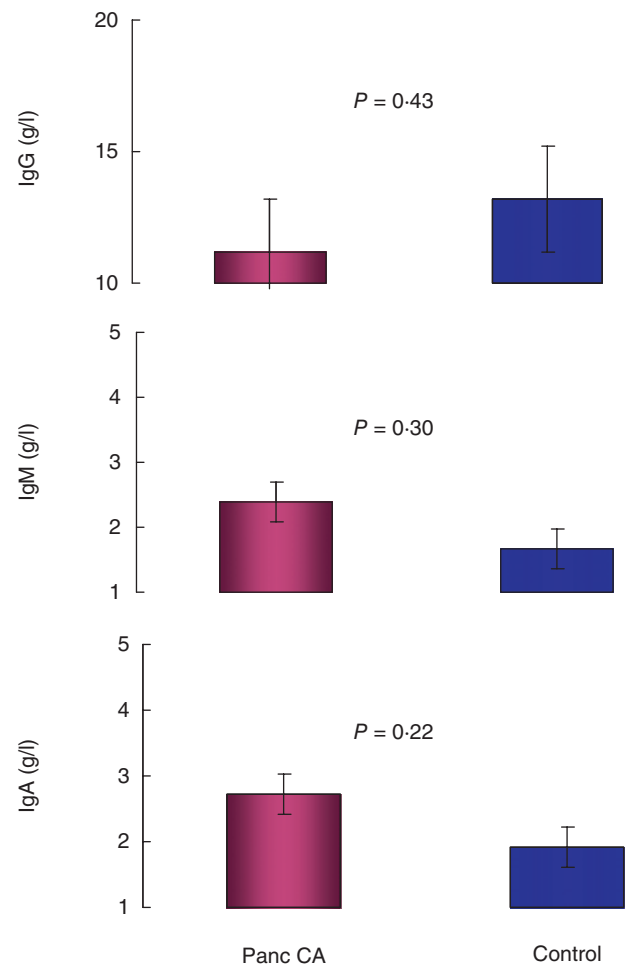


Fig. 1. Serum immunoglobulin (Ig)G, IgM and IgA in pancreatic cancer. Serum obtained from pancreatic cancer patients ($n = 12$) and healthy controls ($n = 15$) were assayed for IgG (top panel), IgM (middle panel) and IgA (bottom panel) by nephelometry (see Materials and methods). Data are expressed as mean g/l ($\pm$ standard error). Significance between groups was determined by Student's *t*-test ($P < 0.05$).

Table 1. Characteristics of pancreatic cancer patients.

Age (range)	65 (38–84)
Sex	58% F
Resection	67%
Dx methods	CT, ERCP, EUS, FNA
Tumour stage	67% $\geq$ IIA–III (T3NxMx)
Mass location	83% head
Allergy Hx	None
Infx Hx	None
Past medical Hx:	HTN (67%)
	DM (50%)
	ESRD (17%)

HTN, hypertension; DM, diabetes mellitus; ESRD, end-stage renal disease; CT, computed tomography scan; ERCP, endoscopic retrograde cholangiopancreatography; EUS, endoscopic ultrasound; FNA, fine needle aspiration.

anti-cancer antibodies, pancreatic cancer cells were incubated with patient and control sera and probed for IgE and IgG. Immunofluorescence microscopy analysis also revealed the presence of anti-IgG and anti-IgE antibodies (data not shown).

In addition to cell surface recognition of IgE antibodies, we investigated the presence of specific IgE anti-pancreatic cancer antibodies against total cell components. HPAC cells were grown to confluence and lysed and the lysates were blotted and probed for the presence of antigen-specific IgE. Interestingly, Western blot analysis revealed a 50 kD band in which was recognized by IgE obtained from pancreatic cancer patient serum, which was not found in normal serum (Fig. 4).

In order to study the function of IgE as an anti-cancer Ig, ADCC assays were performed. Serum obtained from

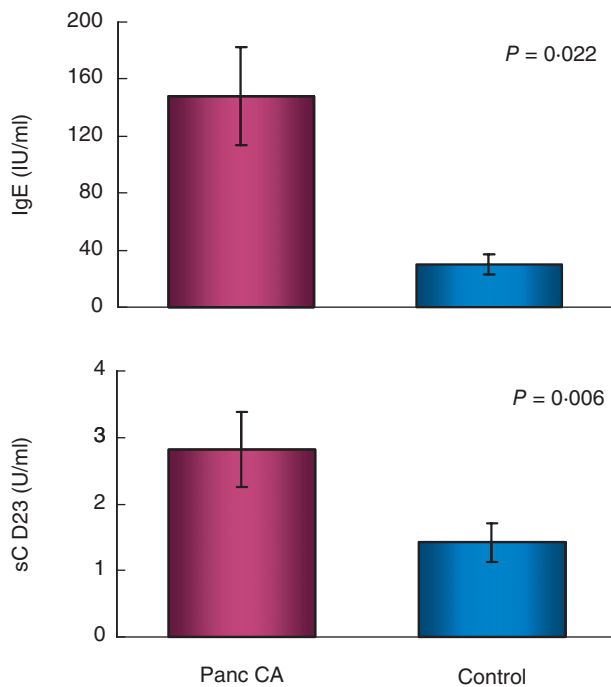


Fig. 2. Serum immunoglobulin (Ig)E and sCD23 in pancreatic cancer. Serum obtained from pancreatic cancer patients ($n = 12$) and healthy controls ($n = 15$) were assayed for IgE (top panel) and soluble CD23 (bottom panel) by enzyme-linked immunosorbent assay. Data are expressed as mean ($\pm$ standard error) (IgE-IU/ml; sCD23-U/ml). Significance between groups was determined by Student's t -test ($P < 0.05$).

pancreatic cancer patients mediated cytotoxicity against HPAC cells. To confirm that this effect was due to IgE, three approaches were utilized: (i) addition of anti-IgE antibody in an effort to neutralize antibody function; (ii) depletion of IgE through immunoaffinity chromatography; and (iii) addition of purified IgE to establish that IgE alone was able to mediate cytotoxicity. Addition of anti-IgE antibody decreased cytotoxicity by 29% when compared with whole serum (Fig. 5). In contrast, addition of anti-IgG antibody, increased cytotoxicity by 43% ($P < 0.05$) (Fig. 5). To understand more clearly the nature of individual IgE responses, each patient's serum was depleted of IgE (immunoaffinity) and depleted serum was assessed for cytotoxicity compared with unfractionated serum. As shown in Fig. 6, patient serum, which was depleted of IgE, showed reduced cytotoxicity whereas IgE depletion of control serum did not differ from that of unfractionated serum. Furthermore, purified IgE obtained from individual patients with pancreatic cancer was also able to mediate cytotoxicity (Fig. 6), whereas purified IgE obtained from controls did not, demonstrating that the cytotoxicity observed with whole pancreatic cancer serum was due, in part, to IgE. In contrast, no cytotoxicity was observed when serum was cultured with target cells alone in the absence of effector cells (data not shown).

Discussion

To our knowledge, this is the first study which demonstrates that serum IgE levels are significantly elevated in patients with pancreatic adenocarcinoma and that serum from these patients contain antigen-specific IgE and mediate an IgE-specific cytotoxic effect against pancreatic cancer cell lines. Although IgE is well known for its role in atopic disease

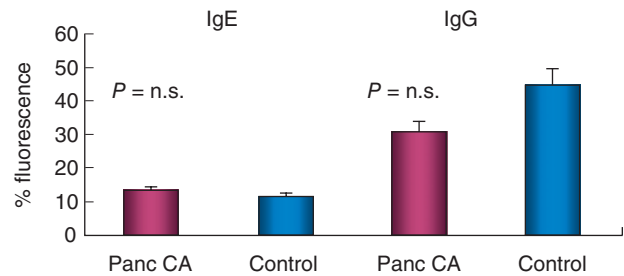


Fig. 3. Presence of pancreatic cancer specific immunoglobulins (Ig) by immunofluorescence. Human pancreatic adenocarcinoma cell lines (HPAC) were cultured in the presence of serum from pancreatic cancer patients and healthy controls. IgE and IgG in serum were detected with fluorescein isothiocyanate-conjugated anti-isotype Ig. Data represent mean $\pm$ standard error of 10 sera per group. Significance between groups was determined by Student's t -test ($P =$ not significant). Similar fluorescence patterns were detected when sera were cultured with other pancreatic cancer cell lines (MiaPaCa-2, pancreatic adenocarcinoma-1).

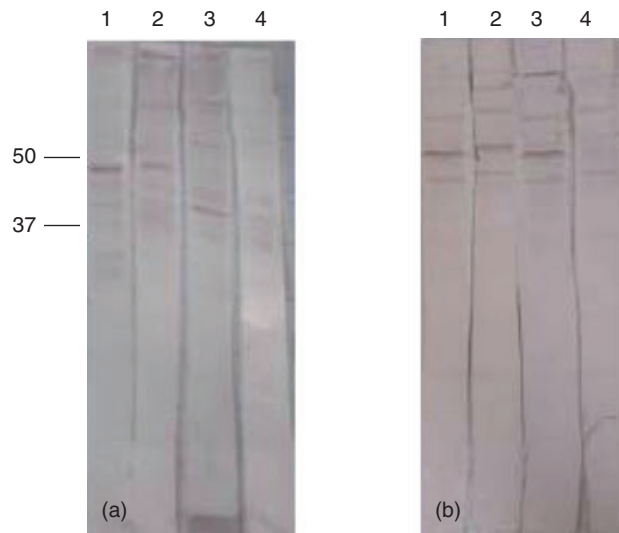


Fig. 4. Antigen-specific antibodies. (a) Human pancreatic adenocarcinoma cell lines (HPAC) lysates were blotted with serum obtained from pancreatic cancer patients (lanes 1 and 2) or healthy controls (lanes 3 and 4) and probed for immunoglobulin (Ig)E and IgG isotypes. (b) Lysates were obtained from MiaPaCa-2 pancreatic cancer cell line and blotted with serum from pancreatic cancer patients (lanes 1–3) or healthy control (lane 4). As shown in both figures, sera from patients with pancreatic cancer contained antibodies (IgG and IgE) which were specific for protein of ~50 kD.

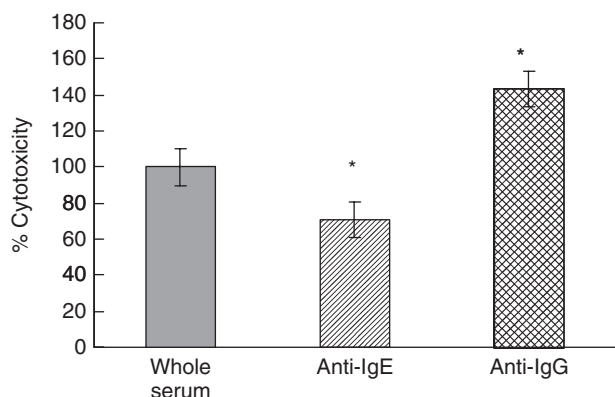


Fig. 5. Immunoglobulin (Ig)E-mediated antibody-dependent cell-mediated cytotoxicity: effect of neutralization. Human pancreatic adenocarcinoma cell lines (HPAC) were cultured in the presence of serum from pancreatic cancer patients and controls ($n = 4/\text{group}$) and cytotoxicity was determined after 18–20 h. In some experiments, anti-human IgE or IgG (10%) was added to serum before culture with target (HPAC) and effector (peripheral blood mononuclear) cells. Data were performed in triplicate with an effector : target ratio of 5 : 1 (using 5×10^3 target cells) and 1% patient serum. Data are expressed as mean percentage of cytotoxicity as determined by lactate dehydrogenase release $\pm$ standard error of anti-IgE or IgG experiments compared with whole serum (cytotoxicity for whole serum alone averaged 17%, range 14–20%). Significance between groups was determined by Student's *t*-test, * $P < 0.05$.

and parasitic infections, new evidence suggests that IgE is a pleiotropic Ig molecule with many more functions. Previous studies in our laboratory have shown that IgE possesses anti-viral activity, in that IgE anti-HIV-1 antibodies were able to

elicit viral clearance and killing through inhibiting virus production and mediating ADCC [16]. Similarly, Karagiannis *et al.* have demonstrated that IgE-specific antibodies can increase survival in an animal model of ovarian carcinoma and elicit anti-cancer effects through ADCC against ovarian cancer cells [19,20].

Immunotherapy is a topic of recent interest in which tumour-specific antigens are needed to identify tumour cells while sparing normal cells. Various immunological strategies in the treatment of pancreatic cancer have been pursued, including stimulation of the patient's native immune system to fight off the malignancy with cytokines as granulocyte-macrophage colony-stimulating factor (GM-CSF) [23], interleukin (IL)-6 [24] and immune response modifiers such as Virulizin [25]. In addition, selective antigens with isotype-specific antibodies such as IgM [26] and IgG [27] have been studied. Earlier studies by Goodale *et al.* determined that Ig levels could be correlated with disease severity in malignancy [28]. Pancreatic fluid obtained from patients with pancreatic cancer was assayed and shown to have increased levels of IgG and IgA, with borderline increases in IgM when compared with healthy controls [29]. Post-mortem examination of the pancreas and other organs from patients with adenocarcinoma demonstrated IgA and some IgM deposits [30], as did pancreato-duodenal fluid obtained from living patients [31]. Here we report the presence of elevated IgE and its receptor sCD23 in serum obtained from pancreatic cancer patients, whereas other Ig isotypes did not differ between patient and control populations. This is in contrast with other above-stated reports which investigated the presence

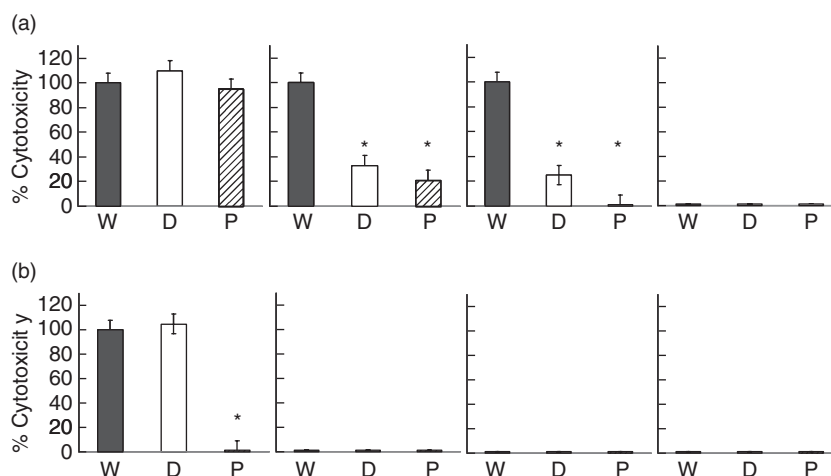


Fig. 6. Immunoglobulin (Ig)E-mediated antibody-dependent cell-mediated cytotoxicity: effect of IgE depletion and purified IgE. Human pancreatic adenocarcinoma cell lines (HPAC) were cultured in the presence of either whole, IgE-depleted (immunoaffinity) serum or purified IgE obtained from (a) pancreatic cancer patients and (b) controls ($n = 4/\text{group}$) and cytotoxicity was determined after 18–20 h. Data were performed in triplicate with an effector : target ratio of 5 : 1 (using 5×10^3 target cells). Purified IgE contained 20–50% of original whole serum IgE content. Data are expressed as mean percentage of cytotoxicity as determined by lactate dehydrogenase release $\pm$ standard error of anti-IgE or IgG experiments compared with whole serum (cytotoxicity for whole serum alone averaged 17%, range 14–20%). Significance between groups was determined by Student's *t*-test, * $P < 0.05$. W: whole serum; D: IgE-depleted serum; P: purified IgE.

of IgE in pancreatic juice, fluid or solid tissue. Our studies investigated Ig levels in serum, providing a non-invasive means for future studies investigating the diagnostic and/or prognostic role of IgE and its soluble receptor, sCD23, in pancreatic cancer. Although we did not study the relationship of IgE or sCD23 and their effects against pancreatic cancer over time, it could be that increased IgE may prognosticate better survival, as has been demonstrated in patients with glioblastoma [21] and other malignancies [32,33]. However, it is also possible that increased IgE levels may portend worsening survival, as has been shown for sCD23 in malignancy [34]. Patients and controls who have had a history of allergy, atopic disease, parasitic or other recent infections were excluded from this study. Although Gandini and colleagues have proposed recently that patients with allergy/atopy have decreased incidence of pancreatic cancer [15], here we investigated the effects of IgE in the absence of variables known to correlate with its increased levels. Our findings that IgE was selectively increased in the absence of these variables demonstrate a unique role for IgE in malignancy which is not contingent on or related to a hypergammaglobulinaemic response, as has been described in other pancreatic diseases [35]. Whether allergic/atopic status provides an additional protective component in malignancy remains to be determined.

Serum obtained from cancer and control patients contained IgE and IgG antibodies which recognized cell surface proteins by flow cytometry analysis and fluorescence microscopy. Identification of tumour-specific antigens is essential in formulating an effective immunotherapy in targeting pancreatic tumour cells. Schmitz-Winnenthal *et al.* have shown tumour-associated antigen expression of HERV-K-MEL to be expressed with a relatively high prevalence, making it a candidate for specific immunotherapy in a large subgroup of pancreatic cancer patients [36]. Our data demonstrate the presence of a 50 kD protein which is recognized uniquely by IgE obtained from patients with pancreatic cancer. The characterization and function of this protein are unknown, and future studies will investigate the role of this protein in pancreatic cancer. Interestingly, studies by Nakasura *et al.* [37] have identified unique Ig responses to pancreatic cancer antigens by serological analysis of recombinant cDNA expression libraries (SEREX). In those studies, antigens recognized by IgE were distinct from those of IgG and were not correlated with allergic status; the nature of the antigens was not identified. Taken together, the presence of unique antigen-specific IgE in pancreatic cancer patients remains an important prospect for further investigation. Future studies will determine the nature of the 50 kD protein and its relationship to malignancy. This may serve as an eventual biomarker or therapeutic intervention for pancreatic cancer.

Our data suggest that antigen-specific IgE antibodies are produced in pancreatic cancer patients and that these antibodies can mediate anti-tumour effects through

mechanisms such as ADCC, which has been described as an effective anti-cancer modality for immunotherapeutics [38]. ADCC mediated by IgG has been described as an anti-cancer mechanism in hepatocellular carcinoma [25], colon and prostate cancer [39], melanoma [40] and pancreatic cancer [41]. Meanwhile, IgE-mediated ADCC has been described in ovarian cancer [19,20], and has been shown to inhibit the growth of colorectal carcinoma cells [42]. Furthermore, IgE was more effective than IgG1 in mediating ADCC and prolonging survival *in vivo* (40 days *versus* 22 days, $P = 0.012$) in a mouse model of ovarian carcinoma [20].

Our studies demonstrate that serum from pancreatic cancer patients were able to mediate ADCC against pancreatic cancer cells. With the addition of either anti-IgE neutralizing antibody or IgE-depleted serum, cytotoxic activity decreased. In contrast, when anti-IgG neutralizing antibody was added, the cytotoxic activity increased. Because IgG is found in greater quantities than IgE, neutralizing the IgG may have allowed more IgE to elicit its cytotoxic effects. The demonstration that purified IgE was able to mediate cytotoxicity alone provides further evidence for a direct IgE effect. The data indicate that the cytotoxicity observed with whole serum was mediated in part by IgE. The fact that cytotoxicity was observed in the presence of both effector and target cells and not with target cells alone supports ADCC rather than complement-mediated cytotoxicity as the mechanism of action. It is well recognized that IgE functions by binding cytophilically to either high or low (CD23) affinity Fc receptors on various leucocytes. Furthermore, the time-frame when ideal cytotoxicity was observed (18–20 h) suggests that PBMC effector cells or select subsets (i.e. monocytes) may mediate ADCC in a manner similar to those observed in parasitic infections [43].

The anti-cancer effects of IgE may be contingent on the presence and concentration of antigen-specific IgE rather than total IgE levels. The content and characteristics of antigen-specific IgE in the context of total IgE levels probably vary from patient to patient. When the effect of serum-mediated cytotoxicity was assessed in four individual patients and controls, 75% of patients' serum-mediated cytotoxicity compared with 25% of controls. Depletion of IgE decreased the cytotoxic responses in 50% of the patients but none in the controls. Furthermore, purified IgE-mediated cytotoxicity in half the pancreatic cancer patients but not in the controls. In one of the patients there were no differences in observed cytotoxicity in whole, IgE-depleted serum or purified IgE. It could be that in this patient, even though IgE was depleted > 95%, sufficient levels of IgE anti-pancreatic cancer antibodies remained to mediate cytotoxicity. This would also explain why purified IgE of this patient mediated cytotoxicity comparable to whole serum; purified IgE from this patient contained high enough levels of IgE anti-pancreatic cancer antibodies to mediate cytotoxicity, whereas IgE purified from the serum from the other patients did not. We have demonstrated previously such individual differences in the potency

of antigen-specific IgE in mediating ADCC in other disease states [44].

The observation that IgE-mediated cytotoxicity was less than those reported for IgG-mediated ADCC in other systems is due probably to the fact that IgE is found 1000-fold less than IgG in serum concentrations. This underscores the potential potency of IgE as an anti-cancer agent. Future utility of IgE containing antibody therapeutics against malignancy will probably provide a very effective modality, as has been demonstrated in ovarian carcinoma [19,20] and non-malignant disease [45].

Conclusions

To our knowledge, this is the first report of increased serum levels of IgE and sCD23 in non-atopic patients with adenocarcinoma. Sera obtained from these patients were able to mediate antibody-dependent cell-mediated cytotoxicity through IgE-specific antibodies to a pancreatic cancer antigen. Therefore, IgE may be an important biomarker for diagnostic or prognostic purposes and, more importantly, it may be useful in directing IgE-mediated therapy.

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