Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6947–6951, July 1995
Immunology

Prolonged survival of pancreatic islet allografts mediated by adenovirus immunoregulatory transgenes

(transplantation/diabetes/tumor necrosis factor/major histocompatibility complex)

SHIMON EFRAT*, GYORGY FEJER†‡, MICHAEL BROWNLEE§¶, AND MARSHALL S. HORWITZ†‖**

Departments of *Molecular Pharmacology, †Microbiology and Immunology, §Medicine, ¶Pathology, and ‖Pediatrics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

Communicated by Matthew D. Scharff, Albert Einstein College of Medicine, Bronx, NY, December 27, 1994

ABSTRACT **The adenovirus (Ad) early region 3 (E3) genes code for at least four proteins that inhibit the host immune responses mediated by cytotoxic T lymphocytes and tumor necrosis factor α. To evaluate the potential use of these immunoregulatory viral functions in facilitating allogeneic cell transplantation, the Ad E3 genes were expressed in pancreatic beta cells in transgenic mice under control of the rat insulin II promoter. Transgenic H-2$^{b/d}$ (C57BL/6 × BALB/c) islets, expressing the Ad E3 genes, remained viable for at least 94 days after transplantation under the kidney capsule of BALB/c (H-2^{d}) recipients. Nontransgenic H-2$^{b/d}$ control islets were rejected as anticipated between 14 and 28 days. Histological analysis of the transplanted transgenic islets revealed normal architecture. Immunohistochemical studies with antisera to islet hormones revealed the presence of both β and non-β islet cells, suggesting a propagation of the immunosuppressive effect of Ad proteins from β cells to other islet cells. The use of viral genes, which have evolved to regulate virus–host interactions, to immunosupress the antigenicity of donor transplant tissue suggests additional ways for prolonging allograft survival. In addition, these findings have implications for designing Ad vectors for gene therapy.**

The protein products of genes from the adenovirus (Ad) early transcription region 3 (E3) inhibit several different pathways of the host immune response that are probably involved in rejection of foreign tissue. One of these viral gene products from the Ad2 or Ad5 serotypes is the 19-kDa glycoprotein (gp19K), which has been shown to bind to the heavy chain of selected class I major histocompatibility complex (MHC) gene products and prevent transport of MHC out of the endoplasmic reticulum (1). Because of the decreased expression of class I MHC on the plasma membrane, the recognition of Ad-infected cells by cytotoxic T cells (CTL) is downregulated (1, 2). The same effects on the surface expression of class I MHC can be observed by stable transfection of the Ad gp19K genes in the absence of viral infection (3). Downregulation of class I MHC on the plasma membrane can be achieved also by deletion of the β_2-microglobulin (β_2m) or prevention of transport of the short peptide sequences that are two essential components of the assembled class I MHC (4–6). Recently, successful prolonged transplantation of allogeneic murine pancreatic islets has been achieved by inactivation of the β_2m gene by homologous recombination in embryonic stem cells and mating this trait to homozygosity in H-2^{b} donor islet cells (7, 8). β_2m-deficient islet cells express no class I MHC K^{b} antigen on their cell surface and only a small amount of MHC D^{b} (8). Treating human islet donor cells with F(ab')$_2$ antibodies against class I MHC has also been successful in prolonging xenograft transplants into mice (9).

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In addition to the gp19K effect on class I MHC, there are three other Ad2 E3 region polypeptides that may have immunoregulatory activity. A protein of 14.7-kDa (14.7K), or the combination of the 10.4-kDa and 14.5-kDa proteins (10.4K and 14.5K, respectively), can inhibit tumor necrosis factor α (TNF) cytolysis of Ad-infected cells (1, 10). Isolated 14.7K has been shown to antagonize the antiviral effects of TNF in a vaccinia model system (11). The 3.5-kb Ad E3 region codes for at least three more polypeptides, in addition to the four described above. Two additional open reading frames could code for other proteins, which have not yet been observed. All of the E3 polypeptides are produced from transcripts generated from a common viral promoter by alternative splicing (1, 10, 12) (Fig. 1).

For the experiments described in this report, the entire E3-coding region from human Ad2 was placed under control of the rat insulin promoter (RIP), and the construct was used to generate transgenic mice in which Ad E3 proteins would be expressed in pancreatic islet cells. These donor transgenic islets survived allogeneic transplantation under the renal capsule for >94 days. This observation suggests that the Ad2 E3 immunoregulatory gene products can be used to prolong the survival of grafts by downregulating specific recognition signals on the foreign tissue without immunosupressing the host.

MATERIALS AND METHODS

Generation of Transgenic Mice Containing the Adenovirus E3 Region. A fragment of Ad2 genomic DNA extending from the *Bsp*EI site at nt 27752 to the *Nde* I site at 31076 was placed under control of the rat insulin II promoter between the *Xba* I and *Sal* I sites in the pRIP-simian virus 40 T antigen (Tag) plasmid (13), by converting the 5′ and 3′ ends to *Nhe* I and *Sal* I linkers, respectively. The resulting construct is deleted of the simian virus 40 Tag sequence, which is replaced with the Ad E3 genomic DNA. This Ad fragment includes the native splice sites and polyadenylylation signals of the E3 genes (Fig. 1) (14, 15). The plasmid DNA was linearized with *Sal* I and microinjected into C57BL/6 × DBA/2/F2 (B6D2/F2) one-cell embryos. Eight founder RIP-E3 animals were generated, of which lineage no. 7, containing ≈35 copies of the transgene and expressing the highest amount of mRNA for the gp19K protein, was studied in detail. Transgenic mice were identified by Southern blotting analysis of tail DNA.

Transplantation of Pancreatic Islets into Allogeneic Recipients. The RIP-E3 B6D2/F2 transgenic founder was back-

Abbreviations: Ad, adenovirus; TNF, tumor necrosis factor α; MHC, major histocompatibility complex; E3, adenovirus early region 3; RIP, rat insulin promoter; gp19K, 19-kDa glycoprotein; Ad 14.7K, 10.4K, and 14.5K, Ad 14.7-kDa, 10.4-kDa, and 14.5-kDa proteins, respectively; β_2m, β_2-microglobulin; Tag, simian virus 40 T antigen.

‡Present address: Department of Microbiology, Semmelweiss School of Medicine, Budapest, Hungary.
**To whom reprint requests should be addressed.

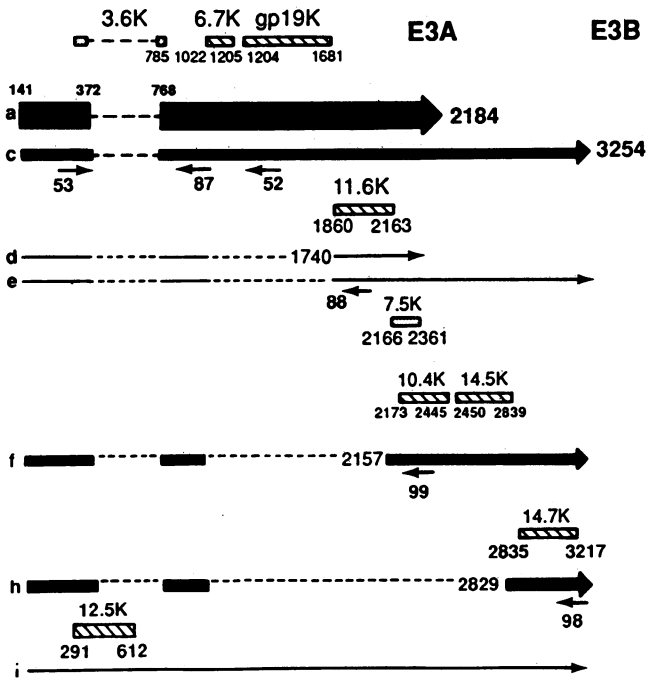

FIG. 1. E3 transcription map and open reading frames. Exons are designated by solid arrows, interrupted by dashed lines representing introns. The thickness of the lines represents the relative amounts of Ad mRNAs (*a–i*) as determined from productive Ad2 infection of KB cells (10). The short arrows represent oligonucleotides used in PCR reactions. The hatched rectangles represent identified proteins, while the stippled rectangles represent putative translational products not yet identified. The coordinates of the mRNAs and proteins are shown in relationship to the E3 transcriptional start site in infected cells. Ad genomic bp 27611 is equivalent to the first base of the E3 transcript. E3A and E3B designate the two polyadenylylation signals. This figure is modified and reprinted with permission from ref. 15 (copyright American Society for Microbiology), in which the sequences of all the PCR primers were reported.

crossed with C57BL/6 mice for two generations and bred to homozygosity at the RIP-E3 locus. A homozygous RIP-E3 H-2^b male was mated with a BALB/c female. The presence of the H-$2^{b/d}$ haplotype in the progeny was confirmed by PCR analysis, utilizing restriction endonuclease polymorphism based on published sequences for H-$2K^b$ and H-$2K^d$ (16). These mice served as islet donors. Islets were isolated by collagenase infusion of the pancreas through the common bile duct and were transplanted under the renal capsule in groups of 10–20 as described (17). At the indicated times after islet transplantation, the kidneys were removed, fixed in 4% (wt/vol) buffered formaldehyde, dehydrated, and embedded in paraffin. Five-micrometer sections were collected on gelatinized slides, deparaffinized, rehydrated, and stained with hematoxylin and eosin or with guinea pig anti-insulin (diluted 1:100) or rabbit anti-glucagon sera (diluted 1:500). The bound antibodies were visualized with horseradish peroxidase-conjugated second antibodies (18).

Reverse Transcriptase-PCR (RT-PCR). Total RNA was purified from ≈100 islets, digested with RNase-free DNase to remove any contaminating genomic DNA, and reverse transcribed using oligo(dT) priming (15). Each PCR reaction used 1% of the cDNA from one animal, 25 pmol of each of the indicated primers, 1 unit of *Taq* polymerase, 250 μM of each dNTP, 2.5 mM $MgCl_2$, 50 mM KCl, gelatin at 10 μg/ml, and 10 mM Tris (pH 8.3). The sample was incubated for 10 min at 80°C, followed by 35 cycles for 1 min each at 94°C, 60°C, and 72°C. The amplified DNA was visualized by electrophoresis in a 1.2% agarose gel containing ethidium bromide.

Detection of Adenovirus Proteins in Cells Containing the E3 Transgene. RIP-E3 mice were crossed with RIP–Tag mice (13), and an insulinoma cell line (βTC-E3) was derived from a double transgenic mouse as described (19). βTC-E3 cells (3×10^6) were labeled with 1 mCi [^{35}S]methionine (1 Ci = 37 GBq) for 5 hr in Dulbecco's modified Eagle's medium lacking methionine but containing 2% dialyzed fetal bovine serum; these cells were harvested from the monolayers, washed free of radiolabel, and lysed in a buffer containing Nonidet P-40, in the presence of protease inhibitors as described (15). Control βTC6 (20) cells without the E3 transgene were similarly processed. L cells were infected with Ad2 (16,000 virions per cell), incubated with cytosine arabinoside (20 μg/ml), labeled from 16 to 21 hr after infection, and processed identically to the βTC cells. Aliquots of the cell lysates (2.2×10^8 cpm for the βTC cells and 1.5×10^7 cpm for Ad2-infected L cells) were immunoprecipitated with an anti-gp19K monoclonal antibody (21) and protein A-Sepharose according to standard protocols. The immunoprecipitates were electrophoresed on 10% PAGE and visualized by autoradiography for 5 hr.

RESULTS

Adenovirus E3 mRNAs Are Expressed in the Islets of Transgenic Mice. Transgenic mice were generated that express the E3 genes in β cells under control of the RIP. The RIP-E3 transgene did not cause any obvious phenotype in the mice, as judged by histologic analysis of pancreas or blood glucose levels during the 24 mo of observation (data not shown). The presence of the various E3 mRNAs (Fig. 1) in the transgenic mouse islets was determined by RT-PCR analysis. Bands of appropriate size were detected by using the common sense primer no. 53 in all reactions, together with the antisense primer no. 87, which detected all E3 mRNAs, no. 52 for the gp19K, no. 88 for the 11.6-kDa protein, no. 99 for 10.4K, and no. 98 for the 14.7K (Fig. 2). The upper band visualized in Fig. 2, lane 2, is of the size predicted for the gp19K primers. The origin of the lower band, which appeared intermittently as an RT-PCR product, has not been further characterized. The doublet of bands for the 11.6-kDa protein (Fig. 2, lane 3) represents two alternate splice sites, one at nt 1740 (Fig. 1) and another previously undetected at nt 1792, which was confirmed by sequencing the cDNA (data not shown).

Generation of Immortalized Ad E3-Containing β Cells for Detection of Expressed Viral Proteins. To obtain an abundant source of β cells, the RIP-E3 mice were crossed with RIP-Tag mice, which express the Tag in β cells and develop insulinomas by 3 mo of age (13). Progeny expressing both transgenes were selected, and insulinomas that developed in the double-transgenic animals were cultured to derive β-cell lines. One of these lines, designated βTC-E3, was used to demonstrate the

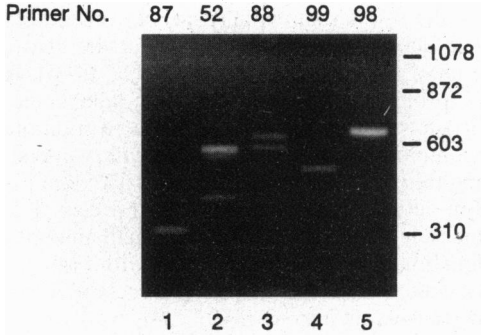

FIG. 2. RT-PCR analysis of E3 mRNAs expressed in RIP-E3 islets. Transgenic islet cDNA was amplified with paired primers no. 53, and each of the indicated antisense primers as described. Size markers are in bp.

presence of the Ad gp19K protein by radioactive labeling with [^{35}S]methionine and immunoprecipitation with a monoclonal antibody that recognizes the Ad gp19K protein (Fig. 3). A radioactive band, which appeared on autoradiographs from extracts of β cells containing the E3 transgene (Fig. 3, lane 2), was not present in a control β-cell line (βTC6) transformed with Tag but lacking the Ad E3 transgene (lane 3). This band was also detected in extracts of Ad2-infected L cells (lane 1). The lowest band in control lane 3 was present in immunoprecipitates of some preparations of pancreatic islets unrelated to the presence of the Ad2 E3 transgene.

RIP-E3 Gene Products Mediate the Long-Term Survival of Transplanted Islets. To assess the effect of the E3 proteins on allograft survival, RIP-E3 islets were transplanted under the renal capsule of allogeneic recipients. RIP-E3 islet donors were generated by crossing RIP-E3 (C57BL/6) mice with BALB/c mice (see *Materials and Methods*). The haplotype of each donor offspring was determined by PCR analysis (data not shown). Donor islets from RIP-E3 [C57BL/6 × BALB/c/F1 (H-2$^{b/d}$)] transgenic animals were transplanted into either BALB/c or C57BL/6 (B6) recipients. As controls, normal nontransgenic H-2$^{b/d}$ islets [either C57BL/6 × DBA/2/F1 (B6D2/F1) done concurrently or C57BL/6 × BALB/c/F1 (B6 × BALB/c/F1) done subsequently] were transplanted into BALB/c or C57BL/6 recipients. Islet survival was monitored by sacrificing the recipient mice and visualizing the donor islets under a dissecting microscope, followed by histologic and immunohistochemical analyses of kidney sections. Transgenic H-2$^{b/d}$ donor islets survived in two of three BALB/c mice dissected at 94 days, which was the last time point examined (Fig. 4). In contrast, nontransgenic C57BL/6 × BALB/c/F1 islets were rejected by BALB/c recipients between 21 and 28 days after transplantation, and nontransgenic B6D2/F1 islets were rejected by all BALB/c recipients between 14 and 21 days after transplantation (Fig. 4). Histologic analysis of the RIP-E3 islets at 91 days after transplantation into BALB/c mice showed no inflammatory response within the islets (Fig. 5*A*); however, mononuclear cells were observed near, but not infiltrating, the islets in some sections (Fig. 5*F*). The transplanted islets contained, in addition to β cells stained for insulin (Fig. 5*C*), α cells that stained for glucagon (Fig. 5*E*) and δ cells that stained for somatostatin (data not shown). In addition to cells that stain exclusively with the antibody to glucagon, there was a low level of diffuse staining throughout the islets that was not seen in the renal parenchyma. Analysis of the control nontransgenic B6D2/F1 islets transplanted into BALB/c recipients showed an intense mononuclear inflammatory response with islet infiltration at 14 days after transplantation (Fig. 5 *B* and *D*). In contrast to the transgenic donor islets that were accepted by BALB/c mice, islets from the same donor were rejected by C57BL/6 animals (Fig. 4), indicating that expression of the E3 transgenes protected against graft rejection in response to the H-2^{b} but not the H-2^{d}-associated antigens presented by these donor cells.

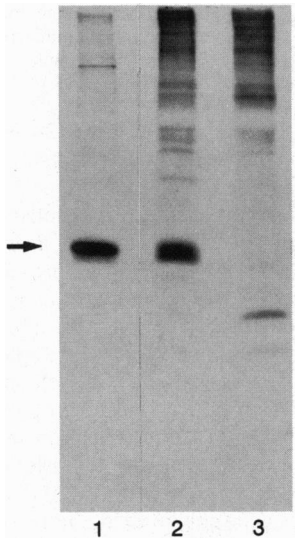

FIG. 3. Expression of the Ad2 gp19K protein in transgenic β cells. Cells were radiolabeled with [^{35}S]methionine, and the gp19K polypeptide was immunoprecipitated as described. Lanes: 1, Ad2-infected L cells; 2, βTC-E3 cells; 3, βTC6 cells. The arrow designates gp19K protein.

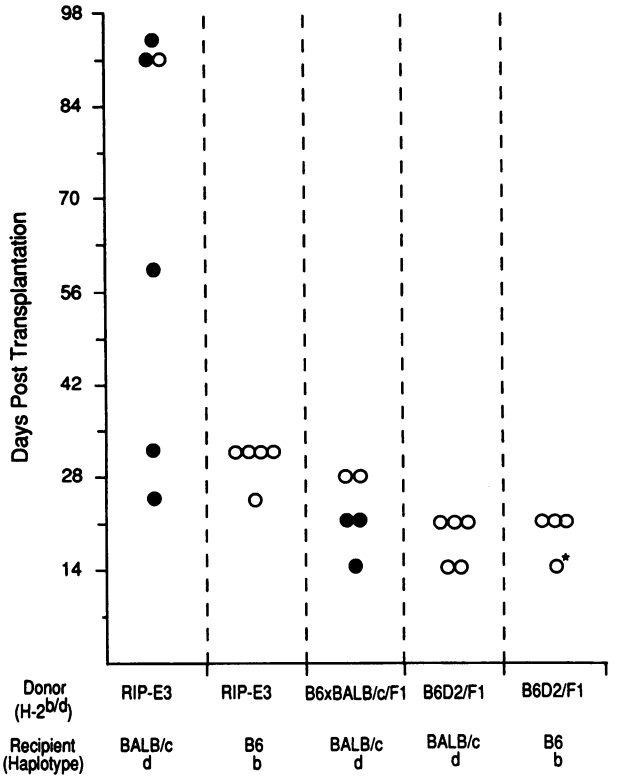

FIG. 4. Survival of RIP-E3 allogeneic islet transplants. Islets were isolated from the pancreas and transplanted under the renal capsule as described. ○, No islets found at dissection; therefore, these represent maximum times to reject transplanted islets. ●, Islets intact at time of dissection; therefore, these numbers are minimal estimates of transgenic islet survival. B6, C57BL/6; B6D2/F1, C57BL/6 × DBA/2/F1; RIP-E3, C57BL/6 × BALB/c/F1 containing the RIP-E3 transgene. *, Animal dissected during active rejection (see Fig. 5 *B* and *D*).

DISCUSSION

Our results demonstrate that expression of the Ad E3 genes in the donor cells prevents allograft rejection for >94 days in the absence of any systemic immunosuppression of the host. Further experiments are required to determine the contribution to successful allogeneic transplantation by each of the viral proteins encoded in the E3 region. Experiments with cultured cells transfected with the Ad gp19K gene have shown that the gp19K protein binds class I MHC heavy chains of different haplotypes with different affinities. For example, the Ad2 or Ad5 gp19K binds best to H-2D^{b} and -K^{d}, less well to H-2L^{d}, and poorly to H-2K^{b}, -D^{d}, -K^{k} and -D^{k} (22, 23). If H-2 binding to Ad gp19K *in vivo* is the same as in tissue culture experiments, the results suggest that H-2D^{b}, but not H-2K^{b}, is important in presentation of antigens in this islet-engraftment model. The observations that the grafts of H-2$^{b/d}$ islets into H-2^{d} mice were successful, whereas the same grafts were rejected by H-2^{b} mice, suggest a key role for the gp19K protein in the allograft

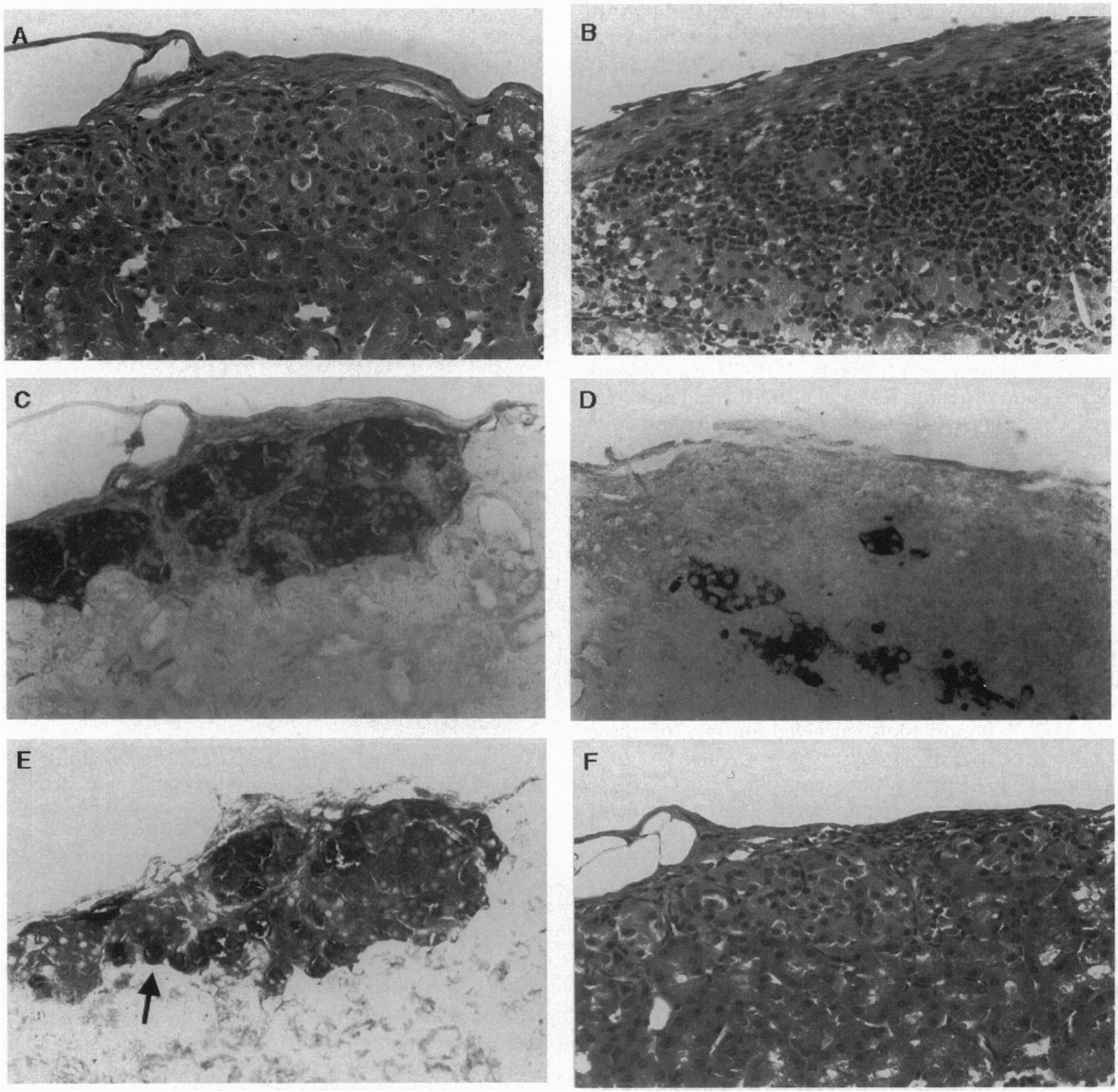

FIG. 5. Survival of allogeneic RIP-E3 islet transplants. Donor RIP-E3 transgenic islets (H-$2^{b/d}$) transplanted for 91 days under the renal capsule of BALB/c (H-2^{d}) recipients were stained with hematoxylin and eosin (*A*) or with antisera to insulin (*C*) or glucagon (*E*, see arrow for example of stained cell). Control B6D2/F1 (H-$2^{b/d}$) islets transplanted under the kidney capsule of BALB/c mice are shown at 14 days after transplantation (*B*), when infiltration with mononuclear cells was well developed, but a few β cells that contained insulin could still be detected (*D*). In another section of kidney transplanted for 91 days with transgenic islets, a thin rim of mononuclear cells between the capsule and the islets was observed (*F*). (× 213.)

survival. The essential role of class I MHC-associated antigen presentation in mediating allograft rejection has been demonstrated also by survival of islet allografts from donor mice deficient in β_2m (7), which is required for surface presentation of most class I MHC molecules. Similar to our own findings, mononuclear cells were observed at the periphery of β_2m-deficient islet allografts, without inflammatory infiltration into the islets. Apparently, mononuclear cells may be attracted to the transplant area but do not recognize the islets as targets for cytotoxicity.

There are conflicting reports about the role of TNF and anti-TNF antibodies in prolonging the engraftment of foreign tissue. Evidence has been presented that TNF infusion can prolong survival of islet xenografts from rats to mice (24); however, a systemic immunosuppressive effect on the recipient animal, rather than a local masking of immunogenicity of the donor cells, could be responsible for the latter results. In contrast, the levels of TNF have been observed to rise both preceeding and during allogeneic graft rejection in humans (25–27), and antibodies to TNF have been observed to prolong allogeneic cardiac transplants in mice (28–30). Expression of TNF in β cells in transgenic mice results in insulitis but does not progress to diabetes (31, 32).

The apparent survival of non-β islet cells suggests a role for a secreted viral product, which extends a protective effect to neighboring cells. The anti-TNF Ad E3 proteins (14.7K, 10.4K, and 14.5K) are not known to be secreted, although the latter two appear on the plasma membrane (10, 33). There are additional protein products of the E3 region (6.7 kDa and 12.5-kDa proteins proven to exist, as well as the 3.5-kDa and the 7.5-kDa proteins proposed but not yet detected), but their functions are not known. An alternative explanation that the Ad E3 transgene is expressed in non-β islet cells is less likely, based on previous experience with the RIP. Although RIP is active in immature cells coexpressing more than one hormone in developing islets, its expression postnatally was shown to be confined essentially to β cells (34). It has not been possible to distinguish by immunocytochemistry between these possibilities because of the high background levels of staining of normal islets with the monoclonal antibody to Ad gp19K.

The anti-TNF effect of Ad 14.7K does not inhibit all TNF functions. Ad 14.7K appears to affect TNF-induced cytolysis of sensitized cells but not to affect the TNF effects on transcription at sites such as the NF-kB cognate sequence (35). Ad 14.7K has been shown to inhibit the release of arachidonic acid by phospholipase A_2, thereby inhibiting a cascade of other inflammatory molecules that can result in apoptosis (36). Additional functions of 10.4K and 14.5K have been described (10). The 10.4K promotes the downregulation of the epidermal growth factor receptor on the cell surface in a process that may also involve the 14.5K (37, 38); other members of the protein kinase family of receptors, such as the insulin receptor and the insulin-like growth factor I receptor, are also downregulated (39). However, analysis of mutants of the 10.4K-encoding gene indicate that control of TNF and cell-surface expression of the tyrosine kinase receptors are separable (10).

The Ad5 gp19K binds to HLA molecules in human cells and downregulates class I MHC expression as it does in the mouse. There also appear to be differences in the affinity of gp19K binding to various HLA types, such that Ad gp19K binds well to A2.1 and B7 but poorly to Aw 68, B27, and BW58 (23). The effect of the Ad gp19K on class I MHC appears to be enhanced by the expression of Ad E1A in human cells (40). Unlike the β_2m gene-targeting approach, Ad E3 genes can be introduced as a dominant genetic alteration into primary human cells in culture or *in vivo* through viral vectors. Potential delivery systems include Ad itself, which is being studied as a vector for insertion of genes into human cells (41). The effects of the E3 region or its isolated genes on pathogenicity or persistence of the Ad vectors is poorly understood (10, 11, 42, 43). There is an emerging body of evidence that immunosuppression of the host response to Ad proteins by cyclosporine administration or the use of congenitally immunodeficient mice can prolong expression of the foreign gene (44). Whether the Ad E3 region itself can be sufficiently immunosuppressive of antigen presentation to prolong vector expression without increasing pathogenicity remains to be answered.

We acknowledge the expert technical assistance of Obaidullah Al Emran, David Fusco–DeMane, Ildiko Gyory, and Dinah Carroll; Kanakatte S. Raviprakash for developing the PCR technique for determining the H-2 haplotype; critical comments on the manuscript by Drs. Betty Diamond, Frank Lilly, and Stanley Nathenson; and grant support from The Juvenile Diabetes Foundation International.

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