

MAJOR histocompatibility complex (MHC) class II antigen expression has been implicated in the pathogenesis of autoimmune type 1 diabetes. In this study we examined the role of various cytokines that may induce MHC class II surface antigen expression, using the rat insulinoma line RIN-5AH as a pertinent model system. As in another study, the ability of IFN- γ to amplify MHC class II antigen expression 4-fold is demonstrated. At the same time we noted a 5-fold increase of these histocompatibility antigens by IL-6. Signal transduction analysis reveals that IL-6-induced MHC class II expression is specifically mediated by the G-protein system (activation of p21^{ras} by IL-6) since mevalonic acid lactone (a G-protein inhibitor) abolishes the action of IL-6. In contrast, IFN- γ , which does not activate p21^{ras}, is not inhibited by protein kinase C (PKC) inhibitors but by those of the G-protein pathway. This finding raises the possibility that IFN- γ induces RIN cells to secrete IL-6 (as shown previously, as well as in this paper) which, in turn, increases class II antigen expression via the G-protein pathway. This action may be unique to IL-6 or in synergy with IFN- γ . Other cytokines such as IL-1 α and β , and TNF- α induce a smaller increase in MHC class II antigens on RIN cells, and appear to activate both the G-protein and the PKC signal transduction pathways to varying degrees. Therefore, injury of pancreatic β -cells and possible induction of autoimmune type 1 diabetes via various cytokines may be caused by IL-6 or IFN- γ , or by their ability to induce MHC class II antigen upregulation.

Key words: Autoimmune diabetes, IFN- γ , IL-1, IL-6, MHC class II antigens, RIN-5AH cells, Signal transduction, TNF- α .

IL-6-mediated MHC class II induction on RIN-5AH insulinoma cells by IFN- γ occurs via the G-protein pathway

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Introduction

Cytokines are intercellular messengers that may mediate diverse actions on different cell types, or even on the same cell type at different cellular states.^{1–3} As such, they have been implicated in many aspects of cellular injury and repair. Thus, in many autoimmune diseases, cytokines are thought to play a pivotal role in perpetuating tissue injury that may be started by unknown environmental insults in genetically susceptible individuals.^{4,5} A whole set of pro-inflammatory cytokines (IL-1, IL-6, TNF- α) have been implicated in the damage to islet β -cells that leads to type 1 diabetes.^{6–12} The involvement of these and other cytokines in the evolution of disease in NOD mice and BB rats (two animal models of the disease) has been well documented.^{13,14} By contrast, nothing has been reported yet on cytokine expression in a number of human pancreases from diabetics who have died at disease

presentation.^{15,16} Furthermore, the action of these cytokines has been documented mostly on whole islet or islet cell preparations as well as whole pancreas preparations, but rarely on isolated β -cells.^{6–8} In order to delineate some of these actions *in vitro* and to study the precise signal transduction pathways by which these proteins mediate their effects, we have chosen the insulin producing rat insulinoma line RIN-5AH in confluent cultures. We have tested IL-1, IL-6, TNF- α and IFN- γ for their actions on this line and their mode of signal transduction.

Materials and Methods

Cells and cell culture: The RIN-5AH cells used in this study were provided by Dr Herbert Oie.^{17,18} This particular line is more β -cell-like, in that it secretes more insulin in response to high glucose, compared to the parental line RIN-5F.

Cells were grown in plastic culture flasks (Flow) in PRMI-1640 medium supplemented with 10% (v/v) foetal calf serum (FCS) and maintained at 37°C in 5% CO₂ atmosphere. Cells were seeded at 10⁵/ml in a total volume of 10 ml of medium and confluence was maintained by changing the medium every 3 days or whenever it was necessary. For surface staining the cells were grown in 12-well plates (Limbro) in 1 ml of medium.

Reagents: For our experiments rat recombinant IFN- γ was purchased from Holland Biotechnology, human IL-6 from R & D Systems, IL-1 α and β were gifts from Dr Steven Gillis of Immunex Corp., and TNF- α from NIBSC, UK. The concentrations used were 250 U/ml for IFN- γ , 1000 U/ml for IL-6, 12.5 U/ml for IL-1 α , 25 U/ml for IL-1 β and 10 U/ml for TNF- α .

Signal transduction experiments required the use of specific monoclonal antibody (MoAb). For this an anti-Pan PKC MoAb (Upstate Biotechnology) was used at a concentration of 1 μ g/ml and an anti-p21^{ras} (Oncogene Sciences) also at 1 μ g/ml. For the same experiments, mevalonic acid lactone (MEV, a G-protein inhibitor) (Sigma) was used at a concentration of 4 mM¹⁹ and sphingosine (SPH, a PKC inhibitor) (Sigma) at a concentration of 50 μ M.²⁰

Induction protocol: After the cells reached confluence, IFN- γ , IL-6, TNF- α and IL-1 α or IL-1 β were added to the cultures. At 48 h after cytokine administration the cells were examined for surface MHC class II antigen expression, or inner surface p21^{ras} and PKC activation.

Indirect immunofluorescence: RIN cells were directly processed for indirect immunofluorescence with mild trypsinization, resuspended in phosphate-buffered saline (PBS) and placed in 96-well plates for examination. For class II antigen expression, the cells were treated with F50 MoAb (class II-FITC conjugated) (Serotec) in 1-step experiments for 45 min at 4°C in the dark. After extensive washings positive cells were counted.

For PKC and p21^{ras} activation, the cells were incubated with 20% ice-cold methanol for 15 min to allow membrane permeabilization, as detection occurred at the inner surface membrane.²¹ Then test MoAbs (1 μ g/ml) were added and incubated for 45 min at 4°C. After washing three times with PBS-BSA-azide, FITC-conjugated goat anti-rat IgG Ab (Tago Inc) was added for another 45 min period at 4°C.

The cells were washed extensively, fixed with 25% glycerol and mounted on slides. Fluorescence was evaluated visually using a Zeiss

(Oberkichen, Germany) fluorescence microscope. Cells with weak or no staining at all were scored as negative. Positive cells were considered to be those showing bright, to very bright, staining.

IL-6 assays: IL-6 activity was measured by its proliferative action on a murine B cell hybridoma (7TD1). Briefly, 7TD1 cells were cultured on 96-well plates (3 \times 10³ cells/well/100 μ l) in RPMI-1640, 10% FCS. Test samples were given in serial dilution. After 72 h incubation at 37°C, cells were pulsed with 1 μ C/well of [³H]-TdRR (NEN) and nuclear incorporation was evaluated after 18 h in an LKB β -counter. The 7TD1 hybridoma is insensitive to IL-1 α , IL-1 β , IL-2, IFN- γ and TNF- α .²²

IL-6 activity was also evaluated by using a standard ELISA method described by Voller *et al.*²³ in which the optical density at 450 nm is determined.

Results

MHC class II antigen induction: We examined the ability of confluent RIN-5AH cells to express MHC class II histocompatibility antigens. Ten U/ml of hrTNF- α increased MHC class II antigen upregulation by 3-fold, while 12.5 ng/ml IL-1 α caused a non-significant increase. In contrast, IL-1 β at 25 ng/ml nearly doubled the density (\sim 30%) of these cell surface molecules. In addition, by employing 250 U/ml of rat recombinant IFN- γ and 1000 U/ml of human recombinant IL-6 (doses found to be non-toxic and sufficient for our experiments; data not shown) we noted that IFN- γ increased MHC class II antigen expression by 4-fold (12 \pm 2% *vs.* 45 \pm 5%), while IL-6 gave a 5-fold increase (12 \pm 2% *vs.* 61 \pm 6%) (Fig. 1) after 48 h treatment. It has been previously shown that IFN- γ increases MHC class II antigen expression on RIN5F cells.¹² It has been also shown that the same cytokine can augment IL-6 production.⁸ Our observations are consistent with our hypothesis that MHC class II antigen induction by IFN- γ could be due to IL-6 release, as reported previously. To investigate this possibility we examined whether IFN- γ induces RIN-5AH cells to secrete IL-6, as described below.

IL-6 production by RIN cells: A sensitive IL-6 bioassay that is mostly specific for this cytokine employing the 7TD1 mouse hybridoma cell line²⁰ was set up in our laboratory. 7TD1 cells are insensitive to IL-1 α , IL-1 β , IL-2, IFN- γ and TNF- α . They grow in the presence of low amounts of IL-6 and have been proven to be the best means of assaying IL-6 release.

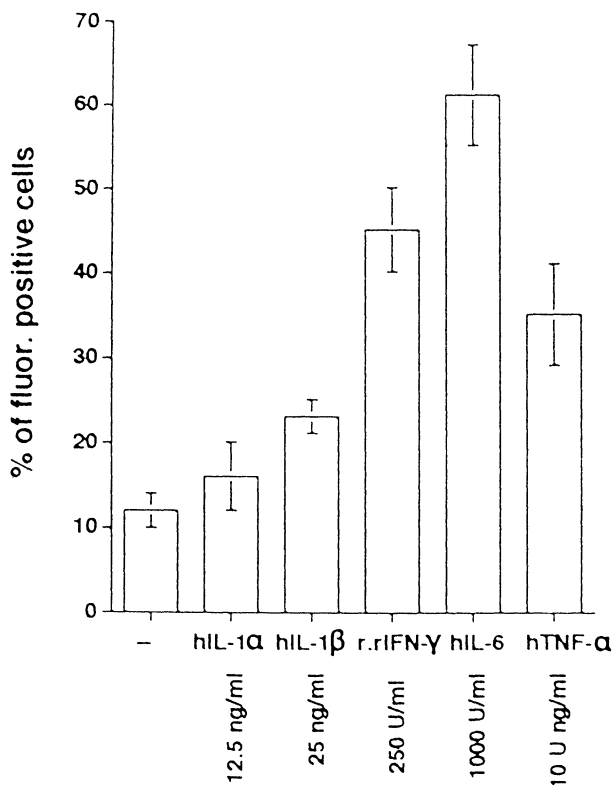


FIG. 1. Induction of MHC class II antigens on the surface of RIN-5AH cells after 48h treatment with specific recombinant cytokines, at the stated doses. Expression was monitored via a fluorescent monoclonal anti-rat class II antibody (see Materials and Methods).

Therefore, after incubating RIN-5AH cells with IFN- γ (250 U/ml) for 48h, supernatants were collected and tested on 7TD1 cells for cell growth (nuclear incorporation of [3 H]-TDR). As shown in Fig. 2, IFN- γ induced IL-6 production. Similar results were obtained by using ELISA (data not shown). Incubation of 7TD1 cells with supernatants of RIN-5AH cells exposed to IL-1 (α or β) or TNF- α showed no IL-6 induction (Fig. 2).

Signal transduction analysis: Once it became clear that MHC class II antigen upregulation may be an indirect effect of IFN- γ , we investigated whether IFN- γ and IL-6 would follow the same signal transduction pathway. If different pathways were to be found, this would mean that the effect of IFN- γ was direct. For this we tested two known pathways, that of PKC and that of G-proteins.^{19,20} The existence of specific pathway inhibitors offers the possibility of analysing such events. For the PKC pathway, we used sphingosine as the inhibitor, whilst for the G-protein pathway, we used mevalonic acid lactone.

To test for activation of the two pathways we employed commercially available specific monoclonal antibodies (pan-PKC and p21^{ras} respec-

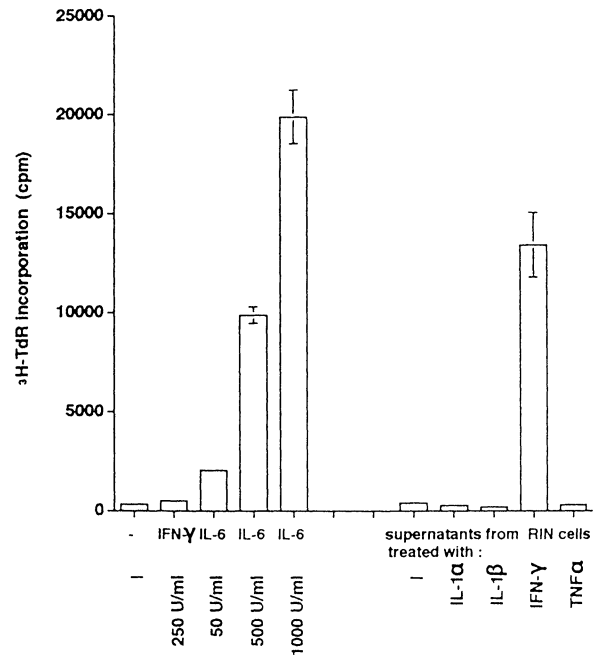


FIG. 2. Bioassay showing IL-6 production by RIN-5AH cells incubated with rIFN- γ . The cells used for the IL-6 bioassay were the 7TD1 cells.

tively). Fig. 3 shows that IL-6 activates inner surface membrane p21^{ras} in 100% of the cells examined, whereas IFN- γ activates only 6% of cells. Furthermore, IL-1 α induces p21^{ras} activation in ca. 25% of the cells (12 \times increase), IL-1 β does so in 15% of the cells and TNF- α in 23% of the cells. In contrast, IFN- γ activates PKC in 68 \pm 5% of the cells whereas IL-6 causes only 13% activation, and the other cytokines are in between (IL-1 α , 35%; IL-1 β 10%; TNF- α 20%) (Fig. 4). So, IL-6 and IFN- γ seem to follow two different signal transduction pathways. However, in order to conclusively arrive at the above statement one has to investigate their inhibition pattern. For this, we used mevalonic acid lactone (a G-protein inhibitor) and sphingosine (a PKC inhibitor) and monitored MHC class II antigen upregulation after IFN- γ and IL-6 treatments.

Fig. 5 shows that sphingosine is unable to block MHC class II antigen induction by either cytokine, whereas mevalonic acid lactone abolishes such events. These results indicate that although IFN- γ activates PKC (Fig. 4), MHC class II antigen expression is due to the subsequent production of IL-6, which in turn acts via the p21^{ras} pathway (Fig. 3). The PKC activity due to IFN- γ shown in Fig. 4 may be activating some other cellular (still unknown) components that are PKC-dependent. For MHC class II antigen induction, IFN- γ clearly induces IL-6 production, which in turn increases MHC class II antigen expression.

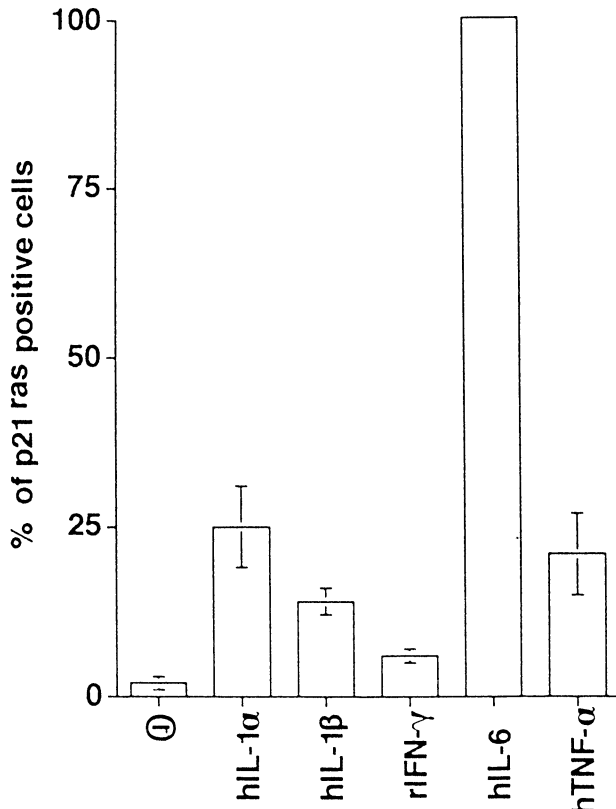


FIG. 3. Extent of activation of the p21^{ras} pathway by the various cytokines used in these experiments.

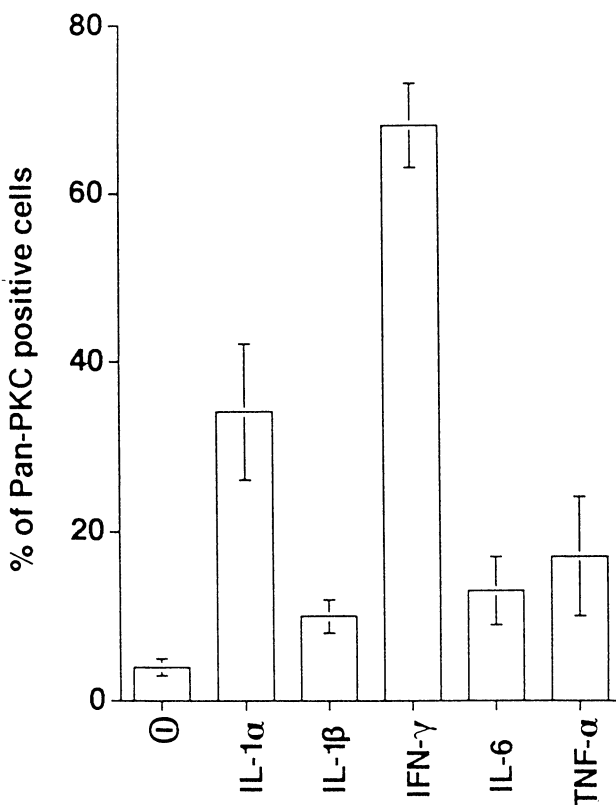


FIG. 4. Extent of activation of the PKC pathway by the various cytokines used in these experiments.

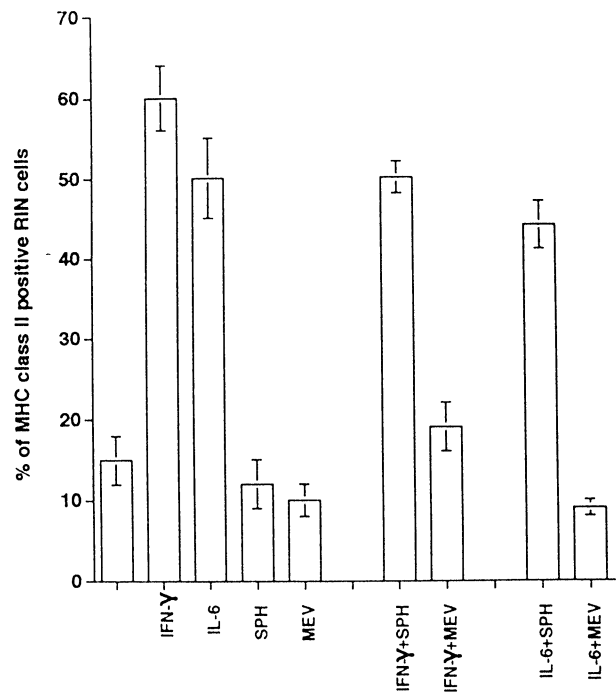


FIG. 5. Inhibition experiments on the expression of MHC class II by RIN-5AH cells after cytokine induction. The PKC inhibitor sphingosine was used at a final concentration of 50 μM. The p21^{ras} inhibitor mevalonic acid lactone was used at a final concentration of 5 mM. Cytokine levels were identical to those of Fig. 4.

Discussion

MHC class II antigen expression plays a pivotal role in the initiation of many immunological reactions, as cellular and humoral responses are MHC class II-dependent. Furthermore, numerous autoimmune states are associated with specific MHC class II loci, and more particularly with certain alleles within each locus.²⁴ In this work, autoimmune type 1 diabetes has been examined with respect to such antigen expression and precisely whether and how MHC class II antigen upregulation participates in the pathogenesis of the disease, since induction of these antigens has been found in β-cells at disease onset.¹⁵ We thus analysed the events taking place during the cytokine signal transduction leading to MHC class II antigen expression. Therefore, the MHC class II negative rat insulinoma cell line RIN-5AH has been used as a model system and various cytokines as inducers.

We found that IFN-γ is able to augment surface MHC class II antigen expression, a finding that has previously been suspected as a causative event for the disease.¹⁵ We, however, investigated whether the action of IFN-γ is direct, since other cytokines are also able to induce MHC class II antigen expression, namely IL-1 and

IL-6 (Fig. 1). Based on the only published report using the RIN model system, i.e. that IFN- γ acts as a regulator for IL-6 release,⁸ we inquired whether IL-6 alone or in synergy with IFN- γ upregulates MHC class II expression, and if so by which pathway(s). To this end we analysed the signals transduced by these two cytokines leading to MHC class II antigen induction. We found that IL-6 activates p21^{ras} and induces MHC class II antigen expression via the G-protein pathway since inhibitors of this route abolish such events. On the other hand, IFN- γ does not activate p21^{ras} but the PKC pathway before inducing class II antigens. Although one would expect inhibition of expression of MHC class II antigens by PKC inhibitors, in the case of IFN- γ it was demonstrated that only a G-protein inhibitor could reverse the action of this cytokine on MHC class II expression.

These results show that induction of MHC class II by IFN- γ and IL-6 are not separate phenomena and that MHC II antigen upregulation is IL-6 dependent and mediated by IFN- γ . It is not clear whether IL-6 alone or in synergy with IFN- γ induces MHC class II antigen expression, especially if we take into consideration the well described inductive capacity of IFN- γ in numerous cellular systems and its role in differentiation. In the RIN-5AH system IFN- γ does not show any antiproliferative effects (data not shown) and does not affect any cellular morphological aspects, as assessed by Giemsa staining (data not shown). Its only apparent function is the production of IL-6 (Fig. 2 and Reference 8), an action here not induced by IL-1 or TNF- α . IFN- γ is also known to enhance the production of IL-6 by endothelial cells *in vitro*,²⁵ and after systemic administration *in vivo*.²⁶

It is possible, however, for IL-6 to be produced by the RIN cells in an autocrine fashion. That is, IL-6 may be synthesized and consumed by the RIN cells very rapidly, a situation that could not be discerned by the systems of IL-6 detection employed here (ELISA assay and bioassay with 7TD1 cells). Regardless of the possible autocrine production in this manner however, our results are consistent with an augmentation of IL-6 levels by IFN- γ . In turn, IL-6 activation of the p21^{ras} pathway (Fig. 3) leads to MHC class II upregulation. As stated above IL-6, either alone or in combination with IFN- γ , does have an inductive action whose final result has been considered by some as a key intermediate in the pathogenesis of type 1 diabetes. Therefore, IL-6 is a new parameter to be investigated in this regard.

To our knowledge this is the first study to analyse signals induced by various cytokines in

this particular cell type. A recent study of the proteins induced by IL-1 in whole neonatal rat islets has revealed the induction and suppression of expression of several proteins after exposure to this cytokine, none of which was identified as a histocompatibility antigen.²⁷ We have also consistently detected the induction of various other proteins on the RIN-5AH clonal cell line by IL-1, TGF- β and their combination (unpublished results), and plan to do the same for IL-6 and IFN- γ . The effects of the latter cytokine have been investigated in depth primarily regarding its MHC class II-inducing ability on immunocytes,²⁸ and to a lesser extent on epithelial cells.²⁹ Although a few reports have shown a similar effect on endocrine cells of the thyroid and the pancreas (mostly the β -cells), there has been no follow-up to such studies.³⁰ In the present work the results are consistent with the use of the G-protein signal transduction pathway in the expression of MHC class II antigens induced by IFN- γ in the RIN-5AH line. IL-6 is also consistently detected in supernatants from such cells treated with IFN- γ , a fact that raises the possibility of IFN- γ upregulating MHC class II expression via IL-6 secretion. This matter requires further investigation by us concerning cause and effect, and experiments are now in progress.

In a study of pancreases from NOD mice with spontaneous autoimmune diabetes it was noted that the islet infiltrating T lymphocytes expressed TNF- α and granzyme A.³¹ A similar study in the other animal model of autoimmune diabetes, i.e. the BB rat, found expression of IL-1, IL-6 and TNF- α in infiltrating T lymphocytes at disease onset.³² The presence of IFN- γ has not been looked for in these or any other studies. By contrast, systemic administration of antibodies to IFN- γ prevents the appearance of the disease in both animal models.^{33,34} Surprisingly, prevention also occurs after systemic administration of TNF- α , IL-4 and IL-10 (reviewed in Reference 5).

Finally, the inductive potential of the other cytokines (IL-1, TNF- α) in regard to MHC class II antigen upregulation on RIN cells appears to be controlled by other mechanisms since their increase in MHC class II antigen expression is not affected by the inhibitors used in this study (data not shown). TNF- α induced MHC class II upregulation by 3-fold, an expression accompanied by simultaneous activation of the p21^{ras} (10-fold) and PKC (4-fold) pathways. By the same token, IL-1 α , although not significantly inducing MHC class II antigens on RIN cells, activates both pathways by 12- and 8-fold respectively. IL-1 β induces MHC class II (from 12 ± 2 to $23 \pm 2\%$ of the cells) and activates only the G-protein pathway (activation of p21^{ras} by 7-fold).

However, the G-protein inhibitor cannot counteract such action showing the existence of other intracellular routes involved in this process.

It is probable that intracellular cross-talk of different pathways mediates MHC class II expression, necessitating a more in-depth study of cytokine signal transduction, before the specific significance to disease pathogenesis can be evaluated. This cross-talk is already demonstrated by us here.

The participation of earlier events prior to G-protein activation seems possible and perhaps Jak- or Jak-like-kinases³⁵ may be responsible for initiating and directing the cascade of intracellular reactions leading not only to MHC class II antigen induction but also to the perpetuation of the set of reactions that may be part of the autoimmune state.

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Note

After this contribution was accepted, a new study on cytokine expression in the pancreata of four newly-diagnosed diabetic subjects that died on presentation appeared (Huang *et al*, *Diabetes* 1995; **44**: 658–664) showing invariably the expression of IFN- α in these pancreata, compared to control pancreata. Most other cytokines tested were either absent or gave no clear signal of presence exclusively in the diabetic pancreas.

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