日本財団補助金による

1998 年度日中医学協力事業報告書

－中国人研究者・医療技術者招聘助成－

財団法人 日 中 医 学 協 会

理事長 中 島 章 殿

H10 年 12 月 15 日

研究室で撮影した本人のスナップ写真、及び発表論文等のコピーを添付

1．招へい責任者 鈴木盛一 ㊞

所属機関 国立小児病院小児医療研究センター実験外科(体外循環) 職名 部長

所在地 〒157-8509 東京都世田谷区大子堂3-35-31 電話 03-3414-8121

招へい研究者氏名 何維

所属機関 中国医科学院基礎医学研究所、中国協和医大兼任教授 職名 教授

研究テーマ マウス混合リンパ球反応における熱ショックタンパク質60に対するab. sera の反応性についての検討

2．日本滞在日程

1998. 6. 28. ～ 1998. 10. 26.

3．研究報告

別紙書式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

論文発表に当っては、日中医学協会－日本財団補助金による旨を明記して下さい。

研究テーマ

マウス混合リンパ球反応における熱ショックタンパク質６０に対するαβ、γδT細胞の反応性についての検討

来日研究者氏名、所属、役職

何維　中国医科学院基礎医学研究所、中国協和医科大学免疫学教室　教授

招聘者氏名、所属、役職

鈴木盛一　国立小児病院小児医療研究センター実験外科生体工学部　部長

要旨

熱ショックタンパク質（Hsp）反応性リンパ球の同種異系（アロ）臓器移植拒絶反応時の関与が最近報告されている。しかし、熱ショックタンパク質の発現機構及び役割について、特に二種類のT細胞、すなわちαβT細胞、γδT細胞のアロリンパ球に対する反応性についてはまだ不明である。そこで、アロ抗原による一方性、二方性マウス混合リンパ球反応におけるHspの発現機構及び役割について検討を行った。正常またはX線照射後リンパ球においては細胞成長因子、抗原、マイトジンなど存在の有無に関わらず、Hsp60またはHsp70分子が細胞表面に発現し、アロ抗原の刺激によりその発現は著しく増強した。αβT細胞はアロ生細胞と共培養時に強く増殖し、また、その増殖性は抗Hsp60抗体によって、31-43%に抑えられた。一方γδT細胞では抗Hsp60抗体の添加によって、完全に増殖が抑えられた。以上の結果から、マウスin virto系においてアロ反応性はHsp60分子とγδT細胞が関連し、αβT細胞の関与は部分的であることを判明した。臓器移植拒絶反応におけるHsp60分子の関与及び役割の機序の解明は臨床において拒絶反応の制御に役に立つと考える。

KEY WORDS

heat shock protein, α β , γ δ T lymphocyte, alloreactive response, transplantation immunology, mixed lymphocyte reaction

研究報告

INTRODUCTION

Heat shock proteins (hsps) are produced by both prokaryotic and eukaryotic cells under a variety of stresses such as sudden elevation of temperature, oxidative radicals, anoxia, alcohol, heavy metals, ischemia, cytokines, infection and inflammation(1-6).This cellular response is presumed to be responsible for protecting stressed cells through reactivating denature protein(7). Besides, many investigations have shown that hsps perform a function as " molecular chaperones" by folding unfolding and translocation of polypetides(7,8,) which is closely related to antigen processing and presentation. (9-13)

For example, hsp70 can interact with unfolding polypeptides so as potentially to facilitate a conformational change in the peptide-MHC complex, which is required for transit of peptide-MHC complex to cellular surface(9). Expression of MHC is promoted by the stimulation via heat stress(13). Antigen presentation can be blacked by antibodies against hsp70(12). These data suggest that hsps belong to antigen-presenting molecules. On the other hand, hsps also seem to be the target antigens recognized by immune system, which can trigger autoimmune response,(5,14) tumor immunity(15), and graft rejection(16-24).

Several investigators have noted that alloreactive lymphocytes are a rather small proportion of T cells within infiltrating lymphocytes in allograft. Therefore, they postulated that graft-infiltrating lymphocytes might posses other specificity to additional antigen(16).They found that rat allograft lymphocytes responded markedly to recombinant mycobacterial hsp65 and hsp70. Many studies have recently revealed that allograft undergoes a stress, in which hsps including the families of hsp60 and hsp70 are upregulated and hsp-reactive lymphocytes can induced in rat, (16,18-20) mouse(17)and human system(21-24). However, there are several questions required to be clarified. Firstly, in several studies the reactivity of lymphocytes infiltrated in allograft tissues to hsps is evaluated by the response to recombinant or non recombinant mycobacterial hsps(16,18,19). This is obviously not so powerful evidence for the interpretation of involvement of hsps in allograft rejection because no hsps derived from same spices could be tested for the response to lymphocytes in these investigations. Recently a group had a promising result that T cells from rejected human kidney allograft respond to human hsp72(23). The conclusion from this report seems to imply that hsp72 is a target antigen which elicit lymphocyte response. Do hsps really therefore serve as alloantigen or antigen presenting molecules in allogeneic lymphocyte reaction? Moreover, although hsp60-reactive ab and $\gamma\ \delta$ T cells have been identified in mice(25) and humans(26), what is difference between the responses of TCR $\alpha\ \beta$ -expressing cells and TCR $\gamma\ \delta$ -expressing cells to allo-stimulator in hsp-reactive pathway? At last, the hsps express generally in within both the cytoplasm and nucleus, however, would hsps express on cell surface under a stressful stimulation by allo-reactivity? In present study, we definitively confirmed the expression of both hsp60 and hsp70 molecules on the cells in murine mixed lymphocyte reaction in vitro. A hsp60-dependant alloreactive response in both cases of $\alpha\ \beta$ T and $\gamma\ \delta$ T cells was found in murine system in vitro.

MATERIALS AND METHODS

Animals

Ten-week-old male inbred BALB/c (H-2d) and C57BL/6 (H-2k) mice, 2 months of age, were purchased from Shizuoka, Laboratory Animal Center (Shizuoka, Japan). All animals had access to water and foods ad libitum and were housed in accordance with institutional animal care policies.

Antibodies and reagents

The following mAbs were used: anti-hsp60 mAb derived from the LK1 hybridoma (mouse IgG1 isotype) has a unique specificity for mammalian hsp60 and avian hsp60, but does not cross react with the bacterial counterpart, or with helminths and spinach. The anti-hsp70 mAb derived from the BRM-22 hybridoma localized both the constitutive (hsp73) and inducible (hsp72) forms of hsp70 in immunoblotting. Both anti-hsp mAbs were purchased from Sigma. The following mAbs or reagents were purchased from PHARMINGEN, USA: Biotin-conjugated hamster anti-mouse anti-TCR $\gamma\delta$ and anti-TCR $\alpha\beta$; biotin-conjugated rat anti-mouse CD19; FITC-conjugated hamster anti-mouse TCR β; R-PE-conjugated hamster anti-mouse TCR $\gamma\delta$, FITC- or R-PE-conjugated hamster IgG isotype standard. FITC-conjugated F(ab')2 rabbit anti-mouse IgG were from SEROTEC. Streptavidin microbeads were purchased from (Miltenyi Biotec, Bergisch Gladbach, Germany).

Preparation of TCR $\alpha\beta$- and TCR $\gamma\delta$-expressing cells. Splenic tissues, obtained from BALB/c or C57BL/6 mice, were gently pressed by slides and passed through a syringe, in which was filled with sterilized tampon, to obtain single-cell suspension. The mononeuclear splenocytes separated from Ficoll-Hypaque centrifugation were then passed through Nylon Fiber Column (Wako Pure Chemical Industries) to obtain T cell-enriched population. Purification of $\alpha\beta$ or $\gamma\delta$ T cells from these BABL/c splenic T cells were done by magnetic activated cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were resuspended in labeling buffer (PBS, pH7.2, 2mM EDTA), to which biotin-conjugated mAbs anti-CD19 (1mg/106 cells) and anti-TCR $\gamma\delta$ or anti-TCR $\alpha\beta$ were added at 1mg/106 cells. The cells were incubated for 20 min on ice and washed twice with labeling buffer. The resuspended cells were then incubated with streptavidin microbeads (10ml/10^7 cells) for 15 min on ice and washed. Cells were then added in a column, which was placed in magnetic activated cell sorter. We collected the entire effluate as a " nonmagnetic" fraction, in which $\alpha\beta$ or $\gamma\delta$ T cells accounted for >90%, as analyzed by fluorescence staining.

Proliferation assay: One-way reaction and two-way reaction of $\alpha\beta$ or $\gamma\delta$ T cells to allogeneic splenocytes. Two-way allo-reactivity: proliferative responses of $\alpha\beta$ or $\gamma\delta$ T cells from spleens of BALB/c mice to splenocytes from C57BL/6 mice were determined in a ^{3H}-TdR incorporation assay. Responder cells (1×10^4 $\alpha\beta$ or $\gamma\delta$ T cells from BABL/c mice) were cultured with 2×10^4 irradiated (20 Gy x-ray) or not irradiated (living) stimulators (splenocytes from C57BL/6 mice) in triplicate in the wells of 96-well V-bottom microtiter plates (Nunc, Wiesbaden, Germany) in a volume of 0.2 ml. The medium was RPMI-1640 supplemented with 10%heat-inactivated FCS, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2 in air for 6 days. Anti-hsp 60 (1:400) or -hsp70 (1:5000) mAb was added to cultures respectively. Controls included responder or stimulator alone, and cultures without anti-hsp60 or -hsp70 mAb. At the indicated time, individual microcultures were pulsed with 1 μ Ci ^{3H}-TdR per well and incubated for another 6 hours. Thereafter, cultures were harvested onto glass fibers and counted for beta emission using a trace-96 direct beta counter (Berthold).

The one-way mixed lymphocyte reaction in a ^{3H}-TdR incorporation assay was also performed. 5×10^4 T cells (responder), which were separated by nylon wool isolation from splenocytes from BABL/c, co-cultured with irradiated (20Gy) 5×10^4 spleen cells (stimulator) from C57BL/6 mice in 96-well round-bottom microplates (Nunc) for 3 days, while expressions of hsp60 and hsp70 on responder, stimulator and responder +stimulator cells were analyzed at day 2 and day3.

Flow cytometry

For phenotypic analysis, the magnetic isolated cells were stained with FITC-anti-TCR β and R-PE- anti- TCR $\gamma\delta$. For hsp expression of analysis, cells were stained with anti-hsp60 or anti-hsp70 respectively for 15 min on ice. After two washing steps, cells were then incubated with FITC-conjugated F(ab')2 rabbit anti-mouse IgG for 15 min on ice and washed twice. The cells were resuspended in PBS with 1% paraformaldehyde. All analyses were measured on a FACScan flow cytometer (Becton Dickinson).

Statistical analysis

Analysis of variance was used to test the differences among all tested groups in proliferation assay.

RESULTS

Expression of hsp60 and hsp70 on cultured cells in one-way mixed lymphocyte reaction in vitroAs illustrated in fig.1, a conventional proliferation of murine splenic T cell (BALB/c) anti allogeneic irradiated splenocyte (C57BL/6) was obviously observed during 3 days of culture in vitro. However, the event in this process we are interested in is whether the response of T cells to allogeneic stimulation could correspond with hsp expression on cells. As shown in fig.2, both freshly isolated responder (BALB/c) splenic T cells and stimulator (C57BL/6) splenocytes could express surface hsp60 molecule in minor population (< 6%, A and B). Responder cells maintained this level of hsp60 expression at day2 (C) and increased at day3 (F) with 25%. The hsp60 expression in irradiated (20Gy) stimulator cells increased with prolonging time of culture (at day2, 43% and at day3, 62%). In the cultures of responder mixed with irradiated stimulator cells, there was increasing positive cell percentage in the whole population accounted for 49% at day2 and 67% at day3 respectively. At same time point, the sequence for the levels of hsp60+ cell percentages is as follows: responder with stimulator (R with S) > stimulator (S) > responder (R). We used a formula to calculate a net increasing percentage in cells from the culture of responder with stimulator. That is, %net increasing = %R with S - [%R (responder) + %S (stimulator)/2]. According the calculation with this formula, we found the net increasing percentages in cultures of responder with stimulator cells were 24.5% at day2 and 23.5% at day3. It indicates that an increasing hsp60 expression exists in cultures of responder with stimulator cells, when compared with responder or stimulator cells at same time. Similar manifestation also happened in the case of hsp70 expression in same test system (fig.2 I to P). Before culture, both responder T cells and stimulator splenocytes expressed hsp70 molecule on their surface in minor population (8%, I and J). After culture, responder cells almost kept this level at day2 (K, 6%) and increased at day3 (N) with 43%. The hsp70 expression in irradiated stimulator cells increased with prolonging time of culture (at day2, 50% and at day3, 24%). The hsp70+ cell percentages in the cultures of responder with irradiated stimulator cells significantly increased (38% at day2 and 58% at day3). Their net percentages were 10% at day2 and 29% at day3.

The alloreactive proliferative responses of $\gamma\ \delta$ T or $\alpha\ \beta$ T cell to allogeneic stimulator cells in one-way or two-way reaction were completely or partially blocked by anti-hsp60 antibody in vitro. To directly address the role of hsp60 and hsp70 in allo-reactivity of $\alpha\ \beta$ T or $\gamma\ \delta$ T cells (BABL/c), we performed the experiment as

illustrated in fig. 4. As noted elsewhere, $\gamma\delta$ T cells were not as good responder as $\alpha\beta$ T cells to alloantigens. In one-way reaction, there was no growth detected in cultures of $\gamma\delta$ T cells plus irradiated allogeneic spleen cells (C57BL/6), which was similar with the cases of all controls, such as $\gamma\delta$ T cells, $\alpha\beta$ T cells, irradiated or not irradiated (living) stimulator cells alone. A detectable proliferation was found in the cultures of $\gamma\delta$ T cells with living allogeneic cells (two-way reaction) during 3 to 4days of incubation in vitro ($P<0.05$, when compared with all controls or $\gamma\delta$ T cells plus irradiated allogeneic stimulator cells), while this response was completely blocked by adding mAb anti-hsp60 to culture ($P<0.05$, $\gamma\delta$ T cells + living stimulator versus (vs) $\gamma\delta$ T cells + living stimulator + anti-hsp60), and was not affected at all by anti-hsp70 ($P>0.05$, $\gamma\delta$ T cells + living stimulator vs $\gamma\delta$ T cells + living stimulator + anti-hsp70). Differentially to $\gamma\delta$ T cells, $\alpha\beta$ T cells demonstrated irradiated allo-lymphocyte-induced proliferation at the top level on day4. We interestingly noted that this allo-reactive response was not influenced by adding both mAbs anti-hsp60 and anti-hsp70 to cultures ($P>0.05$, when compared these groups). However, when the irradiated stimulator was changed into living allogeneic cells, anti-hsp60 antibody could blocked the proliferative allo-reactivity of between $\alpha\beta$ T cells and allogeneic living spleen cells ($P<0.05$, blocked vs not blocked), which was the strongest proliferation in this tested system ($P<0.05$, when compared with other groups except $\alpha\beta$ T cells plus living stimulator with anti-hsp70). Anti-hsp70 antibody did not show any blocking effect on two-way allo-reaction between $\alpha\beta$ T cells and allogeneic living spleen cells. As clearly shown in fig.4, the following proliferative responses during the period from day3 to day5 were at same level ($P>0.05$, when compared with each other): $\alpha\beta$ T cells plus irradiated stimulator with or without mAbs anti-hsp60 or anti-hsp70, $\alpha\beta$ T cells plus living stimulator with mAb anti-hsp60, and $\gamma\delta$ T cells plus living stimulator with or without mAb anti-hsp70. All statistical analyses above were basely on data from the determination at time from day3 to day4.

Expression of hsp60 and hsp70 on cultured $\alpha\beta$ and $\gamma\delta$ T cells and stimulator cells in one-way or two-way mixed lymphocyte reaction in vitro. The both freshly isolated $\alpha\beta$ and $\gamma\delta$ T cells (BALB/c) express hsp60 and hsp70 at similar level to T cells isolated with nylon wool as shown in fig2 (data not shown). At the top of alloreactive proliferation in one-way or two-way mixed lymphocyte reaction at day4 (fig4), we analyzed the expressions of these two hsps on cultured cells (fig.5). After 4days of culture, $\gamma\delta$ T cells alone had 40% of hsp60+ cells (A) and 53% of hsp70+ cells (G) respectively, whereas $\alpha\beta$ T cells alone possessed 26% of hsp60+ cells (D)

and 25% of hsp70+ cells (J) respectively. Among $\gamma\delta$ or $\alpha\beta$ T cells with stimulator (C57BL/6) irradiated splenocytes (one-way reaction), there were 60% (B, $\gamma\delta$) or 38% (E, $\alpha\beta$) of hsp60+ cells, as well as 49% (H, $\gamma\delta$) and 37% (K, $\alpha\beta$) of hsp70+ cells respectively. On the other hand, there were 48% (C) of hsp60+ cells and 43% (I) of hsp70+ cells in cultures with $\gamma\delta$ T cells plus not irradiated stimulator cells, whereas there were 44% (F) of hsp60+ cells and 53% (L) of hsp70+ cells in cultures with $\alpha\beta$ T cells plus not irradiated stimulator cells. We had analyzed cultured cells with $\gamma\delta$ or $\alpha\beta$ T cells plus stimulator with or without irradiation via dual color staining by means of anti-TCR $\alpha\beta$-PE or anti-TCR $\gamma\delta$-PE plus anti-hsp60/-hsp70-FITC, the results showed that all non-$\alpha\beta$ T cells or non-$\gamma\delta$ T cells in the cultures were 100% positive for hsp60 or hsp70 (data not showed).

DISCUSSION

Although hsps have basically intracellular expression under a stress, the expression of both hsp60 and hsp70 molecules on cellular surface were observed in present investigation. We found that there was the expression of both hsp60 and hsp70 on the cells in one-way mixed lymphocyte reaction in vitro. In our experimental system, these hsps expressed at small proportion in responder cells (T cells), at large rate in irradiated stimulator lymphocytes, and at the highest percentage in the responder with irradiated stimulator cells. Some T cells cultured with only medium could express hsp60 and hsp70 on surface. As every immunologist knows, T cells tends to die in vitro if they would not be further stimulated by antigens, mitogens, or T cell growth factor, such as IL-2. If no stimulation for T cells in vitro could serve as a stress to T cell, it would not be any difficulty for us to understand the phenomenon of hsp expression in this condition. Furthermore, irradiated lymphocytes expressed high rates of hsp60 (significantly) and hsp70 molecules in same cultural condition. X-ray irradiation seems to be a stimulus for hsp expressions of the lymphocytes. The highest expression of both hsp60 and hsp70 happened in the case of the mixture of T responder cells with allogeneic irradiated lymphocytes in vitro. After the spontaneous expressions of both hsp60 and hsp70 were excluded, the net increasing rates of these expressions were found in this case. It strongly suggests that an allogeneic stimulus could trigger inducible hsp expression on cellular surface. Based on the analysis above, we have a reason to postulate that hsps might be alloantigens or alloantigen-related molecules to participate in the allo-reactivity of lymphocytes as someone have already presumed it several years ago(16). We then designed a further experimental system in vitro. In the design, we

had stressed the three key points as follows: (1) to check possible different alloreactive responses of $\alpha\beta$ T and $\gamma\delta$ T cells to hsp molecules; (2) both one-way and two-way mixed lymphocyte reaction might be all necessary for the evaluation of the role of hsp in all-reactivity, because the hsps could express on both alloreactive lymphocytes; (3) checking possible blocking effects of anti-hsp antibodies on alloreactive response is important to definite hsp-reactive response in this system. As expected, and noted by someone else(27), it is difficult to induce a response of $\gamma\delta$ T cells against irradiated allogeneic lymphocytes, unlike the situation with $\alpha\beta$ T cells. However, an alloreactive proliferation was found in two-way allogeneic reaction in the mixture of $\gamma\delta$ T cells with living allogeneic lymphocytes. Moreover, this proliferation was completely blocked by anti-hsp60 antibody added to the culture, whereas an increasing expression of hsp60 molecule was detected at same time on the cells of both $\gamma\delta$ T cells cultured alone and $\gamma\delta$ T cells with allogeneic spleen cells. Although hsp60 expressed in 60% of the cells cultured with irradiated allogeneic splenocytes, there was no cell growth detected in this culture yet. However, a complete hsp-reactive alloreactive response of $\gamma\delta$ T cells with allogeneic spleen cells existed in anti-allo-lymphocyte/$\gamma\delta$ T cells in our experimental system. In striking contrast, $\alpha\beta$ T cells responded to irradiated allogeneic spleen cells at similar proliferative level to $\gamma\delta$ T cells with living allogeneic cells, while there was the strongest cell growth in the mixture of $\alpha\beta$ T cells with living allogeneic spleen cells. Alloantigens are obviously good stimuli for the response of $\alpha\beta$ T cells. In blocking test, we found that both anti-hsp60 could partially blocked two-way allo-reactivity with 31-43% inhibition rate at day3 to day5 of culture, but did not affect one-way allo-reactive response. Similarly to the case of $\gamma\delta$ T cells, we also found the expression of both hsp60 and hsp70 on the cells in the cultures of $\alpha\beta$ T cells without or with irradiated or not irradiated allogeneic spleen cells, which were all positive for hsps. In spite of high rate of hsp70+ cells in two-way reaction culture, there was not a blocking effect of anti-hsp70 antibody to be detected in the experimental system. It indicates that hsp70 seems not to be related to allo-reactivity or only to participate in antigen presentation in intracellular environment.

Taken together, our results indicate that alloreactive response in two-way reaction of both $\gamma\delta$ T cells to allogeneic spleen cells could be a complete hsp60-dependant, that is, hsp60 serves as an exclusive alloantigen in our experimental system. On the other hand, the allo-reactivity of $\alpha\beta$ T cells only partially due to hsp60, while MHC class I and II alloantigens might be responsible for remaining effect. If this mechanism could be confirmed in humans both in vivo and in vivo, a strategy based on blocking hsp60-dependant allo-reactivity might be beneficial to the control of allgraft rejection in clinic.

Besides, it is need to clarify the further questions: what is exact role of hsp60 in allo-reactive response? Is it target antigen itself, or an antigen present molecule? The resolution of these question will provide further evidence for the participation of hsp in alloreactive response.

REFERENCES

1. LINDQUIST S. (1986) The heat shock response. Annu Rev Biochem 55, 1151.
2. WELCH W.J. (1987) The mammalian heat shock or stress response: a cellular defense mechanism. Adv Exp Med Biol 225, 287.
3. ELLIS J. (1987) Proteins as molecular chaperonins. Nature 328, 378.
4. LINDQUIST S & CRAIG E.A. (1988) The heat-shock proteins. Annu Rev Genet 22, 631.
5. YOUNG R. & ELLIOT T. (1990) Stress Proteins, infection, and immune surveillance. Cell 59,5.
6. CURRIE R.W., TANGUAY R.M. & KINGMA J.G. (1993) Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. Circulation 87, 963.
7. GEORGOPOULOS C. & WELCH W.J. (1993) The role of the major heat shock proteins as molecular chaperones. Annu Rev Cell Biol 9, 601.
8. BRCKMANN R.P., MIZZEN L.E. & WELCH W.J. (1990) Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Science 248, 850.
9. STERN L.J. & WILEY. (1992) The human class II MHC protein HLA-DR1 assembles as empty ab heterodimers in the absences of antigenic peptide. Cel 68, 465.
10. LAKEY E.K., MARGOLIASH E. & PIERCE S.K. (1987) Identification of a peptide binding protein which plays a role in antigen presentation. Proc Natl Acad Sci USA 84, 1659.
11. VAN BUSKIRK V.A., DENAGEL D.C., GUAGLIARDI LE, BRODSKY FM & PIERCE SK. (1991) Cellular and subcellular distribution of PBP72/74, a peptide-binding protein that plays a role in antigen processing. J Immunol 146,500.
12. VAN BUSKIRK V.A., CRUMP B.L., MARGOLIASH E. & PIERCE S.K. (1989) A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. J Exp. Ed 170, 1799.

13. REES A.D.M., CHIANG H.L., OLSON R.S. & DICE J.F. (1991) Stress-induced modulation of antigen-presenting cell function. Immunology 74, 386.
14. KAUFMANN S. (1990) Heat shock proteins and the immune response. Immunol Today 11, 129.
15. UDONO H. & SIVASTRAVA P. (1991) Heat shock protein 70-associated peptides elicit specific cancer immunity. IJ Exp Med 178, 1391.
16. MOLITERNO R., VALDIVIAL L, PAN F. & DUQUESNOY RJ. (1995) Heat shock protein reactivity of lymphocytes isolated from heterotopic rat cardiac allograft. Transplantation 59, 589.
17. SANDBERG JAN-OLOV, MARGULIS B., JANSSON LEIF, KARLSTEN R & KORSGREN O. (1995) Transplantation of fetal porcine pancreas to diabetic or normoglycemic nude mice. Transplantation 59,1665.
18. QIAN J., MOLITERNO RA, ANN DONOVAN-PELUSO M., et al. (1995) Expression of stress proteins and lymphocyte reactivity in heterotopic cardiac allografts undergoing cellular rejection. Transplant Immunology 3, 114.
19. LIU K., MOLITERNO R.A., FU X-F. & DEQUESNOY R.J. (1997) Identification of two types of autoreactive T lymphocyte clones cultured from cardiac allograft-infiltrating cells incubated with recombinant mycobacterial heat shock protein 71. Transplant Immunology 5, 57.
20. BABA H.A., SCHMID C., WILHELM, M.J., BLASIUS, SCHELD H.H., BOECKER W & DOCKHORN-DWORNICZARK. (1997) Inducible heat shock protein 70 in rat cardiac allograft and its immunohischemical location in cardiac myocytes. Transplantation 64,1035.
21. DAVIS E.A., WANG B.H., STAGG C.A., BALDWIN W.M., BAUMGARTNER W.A., SANFILIPPO & UDELSMAN R. (1996) Induction of heat shock protein in cardiac allograft rejection-a cyclosporine-suppressible response. Transplantation 61,279.
22. ALEVY Y.G., BRENNAN D., DURRIYA S., HOWARD T. & MOHANAKUMAR T. (1996) Increased expression of the HDJ-2 heat shock protein in biopsies of human rejected kidney. Transplantation 61,963.
23. TRIEB K., GRUBECK-LOEBENSTEIN B., EBERL T. & MARGREITER R. (1996) T cells from rejected human kidney allografts respond to heat shock protein 72. Transplant Immunology 4, 43.
24. BABA H.A., SCHMID K.W., SCHMID C., BLASIUS S., HEINECKE A., KERBER S., SCHELD H.H., BOECKER W.& DOCKHORN-DWORNICZARK. (1998) POSSIBLE RELATIONSHIP BETWEEN heat shock protein 70, cardiac

hemodynamics, and survival in the early period after heart transplantation. Transplantation 65,799.
25. BRENNER M.B., MCLEAN J., DISLYNAS D.P., et al. (1986) Identification of a putative second T-cell recepto. Nature322,145.
26. BUCY R.P., CHEN C.L. & COOPER M.D. (1989) Tissue localization and CD8 accessory molecule expression of T gd cells in humans. J Immunol 142, 3045.
27. LANNIER L.L. (1995) Unusual lymphocytes-gd T cells and NK cells. The Immunologist 3, 182.

TITLE:

DIFFERENT ALLOREACTIVE RESPONSES OF MURINE TCRαβ– AND TCRγδ-EXPRESSING CELLS TO HEAT SHOCK PROTEIN 60 IN ALLOGENEIC LYMPHOCYTE REACTION IN VITRO

WEI HE*¶, XIAO-KANG LI*[1], AND S. SUZUKI* *Department of Experiment Surgery & Bioengineering, National Children's Medical Research Center, Taishido, Setagaya-ku, Tokyo 154 Japan and ¶Department of Immunology, Institute of Basic Medicine, Chinese Academy of Medical Sciences, Beijing 100005, PR China

SUMMARY

Heat shock protein (hsp)-reactive lymphocytes and inducible expression of hsps have been recently found to be present in allograft undergoing rejection. It is necessary to check the role of hsp in allogeneic lymphocyte reaction, to analysis the difference between the responses of αβT cells and γδT cells to allo-stimulator in hsp-reactive pathway, and to detect hsp expression on cell surface under a stressful stimulation. In present study, we definitively confirmed increasing expression of both hsp60 and hsp70 molecules on murine cells in a one-way or two-way mixed lymphocyte reaction *in vitro*. T cell or x-ray irradiated lymphocytes could express both hsp60 and hsp70 molecules in the absence of exogenous cell growth factor, antigen or mitogen. Allogeneic stimuli would markedly promote these hsp expressions. αβT cells, not γδT cells, responded to allogeneic irradiated lymphocytes in vitro. The strongest proliferation would be elicited when αβT cells co-cultured with allogeneic living lymphocytes not irradiated. This proliferation was partially (31-43%) blocked by anti-hsp60 antibody. On the other hand, a detectable proliferative response occurred in the mixture of γδT cells with allogeneic lymphocytes not irradiated, which would completely (100%) inhibited by adding anti-hsp60 antibody to the culture. Our results suggest that hsp60-dependant allo-reactivity in murine system *in vitro* is completely related to γδT cells and partially to αβT cells. A strategy based on blocking hsp60-dependant allo-reactivity would be beneficial to the control of allgraft rejection in clinic if the mechanism above would be further confirmed in humans both *in vitro* and in *vivo*.

INTRODUCTION

Heat shock proteins (hsps) are produced by both prokaryotic and eukaryotic

[1] Address correspondence to Dr. Xiao-kang Li, Department of Experiment Surgery & Bioengineering, National Children's Medical Research Center, 3-35-31, Taishido, Setagaya-ku, Tokyo 154 Japan

cells under a variety of stresses such as sudden elevation of temperature, oxidative radicals, anoxia, alcohol, heavy metals, ischemia, cytokines, infection and inflammation.[1-6] This cellular response is presumed to be responsible for protecting stressed cells through reactivating denature protein.[7] Besides, many investigations have shown that hsps perform a function as " molecular chaperones" by folding, unfolding, and translocation of polypetides[7,8], which is closely related to antigen processing and presentation.[9-13] For example, hsp70 can interact with unfolding polypeptides so as potentially to facilitate a conformational change in the peptide-MHC complex, which is required for transit of peptide-MHC complex to cellular surface.[9] Expression of MHC is promoted by the stimulation via heat stress.[13] Antigen presentation can be blacked by antibodies against hsp70.[12] These data suggest that hsps belong to antigen-presenting molecules. On the other hand, hsps also seem to be the target antigens recognized by immune system, which can trigger autoimmune response,[5,14] tumor immunity[15], and graft rejection.[16-24]

Several investigators have noted that alloreactive lymphocytes are a rather small proportion of T cells within infiltrating lymphocytes in allograft. Therefore, they postulated that graft-infiltrating lymphocytes might posses other specificity to additional antigen.[16] They found that rat allograft lymphocytes responded markedly to recombinant mycobacterial hsp65 and hsp70. Many studies have recently revealed that allograft undergoes a stress, in which hsps including the families of hsp60 and hsp70 are upregulated and hsp-reactive lymphocytes can induced in rat,[16,18-20] mouse[17] and human system.[21-24] However, there are several questions required to be clarified. Firstly, in several studies the reactivity of lymphocytes infiltrated in allograft tissues to hsps is evaluated by the response to recombinant or non recombinant mycobacterial hsps.[16,18,19] This is obviously not so powerful evidence for the interpretation of involvement of hsps in allograft rejection because no hsps derived from same spices could be tested for the response to lymphocytes in these investigations. Recently a group had a promising result that T cells from rejected human kidney allograft respond to human hsp72.[23] The conclusion from this report seems to imply that hsp72 is a target antigen which elicit lymphocyte response. Do hsps really therefore serve as alloantigen or antigen presenting molecules in allogeneic lymphocyte reaction? Moreover, although hsp60-reactive αβ and γδT cells have been identified in mice[25] and humans[26], what is difference between the responses of TCRαβ-expressing cells and TCRγδ -expressing cells to allo-stimulator in hsp-reactive pathway? At last, the hsps express generally in within both the cytoplasm and nucleus, however, would hsps express on cell surface under a stressful stimulation by allo-reactivity? In present study, we definitively confirmed the expression of both hsp60 and hsp70 molecules on the cells in murine mixed lymphocyte reaction *in vitro*. A hsp60-dependant alloreactive response in both cases of αβT and γδT cells was found in murine

system *in vitro*.

MATERIALS AND METHODS

Animals

Ten-week-old male inbred BALB/c (H-2^d) and C57BL/6 (H-2^b) mice, 2 months of age, were purchased from Shizuoka, Laboratory Animal Center (Shizuoka, Japan). All animals had access to water and foods ad libitum and were housed in accordance with institutional animal care policies.

Antibodies and reagents

The following mAbs were used: anti-hsp60 mAb derived from the LK1 hybridoma (mouse IgG1 isotype) has a unique specificity for mammalian hsp60 and avian hsp60, but does not cross react with the bacterial counterpart, or with helminths and spinach. The anti-hsp70 mAb derived from the BRM-22 hybridoma localized both the constitutive (hsp73) and inducible (hsp72) forms of hsp70 in immunoblotting. Both anti-hsp mAbs were purchased from Sigma. The following mAbs or reagents were purchased from PHARMINGEN, USA: Biotin-conjugated hamster anti-mouse anti-TCRγδ and anti-TCRαβ; biotin-conjugated rat anti-mouse CD19; FITC-conjugated hamster anti-mouse TCRβ; R-PE-conjugated hamster anti-mouse TCRγδ, FITC- or R-PE-conjugated hamster IgG isotype standard. FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgG were from SEROTEC. Streptavidin microbeads were purchased from (Miltenyi Biotec, Bergisch Gladbach, Germany).

Preparation of TCRαβ- and TCRγδ-expressing cells

Splenic tissues, obtained from BALB/c or C57BL/6 mice, were gently pressed by slides and passed through a syringe, in which was filled with sterilized tampon, to obtain single-cell suspension. The mononeuclear splenocytes separated from Ficoll-Hypaque centrifugation were then passed through Nylon Fiber Column (Wako Pure Chemical Industries) to obtain T cell-enriched population. Purification of αβ or γδ T cells from these BABL/c splenic T cells were done by magnetic activated cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were resuspended in labeling buffer (PBS, pH7.2, 2mM EDTA), to which biotin-conjugated mAbs anti-CD19 (1μg/10^6 cells) and anti-TCR γδ or anti-TCR αβ were added at 1μg/10^6 cells. The cells were incubated for 20 min on ice and washed twice with labeling buffer. The resuspended cells were then incubated with streptavidin microbeads (10μl/10^7 cells) for 15 min on ice and washed. Cells were then added in a column, which was placed in magnetic activated cell sorter. We collected the entire effluate as a " nonmagnetic" fraction, in which αβ or γδ T cells accounted for >90%, as analyzed by fluorescence staining.

Proliferation assay: one-way reaction and two-way reaction of αβ or γδ T cells to allogeneic splenocytes

Two-way allo-reactivity: proliferative responses of αβ or γδ T cells from spleens of BALB/c mice to splenocytes from C57BL/6 mice were determined in a ^{3}H-TdR incorporation assay. Responder cells (1×10^4 αβ or γδ T cells from BABL/c mice) were cultured with 2×10^4 irradiated (20 Gy x-ray) or not irradiated (living) stimulators (splenocytes from C57BL/6 mice) in triplicate in the wells of 96-well V-bottom microtiter plates (Nunc, Wiesbaden, Germany) in a volume of 0.2 ml. The medium was RPMI-1640 supplemented with 10%heat-inactivated FCS, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2 in air for 6 days. Anti-hsp 60 (1:400) or -hsp70 (1:5000) mAb was added to cultures respectively. Controls included responder or stimulator alone, and cultures without anti-hsp60 or -hsp70 mAb. At the indicated time, individual microcultures were pulsed with 1 μ Ci ^{3}H-TdR per well and incubated for another 6 hours. Thereafter, cultures were harvested onto glass fibers and counted for beta emission using a trace-96 direct beta counter (Berthold).

The one-way mixed lymphocyte reaction in a ^{3}H-TdR incorporation assay was also performed. 5×10^4 T cells (responder), which were separated by nylon wool isolation from splenocytes from BABL/c, co-cultured with irradiated (20Gy) 5×10^4 spleen cells (stimulator) from C57BL/6 mice in 96-well round-bottom microplates (Nunc) for 3 days, while expressions of hsp60 and hsp70 on responder, stimulator and responder +stimulator cells were analyzed at day 2 and day3.

Flow cytometry

For phenotypic analysis, the magnetic isolated cells were stained with FITC-anti-TCRβ and R-PE- anti- TCRγδ. For hsp expression of analysis, cells were stained with anti-hsp60 or anti-hsp70 respectively for 15 min on ice. After two washing steps, cells were then incubated with FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgG for 15 min on ice and washed twice. The cells were resuspended in PBS with 1% paraformaldehyde. All analyses were measured on a FACScan flow cytometer (Becton Dickinson).

Statistical analysis

Analysis of variance was used to test the differences among all tested groups in proliferation assay.

RESULTS

Expression of hsp60 and hsp70 on cultured cells in one-way mixed lymphocyte reaction *in vitro*

As illustrated in fig.1, a conventional proliferation of murine splenic T cell (BALB/c) anti allogeneic irradiated splenocyte (C57BL/6) was obviously observed during 3 days of culture in vitro. However, the event in this process we are interested in is whether the response of T cells to allogeneic stimulation could correspond with hsp expression on cells. As shown in fig.2, both freshly isolated responder (BALB/c) splenic T cells and stimulator (C57BL/6) splenocytes could express surface hsp60 molecule in minor population (< 6%, A and B). Responder cells maintained this level of hsp60 expression at day2 (C) and increased at day3 (F) with 25%. The hsp60 expression in irradiated (20Gy) stimulator cells increased with prolonging time of culture (at day2, 43% and at day3, 62%). In the cultures of responder mixed with irradiated stimulator cells, there was increasing positive cell percentage in the whole population accounted for 49% at day2 and 67% at day3 respectively. At same time point, the sequence for the levels of $hsp60^+$ cell percentages is as follows: responder with stimulator (R with S) > stimulator (S) > responder (R). We used a formula to calculate a net increasing percentage in cells from the culture of responder with stimulator. That is, %net increasing = %R with S - [%R (responder) + %S (stimulator)/2]. According the calculation with this formula, we found the net increasing percentages in cultures of responder with stimulator cells were 24.5% at day2 and 23.5% at day3. It indicates that an increasing hsp60 expression exists in cultures of responder with stimulator cells, when compared with responder or stimulator cells at same time. Similar manifestation also happened in the case of hsp70 expression in same test system (fig.2 I to P). Before culture, both responder T cells and stimulator splenocytes expressed hsp70 molecule on their surface in minor population (8%, I and J). After culture, responder cells almost kept this level at day2 (K, 6%) and increased at day3 (N) with 43%. The hsp70 expression in irradiated stimulator cells increased with prolonging time of culture (at day2, 50% and at day3, 24%). The $hsp70^+$ cell percentages in the cultures of responder with irradiated stimulator cells significantly increased (38% at day2 and 58% at day3). Their net percentages were 10% at day2 and 29% at day3.

The alloreactive proliferative responses of γδT or αβT cell to allogeneic stimulator cells in one-way or two-way reaction were completely or partially blocked by anti-hsp60 antibody *in vitro*

To directly address the role of hsp60 and hsp70 in allo-reactivity of αβT or γδT cells (BABL/c), we performed the experiment as illustrated in fig. 4. As noted elsewhere, γδT cells were not as good responder as αβT cells to alloantigens.

In one-way reaction, there was no growth detected in cultures of γδT cells plus irradiated allogeneic spleen cells (C57BL/6), which was similar with the cases of all controls, such as γδT cells, αβT cells, irradiated or not irradiated (living) stimulator cells alone. A detectable proliferation was found in the cultures of γδT cells with living allogeneic cells (two-way reaction) during 3 to 4days of incubation in vitro ($P<0.05$, when compared with all controls or γδT cells plus irradiated allogeneic stimulator cells), while this response was completely blocked by adding mAb anti-hsp60 to culture ($P<0.05$, γδT cells + living stimulator versus (vs) γδT cells + living stimulator + anti-hsp60), and was not affected at all by anti-hsp70 ($P>0.05$, γδT cells + living stimulator vs γδT cells + living stimulator + anti-hsp70). Differentially to γδT cells, αβT cells demonstrated irradiated allo-lymphocyte-induced proliferation at the top level on day4. We interestingly noted that this allo-reactive response was not influenced by adding both mAbs anti-hsp60 and anti-hsp70 to cultures ($P>0.05$, when compared these groups). However, when the irradiated stimulator was changed into living allogeneic cells, anti-hsp60 antibody could blocked the proliferative allo-reactivity of between αβT cells and allogeneic living spleen cells ($P<0.05$, blocked vs not blocked), which was the strongest proliferation in this tested system ($P<0.05$, when compared with other groups except αβT cells plus living stimulator with anti-hsp70). Anti-hsp70 antibody did not show any blocking effect on two-way allo-reaction between αβT cells and allogeneic living spleen cells. As clearly shown in fig.4, the following proliferative responses during the period from day3 to day5 were at same level ($P>0.05$, when compared with each other): αβT cells plus irradiated stimulator with or without mAbs anti-hsp60 or anti-hsp70, αβT cells plus living stimulator with mAb anti-hsp60, and γδT cells plus living stimulator with or without mAb anti-hsp70. All statistical analyses above were basely on data from the determination at time from day3 to day4.

Expression of hsp60 and hsp70 on cultured αβ and γδ T cells and stimulator cells in one-way or two-way mixed lymphocyte reaction *in vitro*

The both freshly isolated αβ and γδ T cells (BALB/c) express hsp60 and hsp70 at similar level to T cells isolated with nylon wool as shown in fig2 (data not shown). At the top of alloreactive proliferation in one-way or two-way mixed lymphocyte reaction at day4 (fig4), we analyzed the expressions of these two hsps on cultured cells (fig.5). After 4days of culture, γδ T cells alone had 40% of $hsp60^+$ cells (A) and 53% of $hsp70^+$ cells (G) respectively, whereas αβT cells alone possessed 26% of $hsp60^+$ cells (D) and 25% of $hsp70^+$ cells (J) respectively. Among γδ or αβT cells with stimulator (C57BL/6) irradiated splenocytes (one-way reaction), there were 60% (B, γδ) or 38% (E, αβ) of $hsp60^+$ cells, as well as 49% (H, γδ) and 37% (K, αβ) of $hsp70^+$ cells respectively. On the other hand, there were 48% (C) of $hsp60^+$ cells and 43%

(I) of hsp70$^+$ cells in cultures with γδT cells plus not irradiated stimulator cells, whereas there were 44% (F) of hsp60$^+$ cells and 53% (L) of hsp70$^+$ cells in cultures with αβT cells plus not irradiated stimulator cells. We had analyzed cultured cells with γδ or αβT cells plus stimulator with or without irradiation via dual color staining by means of anti-TCRαβ-PE or anti-TCRγδ-PE plus anti-hsp60/-hsp70-FITC, the results showed that all non-αβ T cells or non-γδT cells in the cultures were 100% positive for hsp60 or hsp70 (data not showed).

DISCUSSION

Although hsps have basically intracellular expression under a stress, the expression of both hsp60 and hsp70 molecules on cellular surface were observed in present investigation. We found that there was the expression of both hsp60 and hsp70 on the cells in one-way mixed lymphocyte reaction *in vitro*. In our experimental system, these hsps expressed at small proportion in responder cells (T cells), at large rate in irradiated stimulator lymphocytes, and at the highest percentage in the responder with irradiated stimulator cells. Some T cells cultured with only medium could express hsp60 and hsp70 on surface. As every immunologist knows, T cells tends to die *in vitro* if they would not be further stimulated by antigens, mitogens, or T cell growth factor, such as IL-2. If no stimulation for T cells *in vitro* could serve as a stress to T cell, it would not be any difficulty for us to understand the phenomenon of hsp expression in this condition. Furthermore, irradiated lymphocytes expressed high rates of hsp60 (significantly) and hsp70 molecules in same cultural condition. X-ray irradiation seems to be a stimulus for hsp expressions of the lymphocytes. The highest expression of both hsp60 and hsp70 happened in the case of the mixture of T responder cells with allogeneic irradiated lymphocytes *in vitro*. After the spontaneous expressions of both hsp60 and hsp70 were excluded, the net increasing rates of these expressions were found in this case. It strongly suggests that an allogeneic stimulus could trigger inducible hsp expression on cellular surface.

Based on the analysis above, we have a reason to postulate that hsps might be alloantigens or alloantigen-related molecules to participate in the allo-reactivity of lymphocytes as someone have already presumed it several years ago. [16] We then designed a further experimental system *in vitro*. In the design, we had stressed the three key points as follows: (1) to check possible different alloreactive responses of αβT and γδT cells to hsp molecules; (2) both one-way and two-way mixed lymphocyte reaction might be all necessary for the evaluation of the role of hsp in all-reactivity, because the hsps could express on both alloreactive lymphocytes; (3) checking possible blocking effects of anti-hsp antibodies on alloreactive response is important to definite hsp-

reactive response in this system. As expected, and noted by someone else[27], it is difficult to induce a response of γδT cells against irradiated allogeneic lymphocytes, unlike the situation with αβT cells. However, an alloreactive proliferation was found in two-way allogeneic reaction in the mixture of γδT cells with living allogeneic lymphocytes. Moreover, this proliferation was completely blocked by anti-hsp60 antibody added to the culture, whereas an increasing expression of hsp60 molecule was detected at same time on the cells of both γδT cells cultured alone and γδT cells with allogeneic spleen cells. Although hsp60 expressed in 60% of the cells cultured with irradiated allogeneic splenocytes, there was no cell growth detected in this culture yet. However, a complete hsp-reactive alloreactive response of γδT cells with allogeneic spleen cells existed in anti-allo-lymphocyte/γδT cells in our experimental system. In striking contrast, αβT cells responded to irradiated allogeneic spleen cells at similar proliferative level to γδT cells with living allogeneic cells, while there was the strongest cell growth in the mixture of αβT cells with living allogeneic spleen cells. Alloantigens are obviously good stimuli for the response of αβT cells. In blocking test, we found that both anti-hsp60 could partially blocked two-way allo-reactivity with 31-43% inhibition rate at day3 to day5 of culture, but did not affect one-way allo-reactive response. Similarly to the case of γδT cells, we also found the expression of both hsp60 and hsp70 on the cells in the cultures of αβT cells without or with irradiated or not irradiated allogeneic spleen cells, which were all positive for hsps. In spite of high rate of $hsp70^+$ cells in two-way reaction culture, there was not a blocking effect of banti-hsp70 antibody to be detected in the experimental system. It indicates that hsp70 seems not to be related to allo-reactivity or only to participate in antigen presentation in intracellular environment.

Taken together, our results indicate that alloreactive response in two-way reaction of both γδT cells to allogeneic spleen cells could be a complete hsp60-dependant, that is, hsp60 serves as an exclusive alloantigen in our experimental system. On the other hand, the allo-reactivity of αβT cells only partially due to hsp60, while MHC class I and II alloantigens might be responsible for remaining effect. If this mechanism could be confirmed in humans both *in vivo* and *in vivo*, a strategy based on blocking hsp60-dependant allo-reactivity might be beneficial to the control of allgraft rejection in clinic. Besides, it is need to clarify the further questions: what is exact role of hsp60 in allo-reactive response? Is it target antigen itself, or an antigen present molecule? The resolution of these question will provide further evidence for the participation of hsp in alloreactive response.

ACKKNOWLEDGEMENTS

This work was supported by a grant from Japan-China Medicine Friendship

Foundation in 1998.

REFERENCES

1. LINDQUIST S. (1986) The heat shock response. *Annu Rev Biochem* 55, 1151.
2. WELCH W.J. (1987) The mammalian heat shock or stress response: a cellular defense mechanism. *Adv Exp Med Biol* 225, 287.
3. ELLIS J. (1987) Proteins as molecular chaperonins. *Nature* 328, 378.
4. LINDQUIST S & CRAIG E.A. (1988) The heat-shock proteins. *Annu Rev Genet* 22, 631.
5. YOUNG R. & ELLIOT T. (1990) Stress Proteins, infection, and immune surveillance. *Cell* 59,5.
6. CURRIE R.W., TANGUAY R.M. & KINGMA J.G. (1993) Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. *Circulation* 87, 963.
7. GEORGOPOULOS C. & WELCH W.J. (1993) The role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* 9, 601.
8. BRCKMANN R.P., MIZZEN L.E. & WELCH W.J. (1990) Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* 248, 850.
9. STERN L.J. & WILEY. (1992) The human class II MHC protein HLA-DR1 assembles as empty ab heterodimers in the absences of antigenic peptide. *Cel* 68, 465.
10. LAKEY E.K., MARGOLIASH E. & PIERCE S.K. (1987) Identification of a peptide binding protein which plays a role in antigen presentation. *Cproc Natl Acad Sci USA* 84, 1659.
11. VAN BUSKIRK V.A., DENAGEL D.C., GUAGLIARDI LE, BRODSKY FM & PIERCE SK. (1991) Cellular and subcellular distribution of PBP72/74, a peptide-binding protein that plays a role in antigen processing. *J Immunol* 146,500.
12. VAN BUSKIRK V.A., CRUMP B.L., MARGOLIASH E. & PIERCE S.K. (1989) A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. *J Exp. Ed* 170, 1799.
13. REES A.D.M., CHIANG H.L., OLSON R.S. & DICE J.F. (1991) Stress-induced modulation of antigen-presenting cell function. *Immunology* 74, 386.
14. KAUFMANN S. (1990) Heat shock proteins and the immune response. *Immunol Today* 11, 129.
15. UDONO H. & SIVASTRAVA P. (1991) Heat shock protein 70-associated peptides elicit specific cancer immunity. *IJ Exp Med* 178, 1391.

16. MOLITERNO R., VALDIVIAL L, PAN F. & DUQUESNOY RJ. (1995) Heat shock protein reactivity of lymphocytes isolated from heterotopic rat cardiac allograft. *Transplantation* **59**, 589.
17. SANDBERG JAN-OLOV, MARGULIS B., JANSSON LEIF, KARLSTEN R & KORSGREN O. (1995) Transplantation of fetal porcine pancreas to diabetic or normoglycemic nude mice. *Transplantation* **59**,1665.
18. QIAN J., MOLITERNO RA, ANN DONOVAN-PELUSO M., *et al.* (1995) Expression of stress proteins and lymphocyte reactivity in heterotopic cardiac allografts undergoing cellular rejection. *Transplant Immunology* **3**, 114.
19. LIU K., MOLITERNO R.A., FU X-F. & DEQUESNOY R.J. (1997) Identification of two types of autoreactive T lymphocyte clones cultured from cardiac allograft-infiltrating cells incubated with recombinant mycobacterial heat shock protein 71. *Transplant Immunology* **5**, 57.
20. BABA H.A., SCHMID C., WILHELM, M.J., BLASIUS, SCHELD H.H., BOECKER W & DOCKHORN-DWORNICZARK. (1997) Inducible heat shock protein 70 in rat cardiac allograft and its immunohischemical location in cardiac myocytes. *Transplantation* **64**,1035.
21. DAVIS E.A., WANG B.H., STAGG C.A., BALDWIN W.M., BAUMGARTNER W.A., SANFILIPPO & UDELSMAN R. (1996) Induction of heat shock protein in cardiac allograft rejection-a cyclosporine-suppressible response. *Transplantation* **61**,279.
22. ALEVY Y.G., BRENNAN D., DURRIYA S., HOWARD T. & MOHANAKUMAR T. (1996) Increased expression of the HDJ-2 heat shock protein in biopsies of human rejected kidney. *Transplantation* **61**,963.
23. TRIEB K., GRUBECK-LOEBENSTEIN B., EBERL T. & MARGREITER R. (1996) T cells from rejected human kidney allografts respond to heat shock protein 72. *Transplant Immunology* **4**, 43.
24. BABA H.A., SCHMID K.W., SCHMID C., BLASIUS S., HEINECKE A., KERBER S., SCHELD H.H., BOECKER W.& DOCKHORN-DWORNICZARK. (1998) POSSIBLE RELATIONSHIP BETWEEN heat shock protein 70, cardiac hemodynamics, and survival in the early period after heart transplantation. *Transplantation* **65**,799.
25. BRENNER M.B., MCLEAN J., DISLYNAS D.P., *et al.* (1986) Identification of a putative second T-cell recepto. *Nature*322,145.
26. BUCY R.P., CHEN C.L. & COOPER M.D. (1989) Tissue localization and CD8 accessory molecule expression of T γδ cells in humans. *J Immunol* **142**, 3045.
27. LANNIER L.L. (1995) Unusual lymphocytes-γδ T cells and NK cells. *The Immunologist* **3**, 182.

Legends:

Figure 1. The proliferative response of BABL/c splenic T cells (R) freshly isolated by nylon wool column to allogeneic (C57BL/6) irradiated (20Gy) splenocytes (S) in vitro. The data represent the mean value of triplicate wells ±SD from 7 cases of mice

Figure 2. The expressions of hsp60 and hsp70 on cells in one-way mixed lymphocyte reaction *in vitro*. The culture protocol was as same as one in fig.1. Cells were stained with mAbs anti-hsp60 (A to H) and anti-hsp70 (I to P) followed stained with FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgG. The curves with solid areas are FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgG staining alone as isotype control, while ones with empty areas are anti-hsp60- or -hsp70-FITC. Samples: A and I: BABL/c splenic T cells freshly isolated by nylon wool column before culture; B and J: splenocytes from fresh C57BL/6 mouse before culture; after 48 hours (C to E and K to M) or 72 hours (F to H and N to P) of culture in vitro, the following cells were stained: responder cells (BABL/c splenic T cells, C, F, K and N), stimulator cells (irradiated C57BL/6 splenocytes, D, G, L and O) and responder + stimulator cells (E, H, M and P).

Figure 3. Immunofluorescence analysis of murine αβT and γδT cells isolated by magnetic activated cell sorter. The sorted cells were stained with anti-TCRαβ–FITC and anti-TCRγδ-PE. FL1-H: TCRαβ–FITC. FL2-H: TCRγδ–PE.

Figure 4. The alloreactive proliferative responses of αβT or γδT cells to allogeneic stimulator cells in one-way or two-way reaction. αβT or γδT cell (BABL/c) were co-cultured with stimulator allogeneic spleen cells (C57BL/6) with (two-way) or without (one-way) x-ray irradiation (20Gy) for 6 days. The mixed lymphocyte cultured cells were added or not by mAb anti-hsp60 (1:400, ahsp60 in legend) or anti-hsp70 (1:5000, ahsp70 in legend) respectively. Living stimulator in legend means the C57BL/6 splenocytes without irradiation. Data were expressed as mean cpm of triplicate culture from three separate experiments. SD were <10% of mean values.

Figure 5. The expressions of hsp60 and hsp70 on cells in one-way or two-way mixed lymphocyte reaction in vitro. The culture protocol was as same as one in fig.4 and staining was performed at day4 of culture. Cells were stained with mAbs anti-hsp60 (A to F) and anti-hsp70 (G to L) followed stained with FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgG. The curves with solid areas are FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgG staining alone as isotype control, while ones with empty areas are anti-hsp60- or -hsp70-FITC. Samples:

A and G: γδT cells (BABL/c) alone; B and H: γδT cells (BABL/c) with C57BL/6 splenic irradiated (20Gy) cells; C and I: γδT cells (BABL/c) with C57BL/6 splenic cells; D and J: αβT cells (BABL/c) alone; E and K: αβT cells (BABL/c) with C57BL/6 splenic irradiated (20Gy) cells; F and L: αβT cells (BABL/c) with C57BL/6 splenic cells.

Abbreviation:

hsp(s), heat shock protein(s); TCR, T cell receptor; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; R-PE, R-phycoerythrin; Sav-PE, Streptavidin-phycoerythrin.

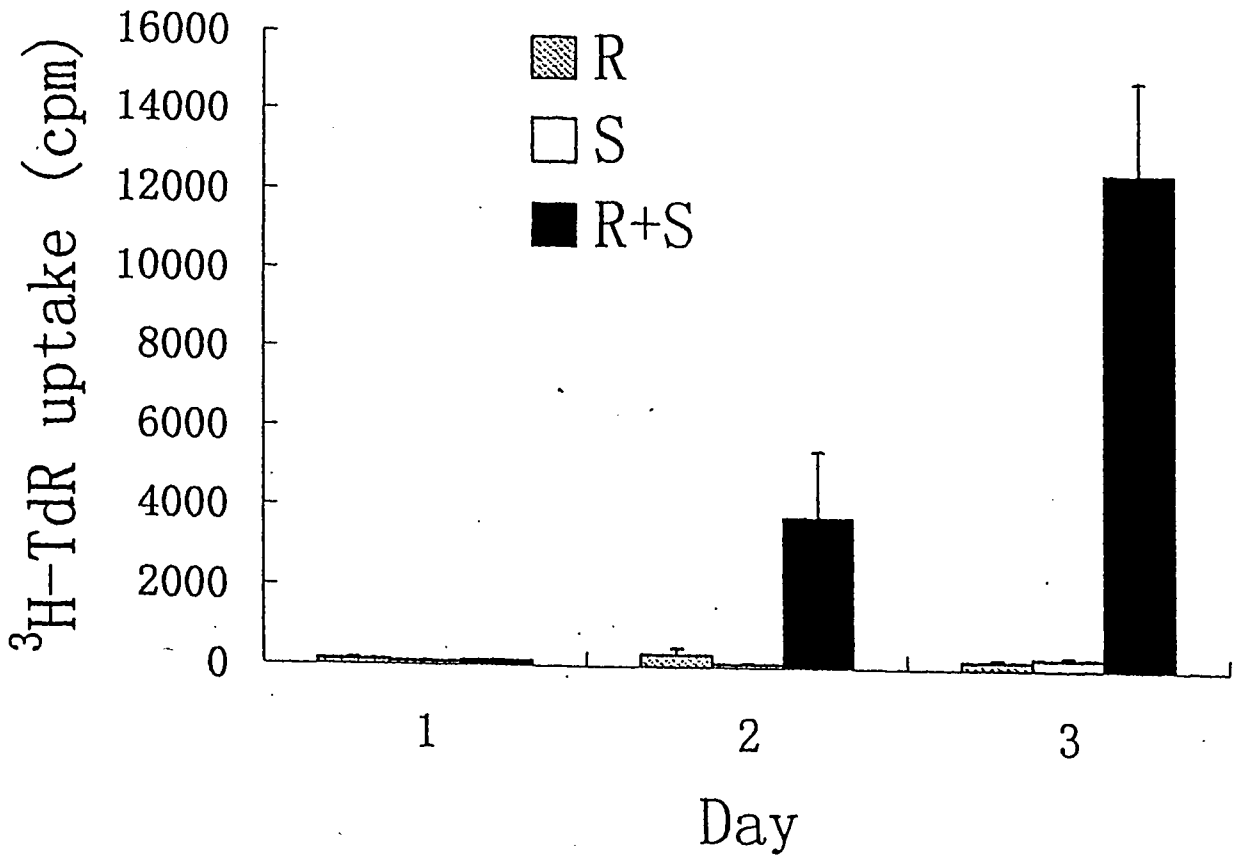

16000
14000
12000
10000
8000
6000
4000
2000
0
^{3}H-TdR uptake (cpm)
R
S
R+S
1
2
3
Day

Figure 1

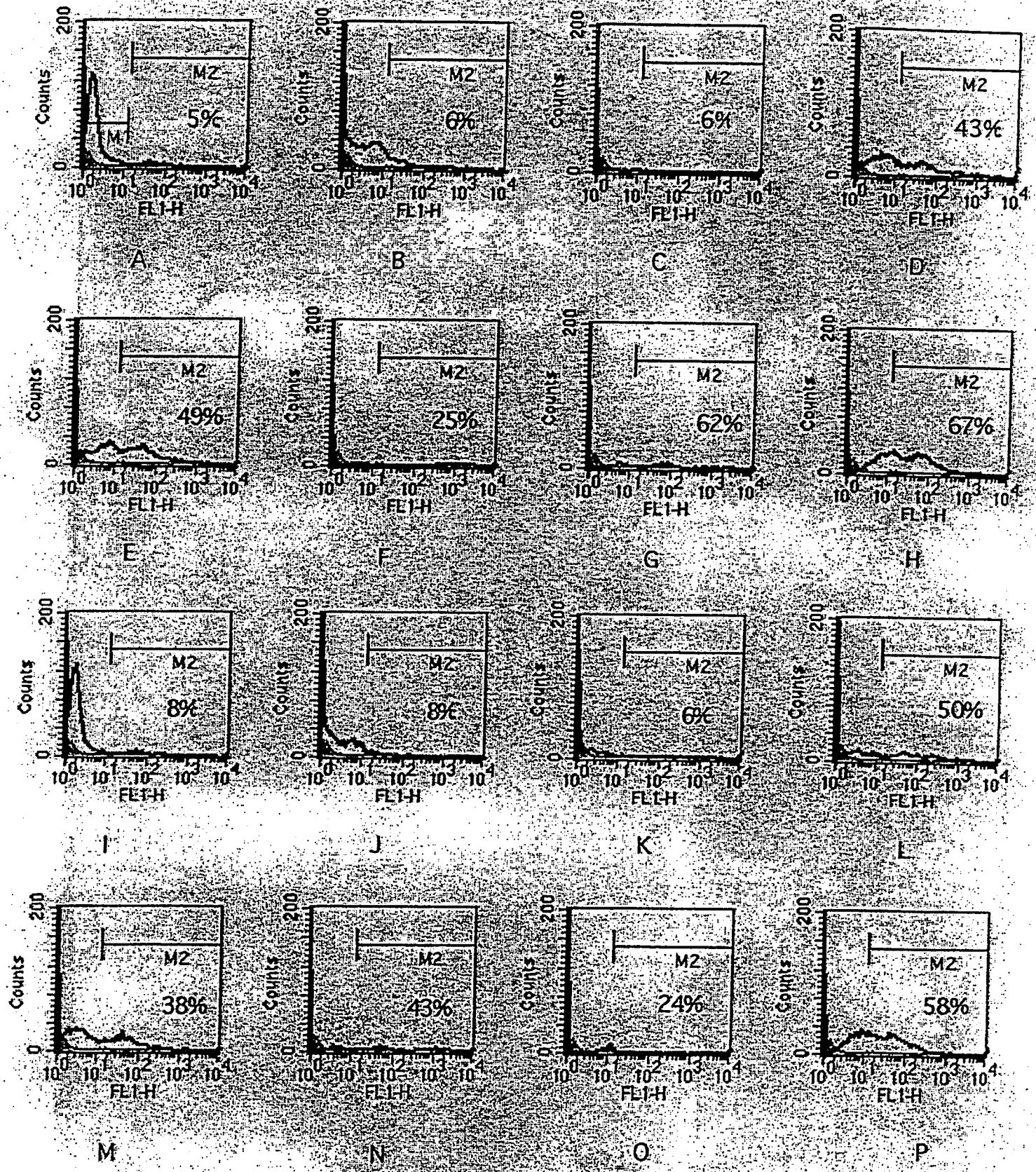
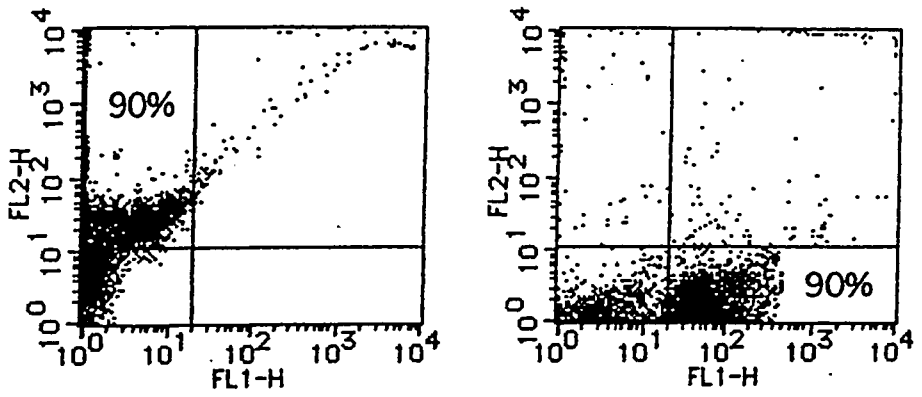

Counts
FL1-H
M1
M2
5%
A
6%
B
6%
C
43%
D
49%
E
25%
F
62%
G
67%
H
8%
I
8%
J
6%
K
50%
L
38%
M
43%
N
24%
O
58%
P

Fig 2


90%
FL2-H
FL1-H
10^0
10^1
10^2
10^3
10^4
90%
FL2-H
FL1-H
10^0
10^1
10^2
10^3
10^4

A B

Figure 3

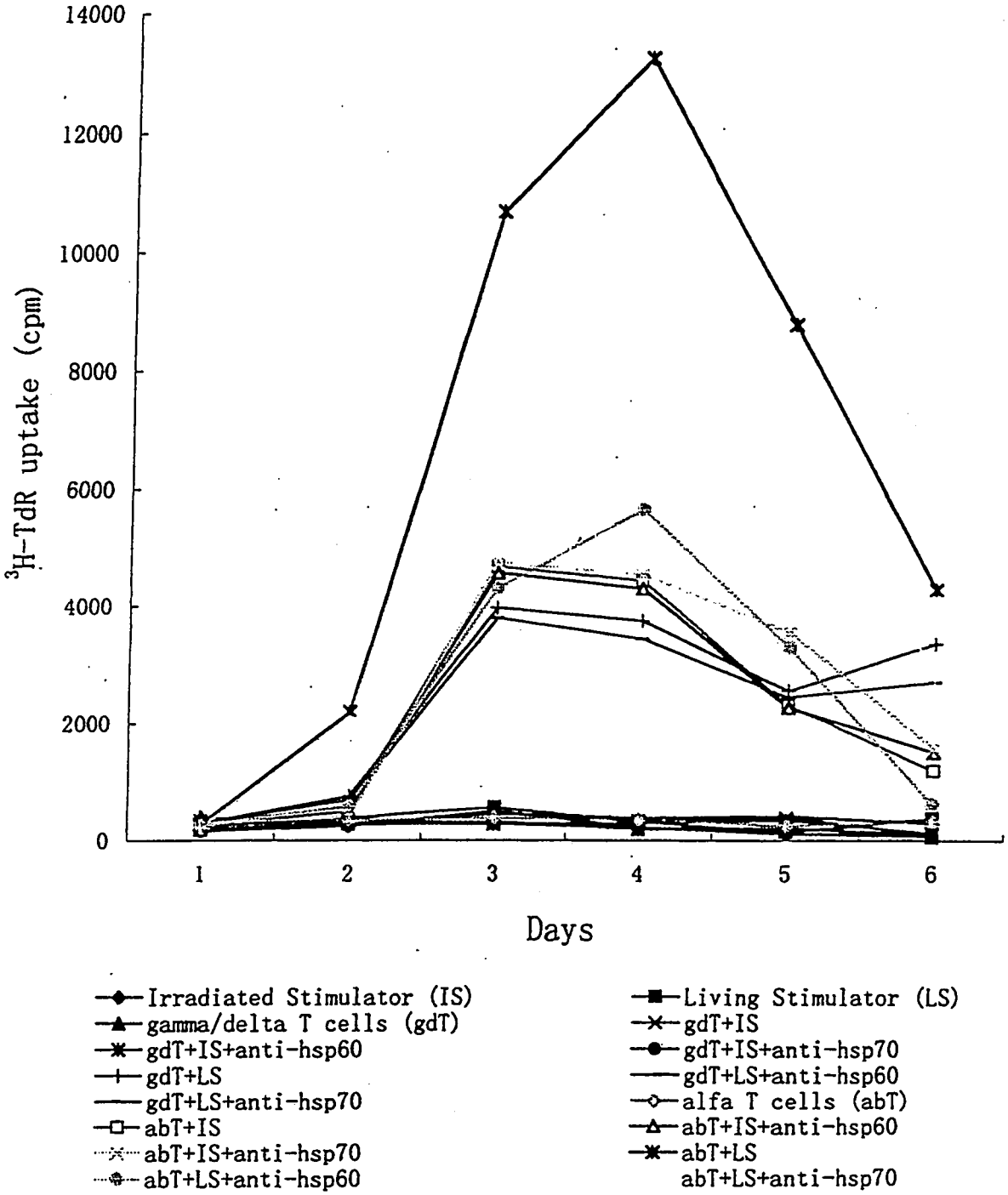

14000
12000
10000
8000
6000
4000
2000
0
^{3}H-TdR uptake (cpm)
1
2
3
4
5
6
Days
Irradiated Stimulator (IS)
gamma/delta T cells (gdT)
gdT+IS+anti-hsp60
gdT+LS
gdT+LS+anti-hsp70
abT+IS
abT+IS+anti-hsp70
abT+LS+anti-hsp60
Living Stimulator (LS)
gdT+IS
gdT+IS+anti-hsp70
gdT+LS+anti-hsp60
alfa T cells (abT)
abT+IS+anti-hsp60
abT+LS
abT+LS+anti-hsp70

Fig 4

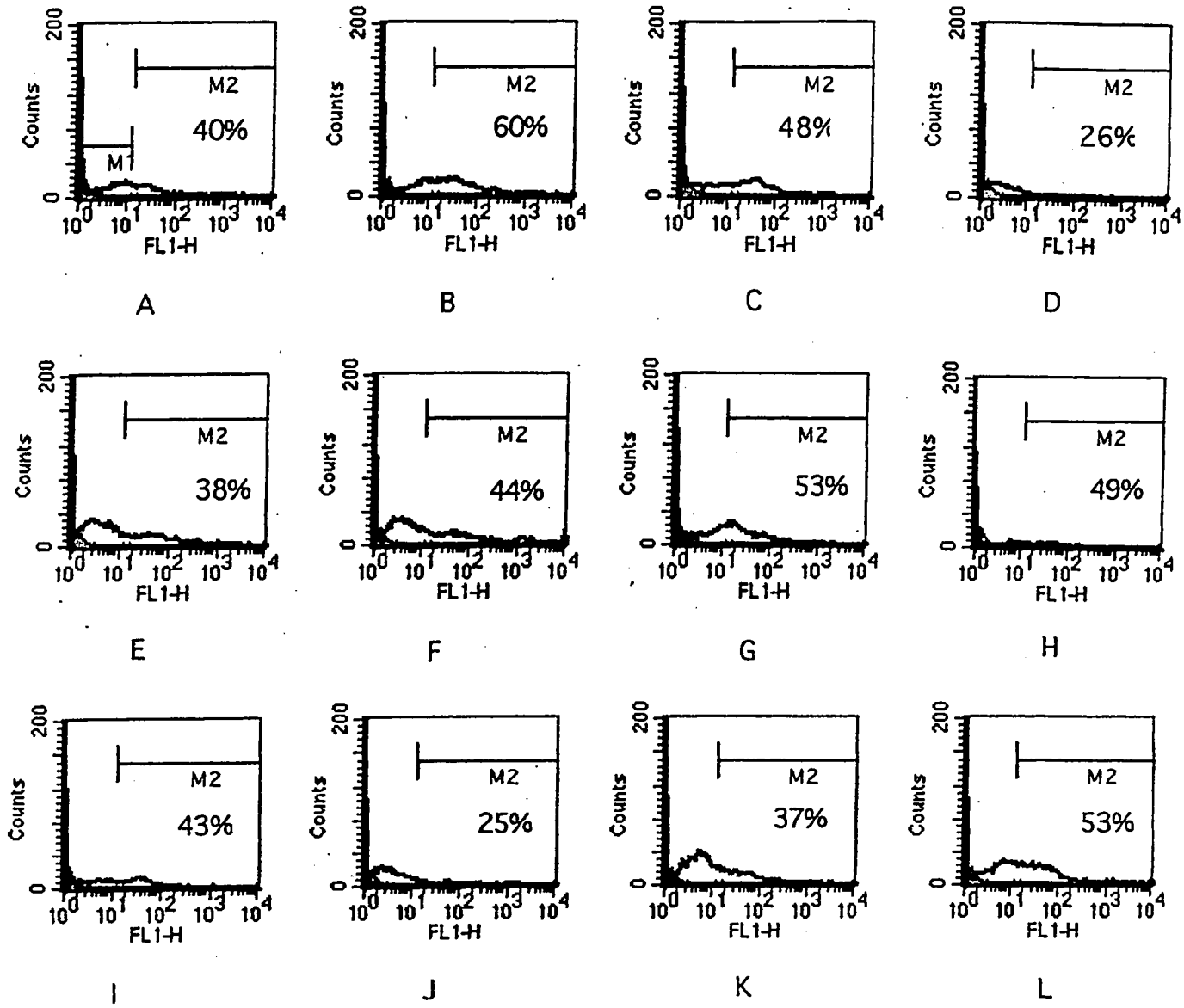

M1
M2
40%
60%
48%
26%
38%
44%
53%
49%
43%
25%
37%
53%
Counts
FL1-H
A
B
C
D
E
F
G
H
I
J
K
L

Fig 5