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# Expression of perforin and granzyme B mRNA in judgement of immunosuppressive effect in rat liver transplantation *

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**Subject headings** liver transplantation; immunosuppression; perforin granzyme B genes; graft rejection

## Abstract

AIM **To explore the expression of perforin and granzyme B genes mRNA to judge the effect of immunosuppression in acute rejection of liver transplantation.**

METHODS **The expression of perforin and granzyme B genes mRNA was examined by reverse transcription-polymerase chain reaction (RT-PCR) in hamster to rat liver grafts under the immunosuppression of cyclosporine or/and splenectomy. Histological findings were studied comparatively.**

RESULTS **Cyclosporine could obviously decrease the cellular infiltration, and completely repress the expression of mRNA for perforin and granzyme B, but could not change severe hepatocyte necrosis and hemorrhage. Splenectomy could significantly lighten hepatocyte necrosis, and completely eliminate hemorrhage, but not affect the cellular infiltration and the expression of perforin and granzyme B genes mRNA. Cyclosporine or splenectomy alone could not prolong the survival time, however, their combination could completely repress the rejection of liver grafts. The survival time of animals were significantly prolonged (**37.1 **days). The architecture of hepatic lobules was preserved. There wes slight cellular infiltration in the portal tracts and no expression of perforin and granzyme B genes mRNA could been seen in three weeks after transplantation.**

CONCLUSION **Perforin and granzyme B genes are valuable in judging the effect of immunosuppression in liver transplantation.**

## INTRODUCTION

Rejection is one of major factors influencing the outcome of the patients after liver transplantation, and acute rejection is more harmful to the grafts and recipients. The cellular immunity has been proved to be a chief mechanism in rejecting liver transplantation, and cytotoxic T lymphocyte (CTL) is the major effector cell, the perforin lytic pathway to granzyme B, plays a critical role in the T-cell immun e response [1]. It is a hot issue of the moment to search for special early markers to judge the effect of immunosuppression in acute rejection of liver transplantation. In this study, the expression of perforin and granzyme B mRNA was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) under the immunosuppression of Cyclosporine (CsA) and splenectomy based on the establishment of a stable and reliable model of hamster to rat concordant xenogeneic orthotopic liver transplantation.

## MATERIALS AND METHODS

### Animals

Female golden hamsters weighing 150 g-180 g were the donors of liver xenografts, and male Wistar rats weighing 230 g-260 g were the recipients. The animals were purchased from Xi Bi Experimental Animal Centre and Shanghai Experimental Animal Centre of Chinese Academy of Sciences.

### *Liver transplantation*

Orthotopic liver transplantation was performed according to simplified three-cuff technoque[2] with some modifications. Donor cholecystectomy was perfo rmed at the time of cuff preparation, without reconstruction of hepatic artery, and splenectomy at the time of transplantation with simple ligation of the splenic hilum and excision. No microscope was

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used for all the operations.

### Experimental groups

Liver xenografts were studied in four groups: A,untreated controls ($n$ = 8); B, treated with cyclosporine 30mg/kg/daily ($n$ = 6); C,treated with splenectomy ($n$ = 6); D, treated with splenectomy and cyclosporine 30 mg • kg • day ($n$ = 7).

### Immunosuppressants

CsA was administered to recipients intramuscularly beginning on the first day of operation, at an interval of 12 hours. Splenectomy was done at the time of transplantation.

### Histology

Postoperative specimens at rejection and specimens taken at sacrifice were fixed in 10% formalin and stained with hematoxylin and eosin.

### RNA extraction

Total RNA was extracted according to the Qiagen kit directory. In brief, after homogenization and lysis with lytic buffer RLT in QIA shredder, same volume of 70% ethanol were added. Samples were then moved into RNeasy spin column and centrifuged for 15sec at 8 000 × g, buffer RW1 and buffer PRE were added and centrif uged for 15 sec at 8 000 × g step by step. Finally, RNA was eluted with diethylpyrocarbonate (DEPC)treated water and centrifuged for 1min at 8 000 × g. The approximate quantity of RNA was determined with an OD 260 nm and the purity was confirmed with an OD ratio of 260 : 280 to be greater than 1.8 in all spec imens. RNA was extracted from rat spenocytes stimulated in culture for 18 hours with PHA (10 mg/L), Con A (10 mg/L) and IL-2 (20 units/mL) for the positive control. The RNA of normal rat liver served as negative control.

### Reverse transcription and polymerase chain reaction

The cDNA synthesis was performed with GIBCOL BRL kit. RNA mixtures were prepared as follows: 2 μg of total RNA and 0.5 μg of oligo (dT) 12-18, were added with DEPC-treated water and diluted to 12 μL, water bathed at 70℃ for 10 min and incubated on ice for at least 1min, then added with 2 μL of 10 × PCR buffer, 2 μL of 25mM $MgCl_2$, 1 μL of 10mM dNTP, and 2 μL of 0.1M DTT and incubated at 42℃ for 5 min. 200U Super Script II RT was added and incubated at 42℃ for 50 min and the reaction was terminated at 70℃ for 15 min and chilled on ice, and finally 1 μL of Rnase H was added to each tube and incubated for 20 min at 37℃. The cDNA was stored at -20℃.

The DNA was amplified using RT-PCR on a Perkin-Elmer 2 400 thermocycler, and RT-PCR primer was designed according to the exon of rat gene sequences. The primer set for perforin was ① 5' GCCATCCTGCGTCTGGACCTG3', ② 5' CATTTGCGGTGCACG ATGGAG3'; primer set for the granzyme was ① GACTTTGTGCTGACTGCTGC TCAC3', ② 5' TTGTCCATAGGAGACGATGCCC GC3'; and for the β-actin: ① 5' TGCTAC ACTGCCA CT CGGTCA3', ② 5' GCATGCTCTGTGGAGCTGT TA3'[3]. The reaction mixture conta ined 2 μL cDNA, 1 μL of 10 mmol/L dNTP, 3 μL of 25 mmol/L $MgCl_2$, 5 μL of 10 × buffer, 1 μL Taq polymerase, 2 μL each of the forward and reverse primer, and 34 μL of dual-distilled water for each 50 μl amplification reaction. Reactions were performed for 30 cycles. The conditions were 94℃ for 3 min prior to cycling, denaturing at 95℃ for 15 sec, annealing at 60℃ for 20 sec and extension at 70℃ for 30 sec. Following amplification, ten μL PCR products were run on a 1.5% agarose gel stained with ethidium bromide, gene specific bands were visualized by phot ography under UV fluorescence.

## RESULTS

### Survival time

Graft survival is shown in Table 1. Groups A, B and C showed rejection in 6-9 days. Groups B and C had rejection with a time course similar to group A ($P$>0.05). Survival in group D was significantly prolonged to 37.1 ± 9.9 days ($P$>0.01).

**Table 1 Survival of hamster to rat liver transplantation**

| Groups | Therapy | Survival (days) | $\bar{x} \pm s$ |
|---|---|---|---|
| A | None | 6,7,7,7,7,7,7 | 6.9 ± 0.4 |
| B | CsA 30 mg • kg • day | 6,7,7,7,8,9 | 7.3 ± 1.0 |
| C | Splenectomy | 6,7,7,7,7,8 | 7.0 ± 0.6 |
| D | CsA 30mg • kg • day+splenectomy | 27,29,30,35,39,46,54 | 37.1 ± 9.9[a] |

[a]$P$<0.01 as compared with groups A, B and C.

### Histological examination

The liver xenografts in group A showed diffuse mononuclear cell infiltration, massive necrosis and interstitial hemorrhage (Figure 1A). In group B, CsA at dose of 30 mg · kg · day obviously decreased cellular infiltration, but severe hepatocyte necrosis and hemorrhage remained unchanged (Figure 1B). Splenectomy (group C) significantly alleviated hepatocyte necrosis and hemorrhage, but did not change diffuse mononuclear cell infiltration (Figure 1C). In group D, CsA and splenectomy abated cellular infiltration and hepatocyte necrosis and hemorrhage, the architecture of the hepatic lobule was preserved, but there was slight cellular infiltration in the portal tracts (Figure 1D).

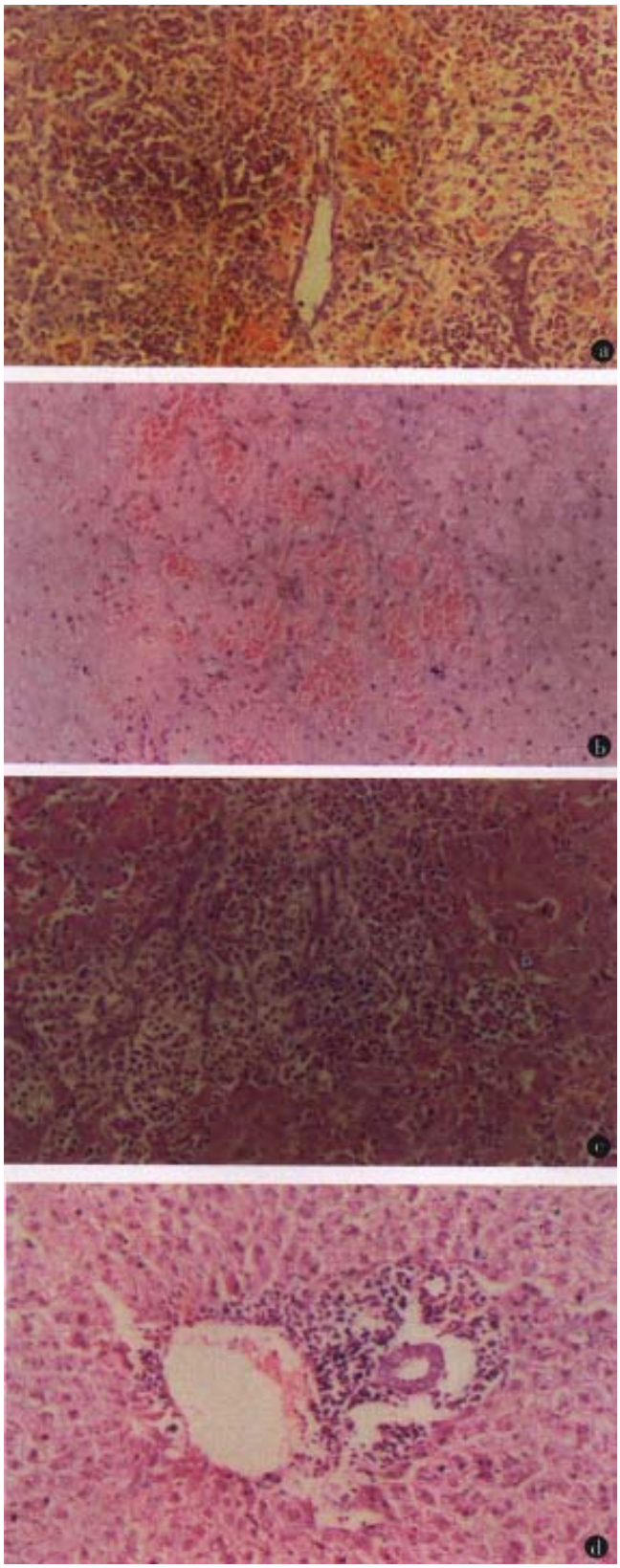

**Figure 1** Histology of liver grafts. (A) Diffuse mono nuclear cell infiltration, massive necrosis and interstitial hemorrhage; (B) Cel lular infiltration obviously decreased, but severe hepatocyte necrosis and hemor rhage unchanged; (C) Splenectomy significantly alleviated hepatocyte necrosis and hemorrhage, but didnot change diffus mononuclear cell infiltration; (D) The architecture of the hepatic lobule was preserved, but there was slight cellular in filtration in the portal tracts.

### *Expression of perforin and granzyme B genes mRNA*

All recipients had the expression of perforin and granzyme B genes mRNA in group A on post-transplantation day (POD) 5; only one (1/6) in group B expressed mRNA of these genes on POD 5; and five recipients (5/6) in group C expressed mRNA of both genes on POD 5. There was no expression of both genes in group D on P OD 5 and 14, but only one recipient (1/7) expressed mRNA of perforin and granz yme B genes on POD 21 (Figure 2).

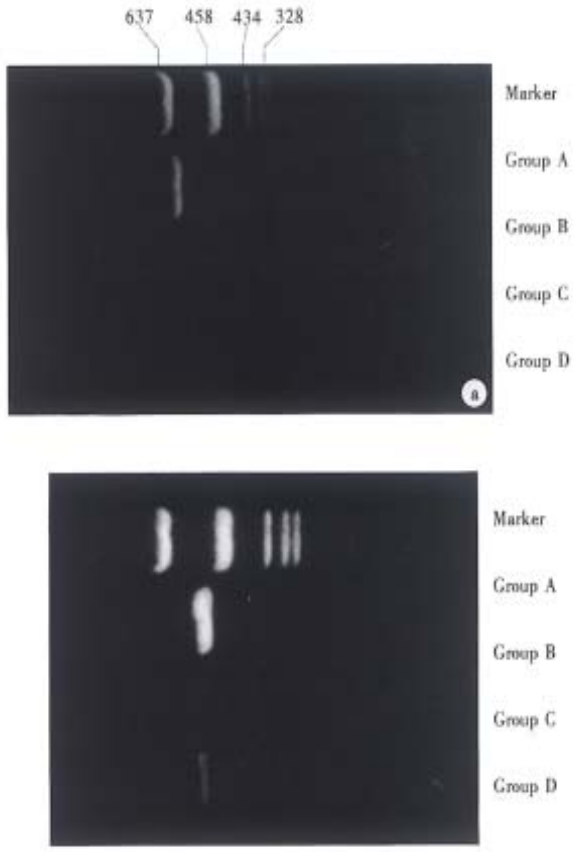


**Figure 2** Expression of perforin and granzyme B genes in liver xenografts on POD 7. A. Perforin; B. Granzyme B.

## DISCUSSION

Gold hamster to rat orthotopic liver transplantation is concordant and heterotra nsplantation and presents with acute rejection. The recipient's survival can not prolong until both cellular and humoral rejection are depressed due to its dua l-immune mechanism. Splenectomy can effectively inhibit antibody formation, obviously abated hepatocyte necrosis and hemorrhage, but is unable to improve the diffuse mononuclear cellular infiltration in the grafted liver. CsA can significantly decrease cellular infiltration, but can not improve hepatocyte necrosis and hemorrhage,

neither of them can prolong the recipient's survival when used alone, but they can inhibit both cellular and humoral rejection, normalize the architecture of the grafted liver, and significantly prolong the recipient's survival to 37.1 days when used in combination. The result is better than that reported abroad[4].

CTL is believed to play an important role in the mechanism of rejection, and effect mechanism of perforin and granzyme B, in spite of the regulatory and effect mechanism underlying the rejection process, remains incompletely understood. Effect of immunosuppression on the expression of perforin and granzyme B mRNA has become a hot topic in recent years. Mueller *et al*[5] analyzed the expression of perforin and granzyme A genes in situ hybridization in cellular infiltrates of MHC mismatched mouse heart transplants both in immunosuppressed recipients treated with CsA and untreated recipients. In untreated grafts, there were many perforin and granzyme A-expressing cells and heart transplants were completely rejected on POD 10. In contrast, CsA treatment significantly decreased the positive cells and prolonged survival of the transplants to 30 days. CsA did not obviously decrease infiltration of $CD8^+$ cells but significantly reduced the number of perforin and granzyme A-positive cells. It shows that CsA treatment mainly depressed the activation of CTL rather than decreased the number of infiltrating cells. Rapamycin can completely block the expression of granzyme B gene in infiltrating cells of grafts, obviously prolong the survival of grafts[6].

Our experimental results show that combined CsA and splenectomy could effectively depress rejection in hamster to rat orthotopic liver transplantation. The architecture of the hepatic lobule was undamaged, and the survival was significantly prolonged, and there were no expression of perforin and granzyme B genes mRNA.

In conclusion, expression of the CTL-associated gene perforin and granzyme B provides two valuable markers to judge the effect of immunosuppression in acute rejection of liver transplantation. But this should be further confirmed clinically.

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