Mixed Lymphocyte Islet Culture (MLIC) and its Use in Manipulation of Human Islet Alloimmunogenicity

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Introduction

Human islets have been transplanted clinically to reverse diabetes, though with poor overall success (reviewed in *Hering, Bretzel* and *Federlin* 1988). Inferring from experimental islet transplantation, it is presently suggested that methods for separating the endocrine from the exocrine pancreas portion and/or serological tissue manipulations prior to islet transplantation should be sought in order to reduce the graft's immunogenicity and thus improve the clinical results (*Gray* and *Morris* 1987; *Gray* 1989). In addition to physical separation procedures, anti-MHC class II (pre)treatment of the graft appears to remain an attractive concept, though thus far insufficiently controlled and with controversial success in vivo (*Faustman, Hauptfeld, Lacy* and *Davie* 1981; *Reece-Smith, McShane* and *Morris* 1984). This situation calls for suitable in vitro test systems to better define manipulative conditions and to help in predicting the in vivo outcome.

One approach toward predicting the result of an in vitro manipulation prior to islet transplantation appears to be the mixed lymphocyte islet culture (MLIC), derived from the one-way mixed lymphocyte culture (MLC) which has been well established to serve this purpose, e.g., in clinical bone marrow transplantation. A few attempts using isolated islets of Langerhans as stimulator population have been reported for the canine (*Roth, Russell, Fuller, Kyriakides, Mintz* and *Miller* 1984), and more recently also for the mouse (*Stock, Asher, Kaufmann* and *Sutherland* 1987) and the rat (*Shizuru, Ramakrishnan, Hunt, Merrell* and *Fathman* 1986; *Lloyd, Cotler, Letai, Stuart* and *Thistletwaite* 1989) systems. However, no data are available thus far for the human system using collagenase-digested islets as stimulator cells. Thus, it was our aim to adapt the MLIC to human islets and to perform a variety of anti-MHC class II (pre)treatment protocols.

Materials and Methods

Human islet isolation

Islets were isolated from HLA-typed cadaver donor pancreata with the collagenase/Ficoll technique of *Gray, McShane* and *Morris* (1984). In good concordance with their observations, the final endocrine tissue content ranged between 10–40% with an islet size variation in the order of approx. 1:5. The viability of the preparation was estimated with the trypan blue dye exclusion test and was usually >95%. Islets were then transferred into a freeze medium (10% dimethylsulfoxide, DMSO, 40% RPMI medium with 5% fetal calf serum, FCS, 50% pure FCS), frozen at –80 °C for 24 h, transferred to liquid nitrogen and stored until further use.

Islet identification

Isolated islets were identified from exocrine tissue debris with the help of the zinc-specific dithizone (diphenylthiocarbazone, DTZ, Sigma Chemie, München) staining technique (*Latif, Noel* and *Alejandro* 1988).

Complementary to the 10–40% endocrine tissue content, as evaluated with the DTZ staining technique, the exocrine tissue content comprised 90–60% of the unpurified islet preparations. For the experiments referred to in this study, the only preparations used for stimulator populations were those that had an exocrine content in the range of 60–75%, i.e., preparations and (see below). Preparation differed from the others in that the degree of enzymatic digestion was high, i.e., the islets were practically free of an exocrine rim (whereas the majority of the islets from the other 3 preparations were surrounded by an exocrine rim as a consequence of enzymatic underdigestion).

Preparation of crude stimulator islets

A crude stimulator islet preparation was obtained from the deep-frozen preparations mentioned above by quick thawing at 37 °C and repeated washings at 500 rpm/10 sec. The final pellet was resuspended 1:2 in complete RPMI (10% human A/B serum, 1% penicillin/streptomycin and 1% L-glutamin). Cell viability was estimated with the trypan blue dye exclusion test and was >90%. Thereafter the crude islet preparation was gently treated with mitomycin C (Sigma chemie, München; 12.5 µg mitomycin C in 1 ml RPMI for about 1.000 islets, 20 min at 37 °C in a water bath) to avoid proliferation of the nonhormonal cells. After careful washing, the crude preparation was diluted with complete RPMI until 100 µl contained approximately 40 islets.

Preparation of responder lymphocytes

Peripheral human blood lymphocytes were isolated from heparinized blood of HLA-typed healthy individuals by density gradient centrifugation. They were washed 3 × at 1.200 rpm in complete RPMI and used at a concentration of 10^7 cells/ml.

Mixed lymphocyte islet culture

10^6 peripheral blood responder lymphocytes from healthy individuals in 100 µl complete RPMI were co-cultured in quadruplicate with approximately 40 crude stimulator islets in 100 µl complete RPMI in a 96-well microtiter culture plate/F-form (Greiner, Nürtingen) for 5 days at 37 °C and 5% CO_2 in air. 16 h prior to cell harvesting at day +6, 1 mCi 3H-methyl-thymidine was added to each culture. The HLA pattern of the stimulator islet donor and the corresponding responder lymphocyte donor are indicated in Table 1.

Table 1 HL pattern of stimulator islet donor (stim) and corresponding responder lymphocyte donor (resp), as used in MLIC experiments.

		HLA pattern				
#10	stim:	A1,	A25,	B8,	—	DR3
	resp:	A2,	A26,	B27,	B38,	DR5
#15	stim:	A24,	—,	B37,	—,	DR1
	resp:	A2,	A11,	B35,	B51,	DR4
#17	stim:	A1,	A3,	B44,	—,	DR7
	resp:	A1,	A3,	B35,	B37,	DR1
#18	stim:	A2,	A24,	B7,	B27,	DR2
	resp:	A2,	A11,	B35,	B51,	DR4

= number of crude islet preparation as inducated in Material and Methods

Inhibition of MLIC by monoclonal MHC class II antibodies TÜ39 and 1aB3

MLIC inhibition experiments were performed with the two HLA-DR/DP-specific monoclonal antibodies (TÜ39 (clonab 39 from Biotest, Frankfurt) and 1aB3 of our own production (*Harpprecht* 1990). Since both antibodies reacted identically in the various morphological and functional tests, they are referred to as anti-MHC class II antibodies. TÜ39 (ascites) was used diluted 1:40 with phosphate-buffered saline (PBS), 1aB3 (culture supernatant) was used undiluted. For preincubation studies, either 5 × 10^7 responder lymphocytes or 1000 crude islets were incubated with 1000 µl anti-MHC II for 40 min/24 °C. Careful repeated washing ensured that no free antibody contaminated the culture medium. In another set of experiments 40 ml anti-MHC II were added permanently to the culture system. The anti-rat MHC class II specific monoclonal antibody MRC-OX17 (diluted 1:40 with PBS) was used as a specificity control.

Results

Variation of crude stimulator islet preparation

To determine the number of crude islets required, increasing numbers, ranging from 5–50, of HLA-mismatched crude stimulator islets (prep. #10, #17 or #18) were added to 10^6 peripheral blood responder lymphocytes. Fig. 1 shows the result of this experiment representatively with crude islet preparation #10. Whereas 5 and 10 crude islets per well did not elicit a significant immune response beyond background reactivity (stimulator index, SI=0.0 and 0.24), a continuous increase occurred between 20 and 40 crude islets (SI=3.0/20 isl.; SI=4.3/30 isl; SI=7.1/40 isl). With as many as 50 crude stimulator islets/well, however, 3H-thymidine uptake began to decrease again (SI=5.6). Crude islet preparations #17

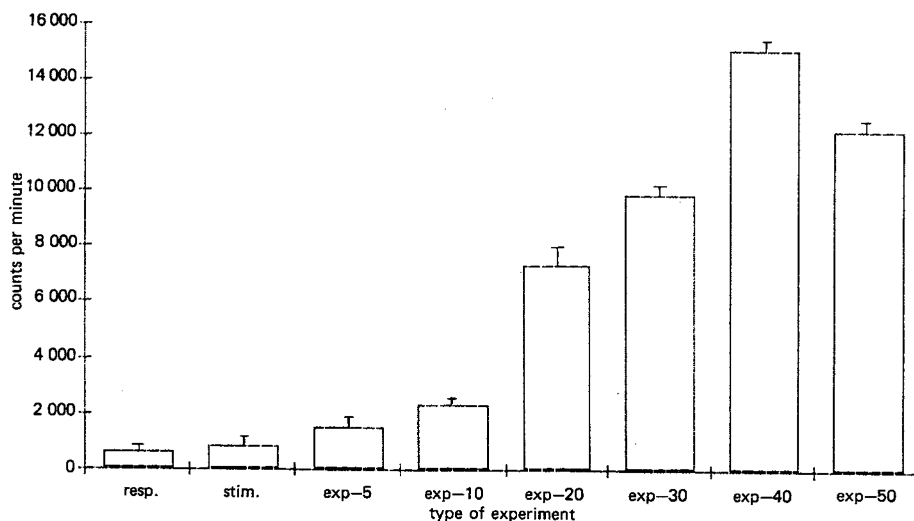

Fig. 1 Human mixed lymphocyte islet culture (MLIC) experiments (exp) with varying numbers (5–50) of HLA-mismatched, mitomycin C-pretreated, crude islet preparations (prep. #10) acting as stimulator cells to 10^6 responder lymphocytes; controls: responder lymphocytes (resp) + medium and crude stimulator islets (stim) + medium; no. of identical experiments = 4.

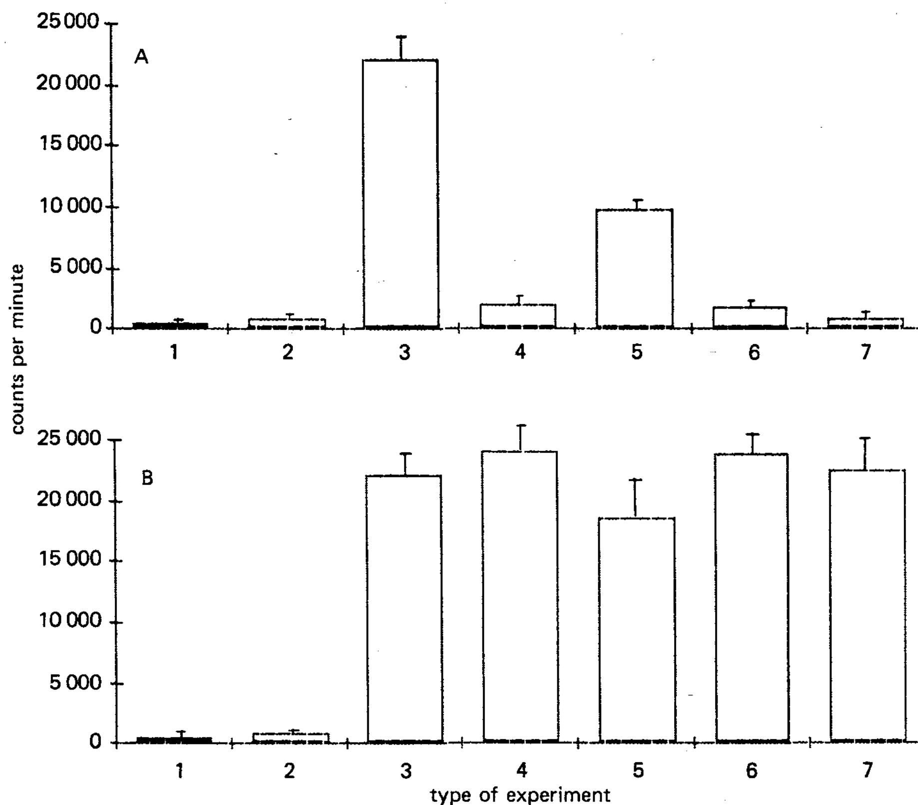

Fig. 2 Inhibition of MLIC reactivity (A) with HLA-DR/DP monoclonal antibody and (B), as a specificity control, with rat MHC class II monoclonal antibody: column 1, responder lymphocytes + medium; col. 2, crude stimulator islets + medium; col. 3, uninhibited allogeneic response; col. 4–col. 7, allogeneic response after varying (pre)treatment protocols (col. 4, pretreatment of responder cells; col. 5, pretreatment of crude stimulator islets; col. 6, pretreatment of both; col. 7, permanent presence of the antibody during culture period). The figure shows the representative results of crude islet preparation #10.

and #18 reacted in a similar fashion.

Inhibition of MLIC response by MHC class II monoclonal antibodies

Having shown that the MLIC test system allowed us to analyze the immunogenicity of the crude human islet graft in vitro, the major manipulative effort to diminish immunogenicity consisted in treating the MLIC components with MHC class II monoclonal antibodies. Four different approaches were chosen, using crude islet preparations and (a) pretreatment of the stimulators, (b) pretreatment of the responders, (c) pretreatment of both stimulators and responders and (d) permanent presence of the antibody during the culture period. Repeated and thorough washings after the preincubation period ensured that no free antibody contaminated the medium during the coculture period. The results of this experiment are shown in Fig. 2A representatively for crude islet preparation #10. Of the four different approaches, (a) had the poorest effect, showing only 58% inhibition (compare column 5 with col. 3). Comparatively more effective was approach (b), pretreatment of the responder cells, with 96% inhibition (compare col. 4 with col. 3). As to be expected, approach (c), pretreatment of both responder cells and crude stimulator islets, led to almost identical results, namely 97% inhibition (compare col. 6 with col. 3). Similarly effective was approach (d) with 100% inhibition when the antibody remained within the culture system until cell harvesting (compare col. 7 with col. 3). Experiments with crude islet preparation led to nearly identical results.

To prove the specificity of the inhibition, a control experiment was carried out with the HLA-DP/DR non-crossreactive mouse anti-rat MHC class II antibody MRC-OX17. As shown in Fig. 2B, of all treatment protocols (col. 4–7), only preincubation of the crude stimulator islets with MRC-OX17 had a mild, but nonsignificant inhibitory effect(col. 5) which remained unidentifiable when islets and responder cells were pretreated at the same time.

Influence of the exocrine tissue content on islet immunogenicity

Since the previous experiments were performed with crude islet preparations, i.e., islets contaminated by 60–75% of exocrine tissue fragments, either free or still attached to the islets as a consequence of enzymatic underdigestion, it was of particular interest to analyze the stimulator capacity of purified islets. For this purpose crude islet preparation was useful as the stimulator population. Purified islets were obtained by handpicking from the crude islet preparation described in Material and Methods, a procedure acceptable for experimental, though not for clinical purposes. The results of this study with 40 islets per experiment are demonstrated in Fig. 3. Compared to the unpurified islets (*Gray* 1989; *Faustman* et al.

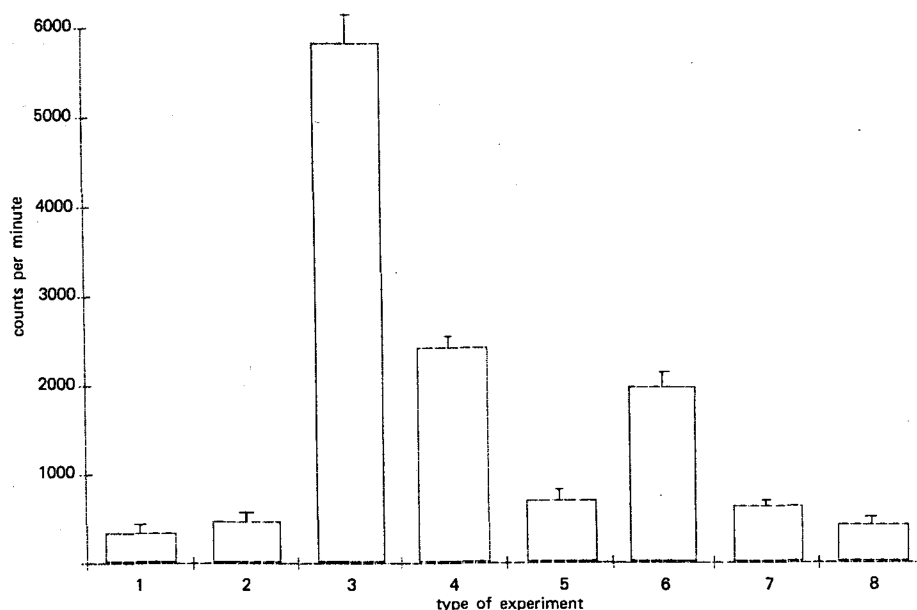

Fig. 3 MLIC with unpurified (column 3–5) and purified (col. 6–8) stimulator islets of crude preparation #15. Purification was performed by additional handpicking. Col. 1, responder lymphocytes + medium; col. 2, crude or purified stimulator islets + medium; col. 3 and 6, uninhibited allogeneic response; col. 4 and 7, pretreatment of crude or purified stimulator islets with anti HLA-DR/DP monoclonal antibody; col. 5 and 8, permanent presence of the antibody during culture period.

1981; *Reece-Smith* et al. 1984), purified islets (*Roth* et al. 1984; *Stock* et al. 1987; *Shizuru* et al. 1986) are significantly less immunogenic and, without further treatment, elicit only 32% of the original immune response (compare *Gray* 1989 with *Roth* et al. 1984). In other words, with regard to islet preparation the exocrine tissue portion contributes 68% of the islet immunogenicity. Having thus decreased islet immunogenicity by a separation procedure in a first step, antibody pretreatment (anti-MHC class II) of the stimulator islets now reduces it to background reactivity (compare *Faustman* et al. 1981 with *Stock* et al. 1987). As to be expected from the previous experiments shown in Fig. 2, permanent presence of the antibody in the culture medium has a similar effect on both types of incubation protocols since reactivity was already at background when the unpurified preparation was used (compare *Reece-Smith* et al. 1984 with *Shizuru* et al. 1986).

Discussion

To summarize the results, we were able to show that the immunogenicity of isolated crude human islet preparations (a) can be effectively measured in vitro with the MLIC and (b) can be inhibited by MHC class II antibody treatment protocols in connection with physical purification procedures. It may be pointed out that we were interested in the immunogenicity of the islet preparations under study rather than endocrinological parameters.

Studies with the MLIC, using normal crude human islet preparations, are reported here for the first time. However, the modification of the MLC into MLIC in the human system was suggested by previous experiments by other authors with canine, rat and mouse islets (*Roth* et al. 1984; *Stock* et al. 1987; *Shizuru* et al. 1986; *Lloyd* et al. 1989) as well as our own successful trials with rat islets (data under publication). But, unlike rodent islets, human islets are difficult to identify within a pancreas digest preparation. Therefore, the exact numbers of crude stimulator islets and, correspondingly, the immune response per well of the microtiter plate varied greatly and led to unsatisfactory results in earlier trials. This problem was easily solved with the DTZ staining technique, which is islet-specific because of the Zinc content of islet cells. It is a simple technique, can be applied to islet preparations in vitro, and apparently is not species-restricted, as similarly positive tests with human, rat and mouse islets show (*Latif* et al. 1988 and unpublished data from our laboratory). Thus, the immunogenicity of defined crude stimulator islet preparations can now be investigated.

The optimum number of about 40 islets in a crude stimulator preparation per well remains clearly below the approximately 50–100 islets used for canine and rodent MLIC (*Roth* et al. 1984; *Stock* et al. 1987; *Shizuru* et al. 1986; *Lloyd* et al. 1989 and our own observations with rats). This may be explained by the fact that, different from handpicked well-digested animal stimulator islets, human islet preparations are still contaminated by exocrine tissue components which usually are transferred into the wells along with the islets and, according to the results reported in Fig. 3, contribute greatly to islet immunogenicity (*Gray* 1989). In this context it may be mentioned that we know from immunohistological studies (manuscript in preparation) that the exo-

crine tissue fragments contain many HLA-DR/DP-positive cells, far more than pure islets. Our present conclusion is that the immunogenicity of viable islet preparations decreases with increasing purity.

Even with improvements in the purification procedures to remove free exocrine tissue debris from crude islet digests, as they are presently under way (*Ricordi, Lacy* and *Scharp* 1989; *Warnock, Ellis, Cattral, Untch, Kneteman* and *Rajotte* 1989; *Winoto-Morbach, Ulrichs, Leyhausen* and *Müller-Ruchholtz* 1989), the immunogenicity of the islet graft is still greatly determined by the extent of the exocrine rim around underdigested islets. Therefore manipulation of islet immunogenicity with MHC class II antibodies appears to be an attractive, and, as our results suggest, successful concept for reducing the effect of present methodological shortcomings. Fig. 3 clearly indicates this, by showing a strong reduction or even abrogation of the allostimulatory capacity, depending on the kind of human islet preparation, following preincubation in such antibodies. This is in line with rat MLIC experiments with purified islets which show a complete reduction of alloimmunogenicity, leaving the hormone-secreting islet cells unaffected, when stimulator islets were pretreated with anti-Ia immunotoxin (*Shizuru* et al. 1986) or MHC class II antibodies and rabbit complement (*Lloyd* et al. 1989). How far this holds for in vivo conditions remains to be studied. It may well be envisaged that the latter require additional immunosuppressive treatment. Even so, from the in vitro studies we can conclude that a substantial decrease in graft immunogenicity should greatly facilitate graft acceptance and survival.

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Abbreviations

DMSO = dimethylsulfoxide
DTZ = diphenylthiocarbazone
FCS = fetal calf serum
RPMI = cell culture medium
MLC = mixed lymphocyte culture
MLIC = mixed lymphocyte islet culture

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