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Human Anti-bovine Mixed Lymphocytes Reaction Is Weaker Than Allogeneic Reaction

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There have not been many studies on the human cellular response to bovine. In this study, we report the first evidence that the human cellular response to bovine is weaker than to allogeneic humans. Human peripheral mononuclear cells were cultured with inactivated bovine peripheral mononuclear cells. Allogeneic mixed lymphocyte reaction (MLR) and human anti-porcine MLR were compared with human anti-bovine MLR. In addition, human cytokine production against bovine was assessed by enzyme-linked immunoabsorbant assay (ELISA) of culture supernatant. The results of ELISA showed that human peripheral mononuclear cells produced preferentially Th1 deviated cytokines (INF-γ and TNF-α) upon stimulation by bovine peripheral mononuclear cells. But the magnitude of the cytokine production was lower than in the allogeneic combination. The examination of MLR showed that human anti-bovine MLR was significantly weaker than allogeneic MLR, and on the same level as the human anti-porcine MLR. These results suggest that the human cellular response to bovine is weaker than to allogeneic humans.

Introduction

Since swine have been considered potential xenogeneic donors, a large body of evidence on the human immune response to swine has been reported, including on the cellular immunity level[1)2)]. The cloning of a certain species of animals by nuclear transfer, however, has opened a new door to xenotransplantation[3)]. The technique of cloning animals has been extensively investigated in bovine rather than in swine, especially in Japan, and generating gene-disrupted animals by nuclear transfer is easier in bovine than in swine. We previously reported the possibility of using bovine as xenogeneic donors[4)].

Once hyperacute rejection (HAR) is overcome, cellular rejection will be encountered, and we therefore thought that it might be of value to elucidate the human cellular response against bovine. In this study, we report the results of mixed lymphocyte reaction (MLR) examined between humans and bovine.

Materials and Methods

The present study was performed with the permission of Tokyo Women's Medical University, Institute of Laboratory Animals (99-142).

Isolation of peripheral blood mononuclear cells

Heparinized human peripheral blood was ob-

tained from healthy blood donors. Bovine peripheral blood was obtained from the same line of bovine we were cloning. Porcine peripheral blood was obtained from the local slaughterhouse. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll density centrifugation. After harvesting the interface, residual red blood cells were lysed with ammonium chloride solution. Washing the PBMC three times with phosphate buffer saline (PBS), they were resuspended with AIM-V medium (Life Technologies, Rockville, MD). Human PBMC were used as responder cells. Allogeneic human PBMC (Group 1), porcine PBMC (Group 2) and bovine PBMC (Group 3) were used as stimulator cells.

Preparation of stimulator cells

For the preparation of stimulator cells, PBMC were resuspended in 5 ml of RMPI 1640 (Life Technologies), and incubated with mitomycin (250 μg/ml, MITOMYCIN Kyowa S, KYOWA Co, Tokyo). After 30-min incubation at 37 ℃, PBMC were washed 3 times with PBS and, finally, PBMC were resuspended in AIM-V medium.

Cell staining and flow cytometry

Approximately, 1×10^6 of responder cells were cultured with the equal number of stimulator cells. Flow cytometry was performed at the day-0 and day-5 of the in vitro culture. Human PBMC were stained as follows. Approximately, 5×10^5 PBMC were stained in 100 μl of staining buffer (Hank's balanced salt solution, 1% heat inactivated normal human serum, 0.1% NaN_3) containing saturating concentration of antibodies directly labeled with fluorescein isothiocyanate (FITC), or phycoerythrin (PE). Anti-human CD 3-FITC, CD14-FITC, CD19-PE, CD56-PE CD25-PE, HLA-class I antibodies were purchased from Pharmingen (San Diego, CA). The cells were stained for 30 min at 4 ℃, washed 3 times, and analyzed by flow cytometry on a Becton-Dickinson FACscan (Mountain View, CA). Propidium iodide was added to each samples (0.5 μg/sample) to exclude dead cells.

Cytokine analysis

Approximately, 5×10^5 of responder cells and stimulator cells were dissolved with 2 ml of AIM-V medium in 24-wells tissue culture plates. After 3 days of incubation at 37 ℃ under a 5% CO_2 atmosphere, 150 μl of culture supernatant was collected and transferred to 96-well tissue-culture plates. Measurement of human IL-2, INF-γ, TNF-α, IL-4 and TGF-β were carried out by ELISA using antibodies from according to the manufacture's recommendations. The results were compared with the results for human PBMC cultured with the same human PBMC (Auto).

^{3}H incorporation assay

Responder cells and stimulator cells were resuspended in AIM-V medium at a concentration of 1×10^6 cells/ml. Responder cells were cultured with an equal number of stimulator cells in a 96-wells flat bottom plates. The plates were incubated at 37 ℃ under a 5% CO_2 atmosphere for 5 days before pulsed with ^{3}H-thymidine (1μ Ci/well, NEN Dupont, Boston, MA). Proliferation was assayed by ^{3}H-thymidine incorporation measured by MATRIX™ 96 (PACKARD, Meriden, CT). The results of Group 1, 2 and 3 were compared with that of MLR of human PBMC which had been cultured with self-PBMC (Group 4).

Statistical analysis

Statistical analysis was performed using Graphpad Prism 2.01 software. ANOVA method was used for the calculation of the statistical significance. P-value less than 0.05 was accepted as a significant difference.

Results

Flow cytometry (Fig. 1)

As shown in Fig. 1, the frequency of CD3 positive cells of Group 3 increased from 40.3 ± 4.5% at day-0 to 61.6 ± 6.8% at day-5. The frequency of

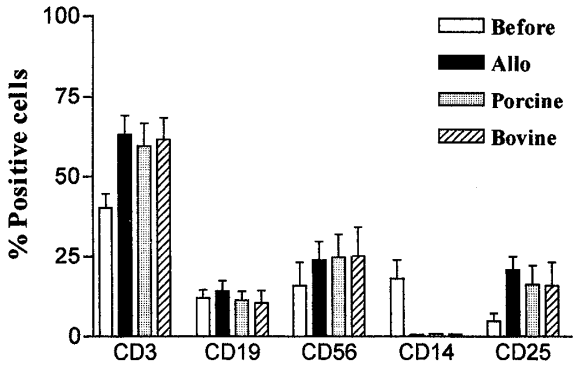

Fig. 1 Phenotypic change of human PBMC
Phenotypic changes of human PBMC before (white) and after co-culture with allogeneic human PBMC (black), porcine PBMC (dotted) and bovine PBMC (hutched) were analyzed with flow cytometry. Human $CD3^+$ T cells tended to express a higher level of CD25 after they were cultured with allogeneic human PBMC, than with xenogeneic bovine PBMC. Numbers were presented with mean ± SD.

CD19 positive cells did not change significantly. The frequency of CD56 positive cells rose from 15.9 ± 7.2 to 25.2 ± 8.9%. The increase in frequency of NK cells was also observed in Group 1 and Group 2. Although HLA class I of human blood donors was typed in all experiments, no correlation between class I disparities and the change in the frequency of NK cells was observed (data not shown). CD14 positive cells were not detected after in vitro stimulation. There were no significant differences in the phenotypic changes between Group 1, 2 and 3. In Group 3, the expression of CD25 was detected on the surface of 15.9 ± 7.2% of the CD3 positive T cells at day-5. Although the number of activated CD3 positive T cells was not statistically lower than that of Group 1, human CD3 positive T cells tended to express higher levels of CD25 in Group 1 than in Group 3.

Cytokine analyses (Fig. 2)

Fig. 2 shows the results of the cytokine production of human PBMC, which had been stimulated with allogeneic and xenogeneic stimulator cells. Although there were no significant differences in the production of IL-2, IL-4, TGF-β between Group 1, 2 and 3, the production of INF-γ and TNFα was significantly higher in Group 1 (109.0 ± 23.9 IU/ml) than in Group 2 or Group 3 (56.1 ± 4.7 and 1.6 ± 0.3 IU/ml, respectively, $p<0.05$).

Human anti-bovine MLR (Fig. 3)

As shown in Fig. 3, anti-bovine MLR (Group 3) was significantly weaker than the allogeneic MLR (Group 1). There was no significant difference between anti-bovine and anti-porcine MLR (Group 2). The experiments were repeated 5 times, using different human, porcine and bovine blood donors, and the human anti-bovine MLR was weaker than the allogeneic MLR in all experiments.

Discussion

Since our ultimate goal is to overcome HAR by producing α1-3 galactosyl transferase knockout bovine, we thought that it was clinically important to obtain some information on the human cellular response to bovine. Previous studies were mainly concerned about xenogeneic human cellular responses to examine whether swine could serve as a donor. The present study, however, is the first on examination of the MLR between human and bovine cells.

The xenogeneic cellular response can be generally divided into two phases: an induction phase and an effector phase. The effector phase of the human cellular response to xenogeneic donors is largely dependent on NK cell mediated killing function[5)6)]. Previous studies on the induction phase of human cellular response against xenogeneic donors, on the other hand, have yielded conflicting results. Some studies reported that human xenogeneic MLR was weaker than allogeneic MLR[2)7)], and other experiments reported the opposite[1)8)]. In the present study, in order to examine the immunogeneicity of bovine PBMC against human PBMC, human anti-bovine MLR was examined, and the results revealed that hu-

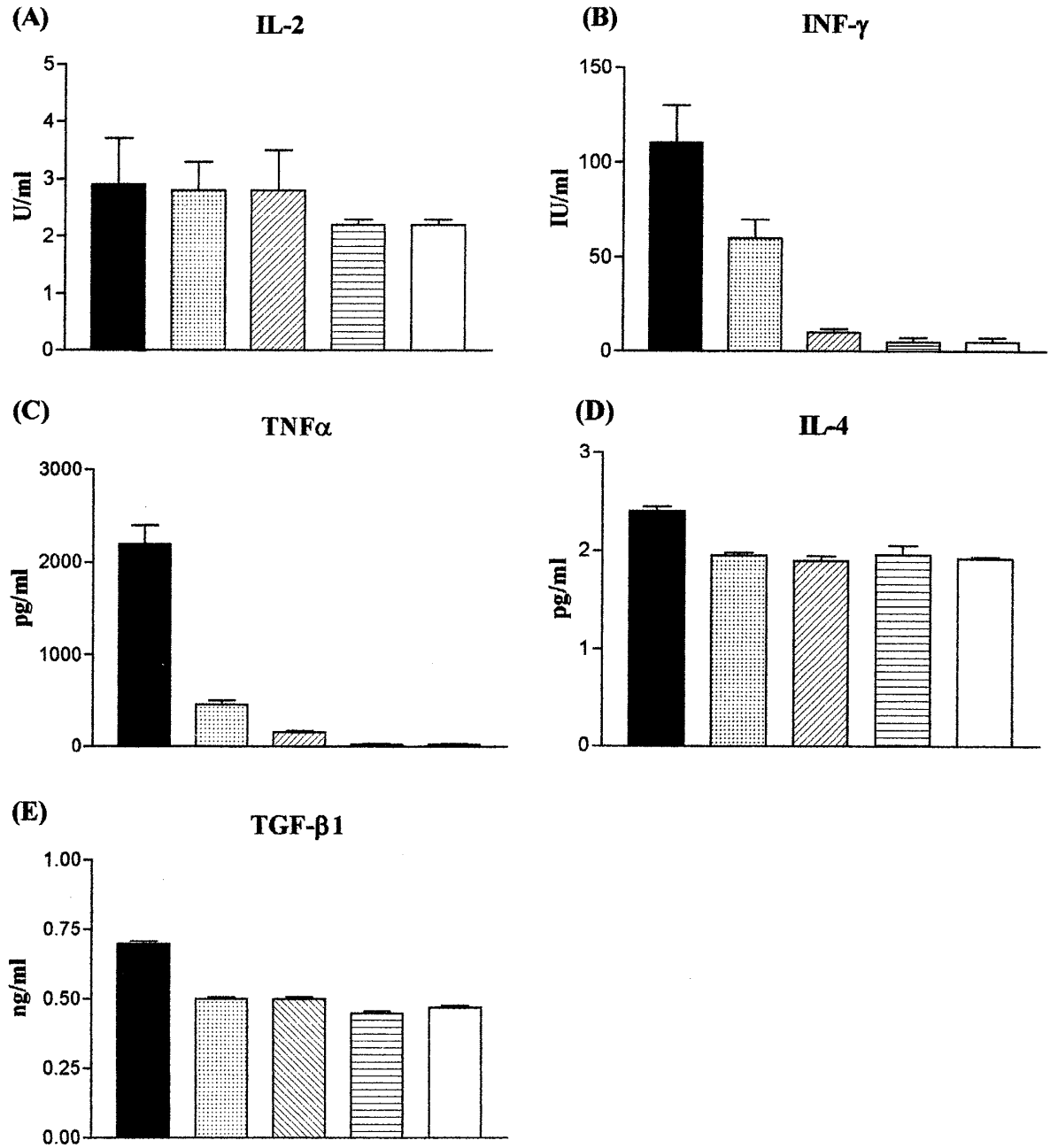

Fig. 2 Cytokine production of human PBMC
Cytokine production of human PBMC against allogeneic human PBMC (black), porcine PBMC (dotted), bovine PBMC (hutched) and self-human PBMC (horizontal) was measured by ELISA. The white bar represents cytokine production of human PBMC without stimulator cells.

man anti-bovine MLR was weaker than human allogeneic MLR.

The most important player in the induction phase may be $CD4^+$ T cells. Upon activation, $CD4^+$ T cells requires the engagement of T cell receptor (TCR) by xenogeneic peptides with self MHC class II antigen (indirect pathway). $CD4^+$ T cells may also recognize xenogeneic peptides with xenogeneic MHC (direct pathway). In both the indirect and the direct pathway, $CD4^+$ T cells require signals through co-stimulatory molecules in addition to signals through TCR. The complex-

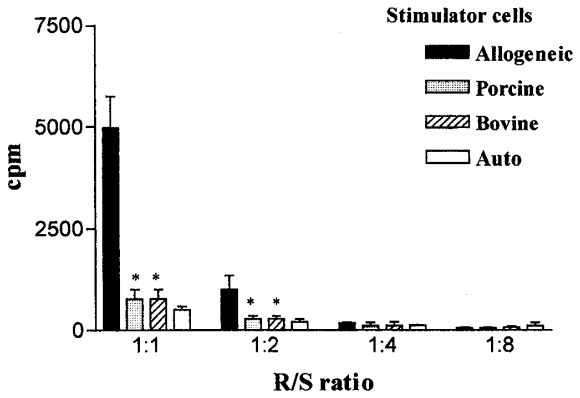

Fig. 3 Results of human anti-bovine MLR
Human anti-bovine MLR (hutched) was compared with allogeneic MLR (black) and anti-porcine MLR (dotted). Human anti-bovine MLR was weaker than allogeneic MLR. (*) ; $p<0.05$.

ity of the human xenogeneic cellular response partially arise from questions of whether human $CD4^+$ T cells can recognize xenogeneic MHC molecules[1)10)], and whether human co-stimulatory molecules can interact with xenogeneic co-stimulatory molecules[10)11)]. Results of the MLR in the present study revealed that human anti-bovine and anti-porcine MLR were weaker than the allogeneic MLR. For the further evaluation, elucidating the effector phase is essential to understand xeno-recognition between human and bovine.

Human PBMC are known to produce a group of cytokines, ie, Th1/Th2, against certain types of targets and there have not been many studies on human cytokine production in response to xenogeneic targets, especially against bovine cells. Previous studies have reported the defective cytokine interactions across the species[12)~14)]. The results of cytokine analysis in our study revealed that human lymphocytes preferentially produced TNFα and INF-γ, but the magnitude of the cytokine production was weaker than that of allogeneic combination. The findings may not be the cause of the defective T cell recognition, but the consequence of the defect in T-cell recognition.

The above findings together with the results of flow cytometry, cytokine production and MLR, indicate that the human cellular response against bovine might be weaker than against allogeneic MLR in the induction phase.

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References

1) **Yamada K, Sachs DH, DerSimonian H**: Human anti-porcine xenogeneic T cell response. Evidence for alleic specificity of mixed leukocyte reaction and for both direct and indirect pathway of recognition. J Immunol **155**: 5249–5256, 1995
2) **Dorling A, Binns R, Lechler R**: Cellular xenoresponses: although vigorous, direct human T cell anti-pig primary xenoresponses are significantly weaker than equivalent alloresponses. Xenotransplantation **3**: 149–154, 1996
3) **Wilmute I, Schnieke AE, McWhir J et al**: Viable offspring derived from fetal and mammalian cells. Nature **385**: 810–813, 1997
4) **Murakami T, Yokoyama T, Sawada T**: Cloning and xenotransplantation. Transplant Now **6**: 723–728, 1998
5) **Bach FH, Winkler H, Ferran C**: Delayed xenograft rejection. Immunol Today **17**: 379–384, 1996
6) **Seebach JD, Yamada K, McMorrow I et al**: Xenogeneic human anti-pig cytotoxicity mediated by activated natural killer cells. Xenotransplantation **3**: 188–194, 1996
7) **Satake M, Kawagishi N, Moller E**: Direct activation of human responder T cells by porcine stimulator cells leads to T cell proliferation and cy-

totoxic T cell development. Xenotransplantation **3**: 198–204, 1996
8) **Kumagai-Braesch M, Satake M, Korsgren O**: Characterization of cellular human anti-porcine xenoreacitivity. Clin Transplant **7**: 273–279, 1993
9) **Murray A, Khodadoust M, Pober J et al**: Porcine aortic endothelial cells activate human T cells: direct presentation of MHC antigens and costimulation by ligands for human CD2 and CD 28. Immunity **1**: 57–63, 1994
10) **Shishido S, Bashoo N, Xu XC et al**: Indirect recognition of porcine xenografts by human $CD4^+$ T cell clones. Transplantation **65**: 706–712, 1998
11) **Shishido S, Naziruddin B, Howard T et al**: Recognition of porcine MHC class I antigens by human CD8+ cytotoxic clones. Transplantation **64**: 340–346, 1997
12) **Moses RD, Winn HJ, Auchincloss H Jr**: Evidence that multiple defects in cell-surface molecule interactions across species differences are responsible for diminished xenogeneic T cell responses. Transplantation **53**: 203–209, 1992
13) **Watier H, Vallee I, Thibault G et al**: Effect of human inflammatory cytokines on porcine endothelial cell MHC molecule expression: unique role for TNF-α in MHC class II induction. Transplant Proc **26**: 1152–1155, 1994
14) **Sultan P, Murray AG, McNiff JM et al**: Pig but not human interferon-γ initiates human cell-mediated rejection of pig tissue in vivo. Proc Natl Acad Sci USA **94**: 8767–8772, 1997

ヒトリンパ球のウシリンパ球に対する免疫学的反応性に関する研究

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異種移植最大の障害は超急性拒絶反応（hyper acute rejection; HAR）であるが，近年トランスジェニック動物が開発され HAR 回避の端緒が開かれた．HAR の克服後にはヒト同種移植と同様，細胞性免疫反応が立ちはだかる．以前より異種移植の有力なドナー候補とみなされてきたブタに比べ，ウシに対するヒトの免疫反応に関する報告は少ない．そこで我々は，ヒト末梢血単核球（peripheral blood mononuclear cells; PBMC）に対するウシ PBMC の免疫原性を検討するため，ヒト PBMC を responder，ヒト（アロ），ウシ，ブタ PBMC を stimulator としてそれぞれ混合培養し，フローサイトメトリー，リンパ球混合試験（mixed lymphocyte reaction; MLR）を施行し，またヒト PBMC のサイトカイン産生能を測定した．

培養開始当日，および 5 日後のヒト PBMC のフローサイトメトリーでは CD3 陽性（T）細胞は，ウシに対しては 40.3±4.5% から 61.6±6.8% へと増加した．CD19 陽性（B）細胞には明らかな変化を認めなかったが CD56 陽性（NK）細胞は 15.9±7.2% から 25.2±8.9% へと増加した．これら phenotype の変化は，stimulator がヒト，ブタと代わっても大きな差異は認めなかった．MLR においては，対ヒトに比較すると対ウシ MLR は低値であり，対ブタ MLR とほぼ同等であった．また，サイトカイン産生においては Th1 細胞由来のサイトカイン優位であるものの，対ヒトに比べると低値であった．

以上，ヒトのウシに対する細胞性免疫反応はブタと同様，ヒト（アロ）に対する反応より弱いことが示唆された．