Differential effects of genistein on apoptosis induced by fluoride and pertussis toxin in human and rat pancreatic islets and RINm5F cells

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Abstract

Clonal pancreatic β-cell lines have been used widely for the study of the factors involved in the regulation of apoptosis but it has not been firmly established that the response of normal islets mirrors that found in transformed β-cells. In the present work, the role of pertussis toxin (Ptx)-sensitive G-proteins in the control of β-cell apoptosis was studied in isolated rat and human islets of Langerhans and compared with the clonal β-cell line, RINm5F. Annexin-V and deoxycarboxyfluoroscein diacetate staining was used to identify viable, apoptotic and necrotic cells directly, under fluorescence illumination. Treatment of human and rat islet cells with the G-protein activator fluoride (NaF; 5 mM) caused a marked increase in apoptosis that was further potentiated in islets pretreated with Ptx. The tyrosine kinase inhibitor genistein (100 µM) also increased islet cell apoptosis and the combination of 100 µM genistein and 5 mM NaF did not lead to any diminution of the apoptotic response. This latter effect was quite different from that seen in RINm5F cells where the combination of 100 µM genistein and 5 mM NaF resulted in much less apoptosis than was observed with either agent alone. In islets treated with a lower concentration of genistein (25 µM; that did not, itself, increase cell death), the drug attenuated NaF-induced apoptosis and also blocked the enhancement mediated by Ptx. These results revealed that human (and rat) islets are equipped with a Ptx-sensitive pathway that may be regulated by tyrosine phosphorylation and is anti-apoptotic. However, they also define conditions under which marked differences in response between RINm5F cells and normal islets were observed and they suggest that care should be taken when extrapolating data obtained with clonal cell lines to the situation in normal islet cells.

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Introduction

There is mounting evidence that inappropriate activation of apoptosis plays a role in the process of β-cell loss associated with the development of type 1 and type 2 diabetes in man (reviewed by Mauricio & Mandrup-Poulsen 1998, Signore et al. 1998, Sjoholm 1998, Mountz et al. 1999, Roche et al. 2000, Mandrup-Poulsen 2001). Thus, it is important that the factors involved in the regulation of β-cell apoptosis are defined and an increasing number of studies are attempting to achieve this objective. However, this can be difficult using primary human islets of Langerhans since the tissue is normally available in only limited quantity and quantitation of β-cell apoptosis is hampered by the spatial organisation of the endocrine cells within the islet structure and by the presence of other cell types. For these reasons, many workers have chosen to use cultured rodent β-cell lines as model systems for the study of the control of apoptosis (Loweth et al. 1996a, 1997, Di Matteo et al. 1997, Bai et al. 1999, Nakata et al. 1999, Olejnicka et al. 1999, Tejedo et al. 1999, Ahmad et al. 2000, Ammendrup et al. 2000, Bonny et al. 2000, Krautheim et al. 2000, Sjoholm et al. 2000, Baker et al. 2001). The implicit assumption in such studies is that the responses observed are relevant to the aetiology of diabetes and that the cell lines respond in a similar manner to normal human islets of Langerhans. In the majority of cases, however, the validity of this conclusion has not been fully substantiated and we have shown that significant differences can exist (Loweth et al. 1996b).

One of the most widely used β-cell lines is the RINm5F cell which was originally derived from a radiation-induced β-cell tumour in the rat and has been in use for many years as a convenient source of cultured β-cells (Gazdar et al. 1980). In previous studies, we have employed RIN5mF cells to investigate the role of hetero-trimeric G-protein-dependent pathways in the control of β-cell apoptosis and have shown that these cells express an anti-apoptotic pathway that is under the control of a pertussis toxin (Ptx)-sensitive G-protein (Loweth et al. 1997). In this study, we have examined the role of Ptx sensitive G-proteins in the control of β-cell apoptosis in normal human islets and compared the responses observed with those reported in RINm5F cells.
1996a, Elliott et al. 2001). A similar pathway also exists in normal rat islets (Loweth et al. 1996a) suggesting that, at least in this respect, RINm5F cells are an appropriate model system. However, it is not known whether this mechanism is operative in human islet cells.

In recent work, we have demonstrated that the Ptx-sensitive anti-apoptotic pathway present in RINm5F cells may involve the regulation of one or more tyrosine kinase enzymes (Elliott et al. 2001). In the present study, we have extended these experiments to examine the effects of the tyrosine kinase inhibitor, genistein, on cell viability in isolated human islets of Langerhans. The results reveal that significant differences exist between responses to genistein in RINm5F cells and human (and rat) islets and indicate that caution should be exercised when extrapolating from data obtained with β-cell lines.

**Materials and Methods**

**Islet isolation and culture**

Islets were isolated from Wistar rats (body weight 180–220 g) allowed free access to food and water, by collagenase digestion of the pancreas as described previously (Loweth et al. 1996a). Islets from heart-beating cadaver organ donors were isolated in the islet-transplant laboratory, University of Leicester (Leicester, UK) and transported to our laboratory in tissue culture medium (Lacey et al. 1990, 1993, Loweth et al. 1998). They were then cultured for a further 2–3 days in RPMI-1640 medium containing glucose (11·1 mM), l-glutamine (300 µg/ml), sodium penicillin G (100 U/ml), streptomycin sulphate (100 µg/ml) and 10% (v/v) foetal bovine serum. During the culture period the islets remained free floating in the medium and did not become attached to the culture dish.

RINm5F cells were cultured in RPMI-1640 tissue culture medium supplemented with glucose (11·1 mM), l-glutamine (300 µg/ml), sodium penicillin G (100 U/ml), streptomycin sulphate (100 µg/ml) and 10% foetal calf serum and maintained in a 95% air:5% CO2 atmosphere with 100% humidity at 37 °C (Elliott et al. 2001).

Stock solutions of test reagents were prepared in dimethyl sulphoxide (DMSO) and were added directly to the culture medium, as appropriate. Control cultures received DMSO alone and the final concentration did not exceed 0·1%.

**Detection and quantitation of apoptosis**

Human or rat islets were treated with test reagents for the appropriate period of time then harvested by centrifugation (600 g; 2 min) and washed with phosphate-buffered saline (PBS; 500 µl). They were digested with trypsin–EDTA (100 µl) for 3–5 min until dispersed into single cells and the cells were collected by centrifugation (1000 g; 3 min). The trypsin was removed and the cells were washed twice with PBS. The cell pellet was resuspended in approximately 40 µl PBS. In order to prevent loss of sample, the resuspended islet cells were placed inside a circle of clear varnish painted onto poly-lysine-coated slides. The slides were air dried for 10 min and the excess PBS removed. The samples were washed twice with 50 µl binding buffer (10 mM Hepes, 140 mM NaCl, 2·5 mM CaCl2, pH 7·5) before addition of 35 µl deoxy-carboxyfluorescein diacetate together with Cy3-conjugated-annexin V in binding buffer. The cells were then incubated for 10 min in the dark. The slides were washed five times with 50 µl binding buffer and the numbers of viable, apoptotic and necrotic cells determined directly by cell counting under a fluorescence microscope.

In the case of RINm5F cells, they were seeded at 5000 cells per chamber in a multichamber slide and incubated overnight before treatment. The cells were treated with test reagents before the medium was removed and they were washed twice with PBS. A similar procedure for annexin-V/deoxycarboxyfluorescein diacetate staining was used as described above for islets, except that the wash steps were increased to 200 µl.

**Materials**

The following reagents were obtained from Sigma Chemical Co. Ltd, Poole, Dorset, UK: annexin V–Cy3 apoptosis detection kit, collagenase type XI, dimethyl sulphoxide, genistein, Ptx, RPMI-1640 and sodium fluoride. Foetal bovine serum, trypsin/EDTA, l-glutamine, penicillin G and streptomycin sulphate were obtained from Gibco BRL, Paisley, Strathclyde, UK. All other reagents were of analytical grade quality.

**Statistics**

Differences between mean values were assessed by analysis of variance and were considered significant when P<0·05. All experiments were performed in replicates of four and were repeated on at least two separate occasions.

**Results**

Previous studies have shown that NaF causes a time- and dose-dependent loss of viability in both RINm5F cells and normal rat islets (Loweth et al. 1996a, Elliott et al. 2001). The pattern of DNA fragmentation suggested that this is mediated, at least in part, by increased apoptosis of the cells. In the present work, we have employed a more stringent analysis to identify the mode of cell death in NaF–treated cells, by examination of annexin-V and carboxyfluorescein staining under fluorescence illumination. Although time consuming, this method has the advantage that it allows the simultaneous quantitation of
the total number of viable, necrotic and apoptotic cells under each experimental condition. Use of this technique confirmed that exposure of RINm5F cells to 5 mM NaF for 24 h led to a marked increase in the number of cells undergoing apoptosis (Fig. 1), whereas the number of necrotic cells was increased only marginally. Incubation of RINm5F cells with 100 µM genistein (a concentration used in many previous studies of islet cell function (e.g. Sorensen et al. 1994, Jonas et al. 1995, Kwon et al. 1995, Mitchell et al. 2001) also increased apoptosis significantly (Fig. 1). Strikingly, however, incubation of RINm5F cells with both 5 mM NaF and 100 µM genistein together was associated with a dramatic reduction in the extent of apoptosis such that cell viability was restored to a level similar to that seen in control cultures (Fig. 1).

In order to investigate whether a similar phenomenon occurs in primary tissue, human islets were isolated and maintained in free-floating culture in the presence or absence of NaF and genistein for 24 h, prior to measurement of the extent of cell viability. As observed in RINm5F cells, exposure of human islets to either 5 mM NaF or 100 µM genistein alone induced a significant increase in apoptosis with little change in the number of necrotic cells. However, in marked contrast to the situation in RINm5F cells, the combination of 5 mM NaF and 100 µM genistein failed to prevent apoptosis in human islets but, rather, enhanced it still further (Fig. 2).

To confirm that this effect was not related to the species difference between RINm5F cells and human islets, similar studies were repeated with isolated rat islets (Table 1). The responses observed were equivalent to those seen in human islets (but different from those in the RINm5F cell) in that NaF and genistein each caused an increase in apoptosis when added to rat islets individually and they further increased this response when used in combination (Table 1).

To clarify the situation further, an additional series of studies was undertaken in which human islets were treated with a lower concentration of genistein (25 µM) in the absence and presence of 5 mM NaF (Table 2). At this concentration, previous studies have established that genistein does not significantly increase cell death in RINm5F cells, although it attenuates the increase due to NaF (Elliott et al. 2001). In human islets, 25 µM genistein alone did not induce apoptosis but it abolished the increase mediated by 5 mM NaF (Table 2).

The experiments were then extended to compare the ability of Ptx to enhance the level of apoptosis in RINm5F cells and in normal human islets. As observed previously (Loveth et al. 1996a, Elliott et al. 2001), treatment of RINm5F cells with Ptx did not cause any direct loss of viability but the agent elicited a marked potentiation of the effect of 5 mM NaF (Fig. 3). The increase in apoptosis induced by 100 µM genistein in RINm5F cells was not altered by Ptx, suggesting that different mechanisms account for the actions of NaF and genistein. When RINm5F cells were exposed to Ptx and then incubated with both NaF and genistein together, the extent of apoptosis was much less than that caused by NaF alone in Ptx-treated cells (Fig. 3).

The responses of isolated human islets to these various incubation conditions were compared with those observed in RINm5F cells (Fig. 4) and it was revealed that, as
previously reported in rat islets (Loweth et al. 1996a), pretreatment with Ptx resulted in a significant potentiation of NaF-induced apoptosis. Genistein (100 µM) was still able to increase apoptosis in human islets exposed to Ptx and, by contrast with the situation in RINm5F cells, the combination of 5 mM NaF and 100 µM genistein also elicited a marked increase in apoptosis under these conditions (Fig. 4). However, when the concentration of genistein was reduced to 25 µM, the enhancement of NaF-mediated apoptosis by Ptx was entirely prevented (Fig. 5).

### Discussion

In each of the experimental preparations used in the present work (rat islets, human islets and RINm5F cells), exposure to either 5 mM NaF or 100 µM genistein caused a significant loss of cell viability. Annexin-V and carboxyfluorescein staining confirmed that this was due principally to an increase in apoptosis and revealed that the number of necrotic cells was increased to only a minor extent. The concordance of these results suggests that human islets, rat islets and RINm5F cells responded to treatment with 5 mM NaF or 100 µM genistein alone, in a broadly similar manner.

Previous studies have indicated that the ability of NaF to promote islet cell apoptosis is mediated, at least in part, by a G-protein-dependent mechanism since the effect is attenuated by the aluminium chelator, desferrioxamine, an agent known to deplete the levels of [aluminium tetrafluoride]− required for activation of G-proteins by NaF (Loweth et al. 1996a). This is consistent with other

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### Table 1 Effects of 5 mM NaF or/and 100 µM genistein on the viability of rat islet cells.

<table>
<thead>
<tr>
<th></th>
<th>Viable</th>
<th>Necrotic</th>
<th>Apoptotic</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>95 ± 1</td>
<td>1 ± 0 ± 2</td>
<td>0 ± 6 ± 05</td>
</tr>
<tr>
<td>NaF</td>
<td>78 ± 2*</td>
<td>1 ± 0 ± 3</td>
<td>21 ± 2**</td>
</tr>
<tr>
<td>Genistein</td>
<td>75 ± 1*</td>
<td>1 ± 0 ± 3</td>
<td>22 ± 2**</td>
</tr>
<tr>
<td>NaF + genistein</td>
<td>62 ± 3*</td>
<td>2 ± 0 ± 5</td>
<td>32 ± 3**</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.

isolated rat islets were exposed to 5 mM NaF or/and 100 µM genistein, as shown, for 24 h. After this time the islets were harvested, the cells dispersed with trypsin/EDTA and stained with annexin-V and deoxycarboxyfluorescein diacetate. The number of viable, necrotic and apoptotic cells was counted and expressed as a percentage of the total. *P<0.01 relative to untreated cells; **P<0.001 relative to untreated cells; ***P<0.005 relative to untreated cells; ###P<0.0005 relative to untreated cells.

### Table 2 Effects of 5 mM NaF or/and 25 µM genistein on the viability of human islet cells.

<table>
<thead>
<tr>
<th></th>
<th>Viable</th>
<th>Necrotic</th>
<th>Apoptotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>90 ± 1</td>
<td>50 ± 0 ± 5</td>
<td>3 ± 0 ± 5</td>
</tr>
<tr>
<td>NaF</td>
<td>84 ± 2*</td>
<td>60 ± 0 ± 7</td>
<td>9 ± 1 ± 0 §</td>
</tr>
<tr>
<td>Genistein</td>
<td>88 ± 2</td>
<td>60 ± 0 ± 6</td>
<td>4 ± 0 ± 5</td>
</tr>
<tr>
<td>NaF + genistein</td>
<td>91 ± 2</td>
<td>4 ± 0 ± 5</td>
<td>4 ± 0 ± 5 ***</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.

isolated human islets were exposed to 5 mM NaF or 25 µM genistein, as shown, for 24 h. After this time the islets were harvested, the cells dispersed with trypsin/EDTA and stained with annexin-V and deoxycarboxyfluorescein diacetate. The number of viable, necrotic and apoptotic cells was counted and expressed as a percentage of the total. *P<0.01 relative to untreated cells; **P<0.001 relative to untreated cells; ***P<0.005 relative to untreated cells.
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Figure 4 Effects of Ptx on apoptosis induced by 5 mM NaF and 100 µM genistein in human islets. Isolated human islets were exposed to 5 mM NaF or/and 100 µM genistein (Gen), as shown, for 24 h. After this time the islets were harvested and dispersed into single cells with trypsin/EDTA. The cells were stained with annexin-V and deoxycarboxyfluorescein diacetate and the number of apoptotic cells was counted and expressed as a proportion of the total cell number. *P<0.01 relative to NaF in the absence of Ptx. Values are means ± S.E.M.

Figure 5 Effects of Ptx on apoptosis induced by 5 mM NaF and 25 µM genistein in human islets. Isolated human islets were exposed to 5 mM NaF or/and 25 µM genistein (Gen), as shown, for 24 h. After this time the islets were harvested and dispersed into single cells with trypsin/EDTA. The cells were stained with annexin-V and deoxycarboxyfluorescein diacetate and the number of apoptotic cells was counted and expressed as a proportion of the total cell number. *P<0.05 relative to NaF in the absence of Ptx. **P<0.01 relative to NaF in the absence of genistein and Ptx, ***P<0.01 relative to NaF plus Ptx in the absence of genistein. Values are means ± S.E.M.

Indeed, evidence from other studies is consistent with this possibility (Mabley et al. 1997, Harrison et al. 1998, Zhang et al. 1998, Welsh et al. 2000). However, recent results have suggested that the pro-apoptotic effects of 100 µM genistein are more likely to be caused by inhibition of topoisomerase II than tyrosine kinase, since its effects are blocked by agents interacting with topoisomerase II (Elsea et al. 1997, Elliott et al. 2001). In confirmation of this, the present studies show that exposure of human islets to a lower concentration of genistein (25 µM) did not cause an increase in apoptosis despite the fact that genistein would be expected to inhibit tyrosine kinases under these conditions (Elliott et al. 2001). Hence, any deductions about the involvement of a genistein-sensitive tyrosine kinase in the maintenance of the basal level of viability in islet cells should be made with caution. By contrast, it may reasonably be concluded that the pro-apoptotic effect of NaF in both RINm5F cells and human islets involves the activation of a genistein-sensitive tyrosine kinase since, in both cases, the response to NaF was significantly attenuated in cells treated with 25 µM genistein. In an attempt to confirm this directly, we examined the extent of protein tyrosine phosphorylation under these conditions (with anti-phosphotyrosine antisera) but were not able to detect any specific changes. This may be because the critical substrate represents only a minor component of the total complement of tyrosine phosphorylated proteins within the β-cell.

In these respects, the effects seen in normal human islets were very similar to those found in RINm5F cells. However, when higher concentrations of genistein were employed in combination with 5 mM NaF, the responses of human islets and RINm5F cells diverged markedly. Whereas exposure of RINm5F cells to 5 mM NaF and 100 µM genistein led to attenuation of apoptosis, these agents caused an additional increase in the apoptotic death of human islet cells. This was not due to the species difference since normal rat islets responded in a similar manner to human islets. It seems likely, therefore, that the fully differentiated cells present in normal islets are more sensitive to the non-tyrosine kinase-mediated actions of genistein (e.g. inhibition of topoisomerase II) than the insulinoma-derived RINm5F cells. In support of this, the extent of apoptosis induced by exposure to 100 µM genistein alone was much greater in both rat and human islets (up to 25% of the total cell population within 24 h) than that found in cultures of RINm5F cells (<10% of the population in 24 h).

To clarify these issues further, both RINm5F cells and normal islets were treated with Ptx and the responses compared. Ptx has been shown to potentiate apoptosis in RINm5F cells exposed to a number of different agents (including NaF, interleukin-1β and chemical nitric oxide donors) suggesting that, in these cells, a Gi/Go-dependent pathway operates to limit the effectiveness of certain pro-apoptotic stimuli (Elliott et al. 2001). A similar

emerging evidence implicating G-protein-dependent mechanisms in the control of β-cell apoptosis (Kowluru & Morgan 2001) but does not exclude the possibility that other, less specific, effects of NaF may also contribute to the response.

Since genistein is a potent inhibitor of certain tyrosine kinase enzymes (Akiyama et al. 1987) the observation that 100 µM genistein promotes the apoptosis of primary islet cells might indicate that the regulation of β-cell survival involves the control of a critical tyrosine kinase activity.

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A high concentration of genistein (100 µM) increases apoptosis in RINm5F cells and normal islets. This response is attenuated by 25 µM genistein, suggesting that a Ptx-sensitive G-protein normally opposes the pro-apoptotic effects of tyrosine kinase activation. A lower concentration of genistein (25 µM; sufficient to inhibit tyrosine kinase activity but not topoisomerase II; Elliott et al. 2001) prevented the enhancement of apoptosis caused by NaF and Ptx in both human islets and RINm5F cells. This confirms that human and rat β-cells each express a genistein-sensitive tyrosine kinase that is pro-apoptotic and whose effects are subject to regulation by a Ptx-sensitive G-protein. In drawing this conclusion, it is also important to note that Ptx did not cause any loss of viability in the absence of an apoptotic stimulus in either human islets or RINm5F cells. This implies that the G1/G0-dependent anti-apoptotic pathway operates most effectively under conditions when apoptosis is actively promoted and that it is not required to maintain islet cell viability under resting conditions.

When considering the overall implications of the current results, it should be borne in mind that, unlike RINm5F cells, rat and human islets are not homogeneous populations of insulin-secreting β-cells. Hence, it is possible that variations in the responses of the individual cell types could have influenced the results obtained. However, this explanation is unlikely to account completely for the differences observed, since whole islets and RINm5F cells responded to NaF, genistein and Ptx in a broadly similar manner under most circumstances and only displayed markedly divergent responses when the stimuli were combined. This, in turn, suggests that the most striking differences occur in the mechanisms regulating aspects of “cross-talk” between the various pathways involved in the control of apoptosis in β-cells.

In conclusion, therefore, the results obtained in this work imply that, although there are a considerable number of similarities between the responses of RINm5F and normal islet cells to defined apoptotic stimuli, conditions exist under which significant differences are seen. The principal similarities and differences between the apoptotic responses to NaF, genistein and Ptx are summarised in Table 3. The results emphasise that conclusions drawn about the regulation of apoptosis in cultured RINm5F cells exposed to pharmacological agents should be confirmed with cells from normal islets before direct extrapolations are made from one to the other.

Acknowledgements

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References


Table 3 Similarities and differences between the apoptotic responses of RINm5F cells and normal islets to fluoride, genistein and Ptx

<table>
<thead>
<tr>
<th>Similarities between normal islets and RINm5F cells</th>
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<tr>
<td>Fluoride (5 mM) markedly increases apoptosis in RINm5F cells as well as in human and rat islets, consistent with the presence of a G-protein-dependent pathway regulating apoptosis.</td>
</tr>
<tr>
<td>Fluoride-induced apoptosis is attenuated by 25 µM genistein in both RINm5F cells and normal islets, implying the involvement of a tyrosine kinase in the pro-apoptotic action of fluoride.</td>
</tr>
<tr>
<td>Fluoride-induced apoptosis is enhanced by Ptx in both RINm5F cells and normal islets. This response is attenuated by 25 µM genistein, suggesting that a Ptx-sensitive G-protein normally opposes the pro-apoptotic effects of tyrosine kinase activation.</td>
</tr>
<tr>
<td>A high concentration of genistein (100 µM) increases apoptosis in RINm5F cells and normal islets. This is unlikely to be mediated by a change in the activity of a tyrosine kinase but probably reflects inhibition of topoisomerase II.</td>
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</table>

<table>
<thead>
<tr>
<th>Differences between normal islets and RIN5F cells</th>
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<tbody>
<tr>
<td>Upon exposure to 100 µM genistein alone, the proportion of cells entering apoptosis is much greater in islets than in RINm5F cells. This is consistent with a differential sensitivity to inhibition of topoisomerase II.</td>
</tr>
<tr>
<td>Fluoride-induced apoptosis is attenuated by 100 µM genistein in RINm5F cells but is further increased in rat and human islets.</td>
</tr>
<tr>
<td>The enhancement of fluoride-induced apoptosis by Ptx is blocked by 100 µM genistein in RINm5F cells but not in islets.</td>
</tr>
<tr>
<td>The differential responses to genistein seen in islets and RINm5F cells suggest that the interactions between tyrosine kinase- and topoisomerase II-mediated apoptotic pathways are quite different in clonal β-cells compared with normal islets.</td>
</tr>
</tbody>
</table>


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