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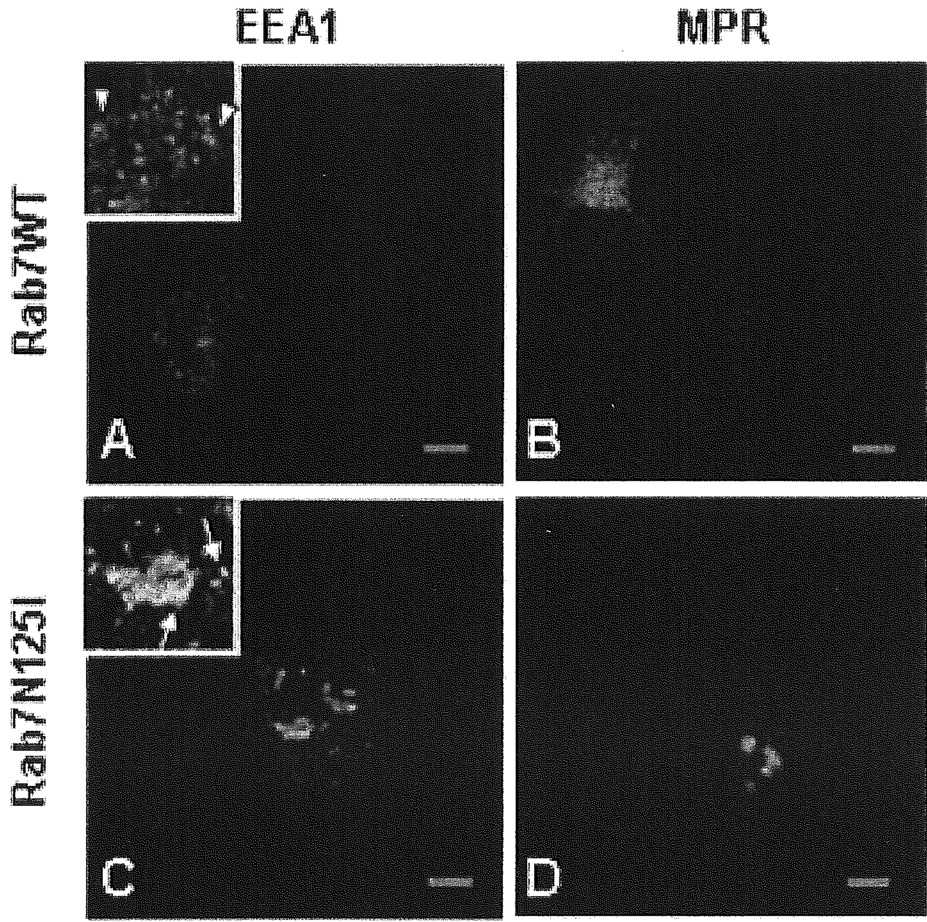

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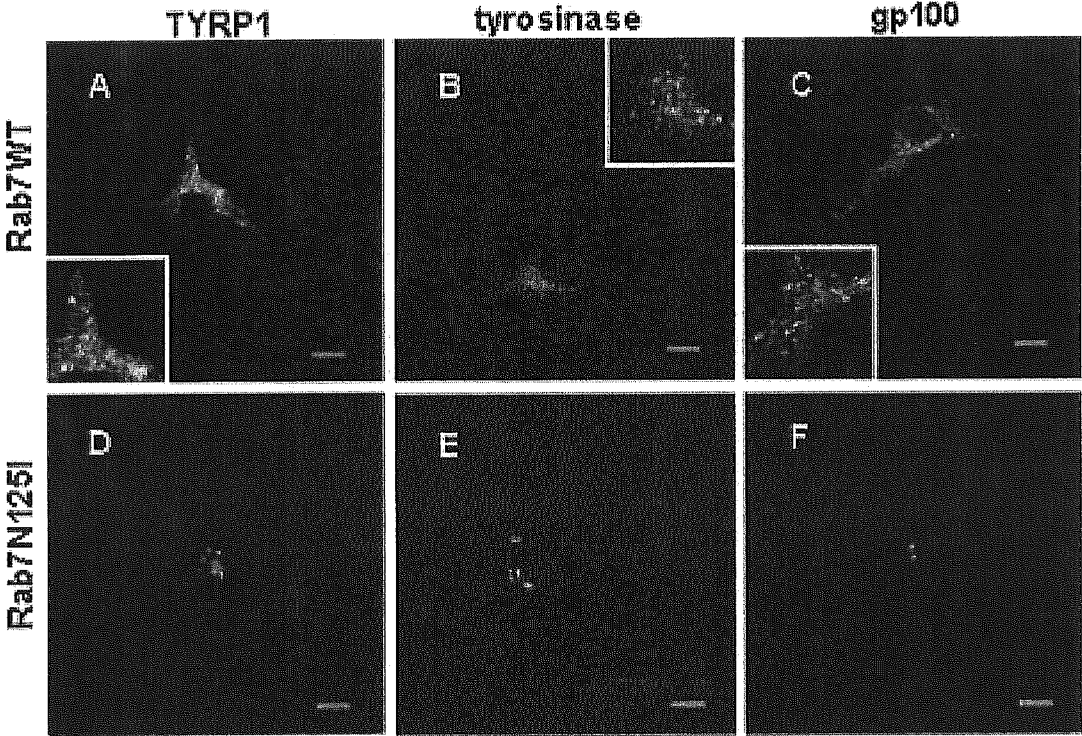

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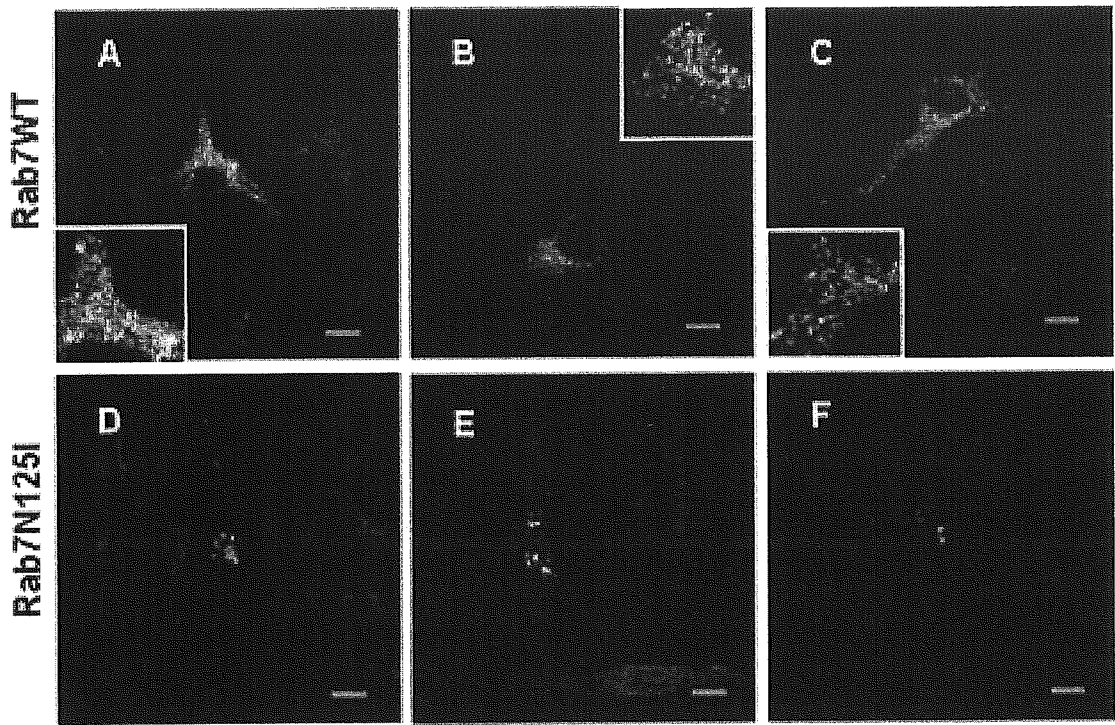

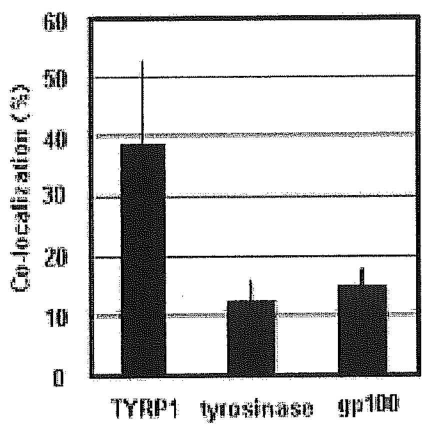

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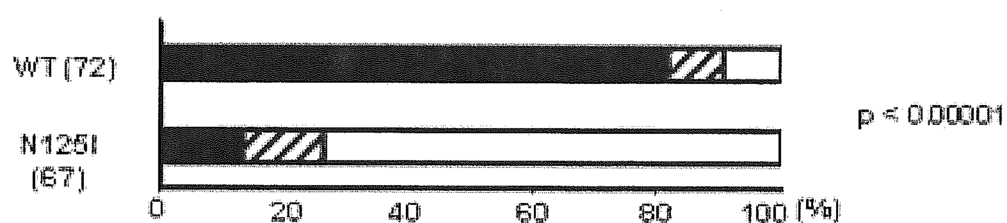
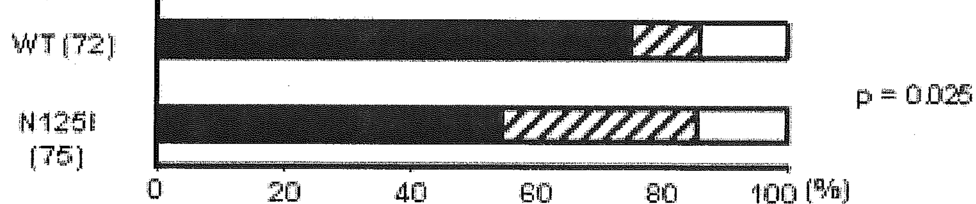
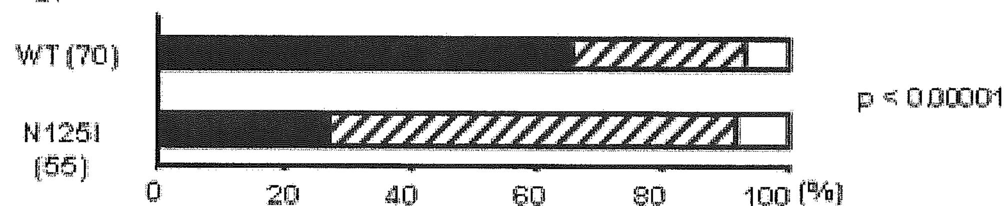
A TYRP1



B tyrosinase



C gp100



perinuclear peripheral agranular

Figure 4
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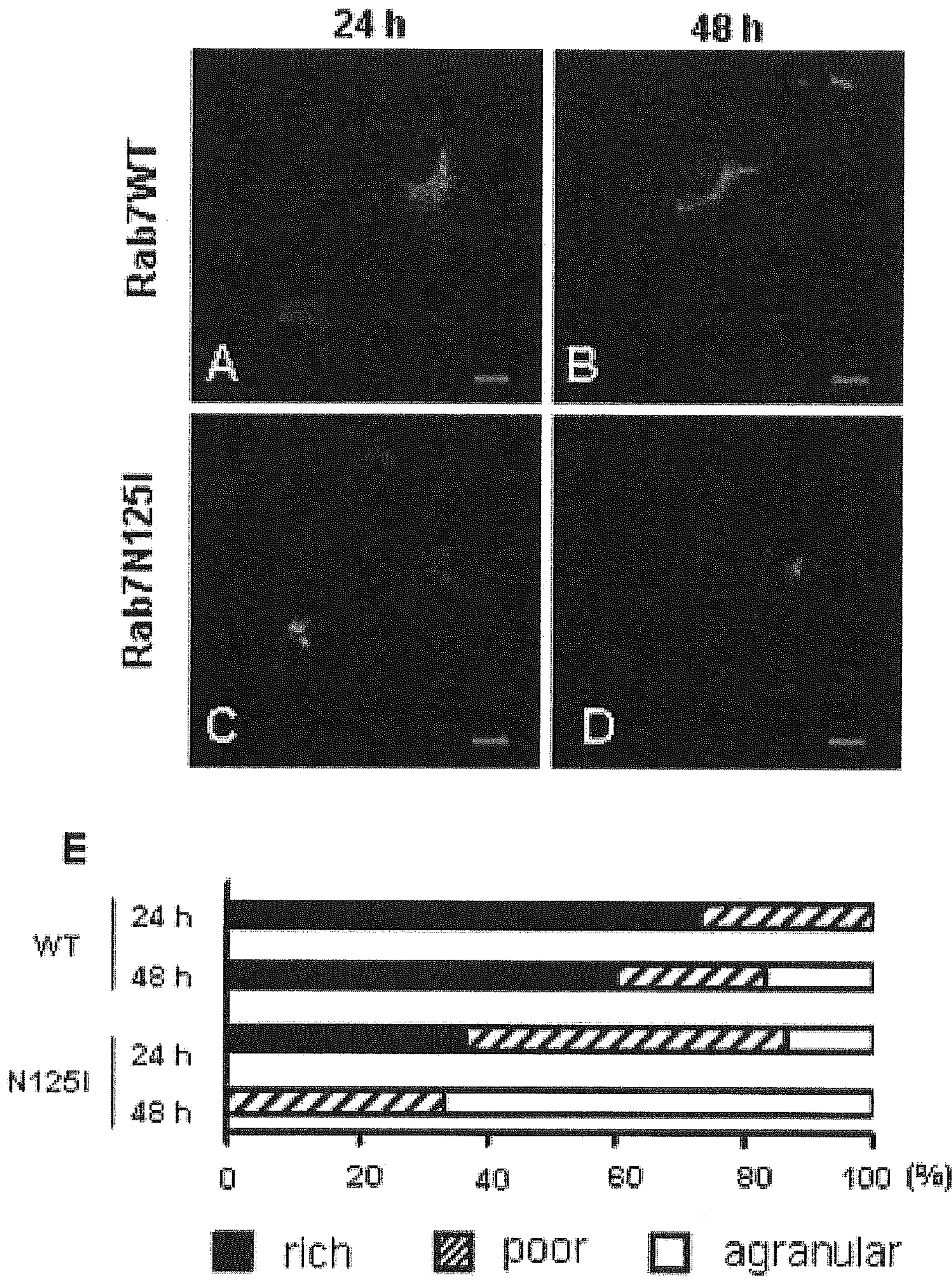

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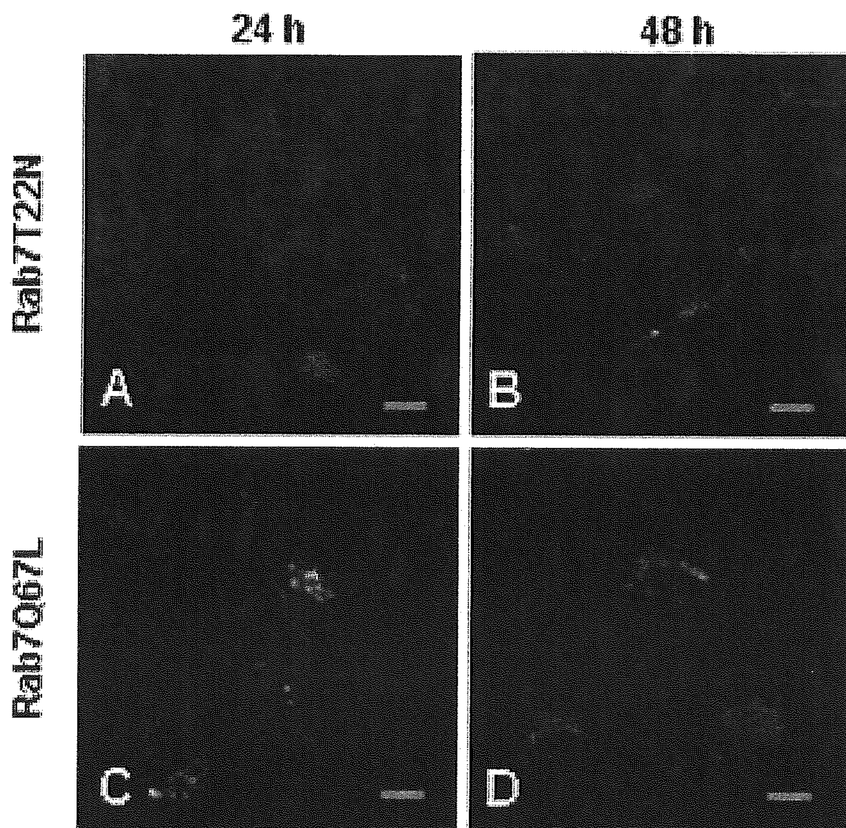

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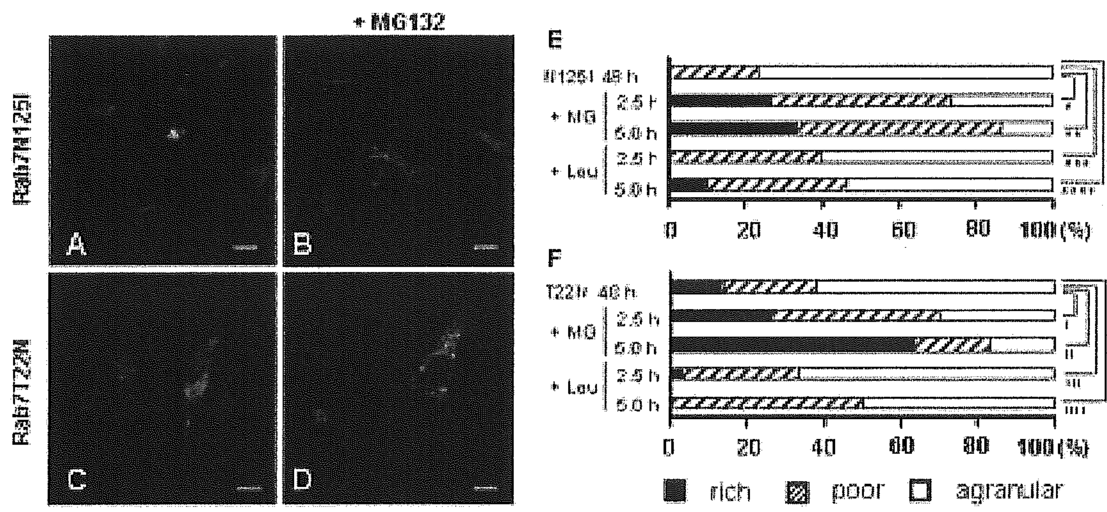

ELSEVIER

Mechanism of the Immunosuppressive Effect In Vivo of Novel Immunosuppressive Drug β-SQAG9, Which Inhibits the Response of the CD62L$^+$ T-Cell Subset

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ABSTRACT

Introduction. We synthesized sulfo-glycolipid, β-SQAG9 (designate □ β-SQAG9 liposome, because it efficiently forms a liposome structure) that possessed immunosuppressive effects such as inhibition of T-cell responses in human allogeneic MLR and skin allograft survival in rats, and bound to CD62L (L-selectin) in vitro. In this study, we further investigated the immunosuppressive mechanism in vivo by β-SQAG9 liposome in a skin-allografted rat model.

Methods. ACI rats (RT1^a) were grafted skin of LEW rats (RT1^1) treated with PBS or β-SQAG9 liposome IV once a day for 7 days. Subsequently, we investigated the population of T cells and CD62L$^+$ T-cell subset in the spleen, axillary lymph nodes (ALNs), and peripheral blood of skin-allografted rats by two-color flow cytometry.

Results. Five of 11 (45.5%) rats that were treated with 50 mg/kg β-SQAG9 liposome showed graft survival and another showed moderate rejection in graft. The CD62L$^+$ T-cell subset population in ALNs of β-SQAG9 liposome-treated rats decreased in a dose-dependent manner. No significant difference in the T-cell population was observed between the β-SQAG9 and control groups. These data suggest that β-SQAG9 could bind to the CD62L$^+$ T-cell subset in vivo as well as in vitro and affect T-cell migration, which might lead to T-cell tolerance in vivo.

WE PREVIOUSLY REPORTED that the synthetic 3-O-(6-deoxy-6-sulfono-β-D-glucopyranosyl)-1,2-di-O-acylglycerol (β-SQDG(18:0)), which was derived from sulfoquinovosyl diacylglycerol of sea urchin, possessed immunosuppressive effects, namely inhibition of T-cell responses in human allogeneic MLR and skin allograft survival in rats.[1,2] Subsequently, it was demonstrated that β-SQAG9, which had been newly synthesized from β-SQDG(18:0) to improve its structural stability in aqueous solution but having the same biological activities, bound CD62L (L-selectin) in vitro.[3] β-SQAG9 efficiently forms a liposome structure that bound L-selectin on the cell surface of the CD62L$^+$ T-cell subset but might not be incorporated into cells. In this study, therefore, we investigated the CD62L$^+$ T-cell subset population in the spleen, axillary lymph nodes (ALNs), and peripheral blood of skin-allografted rats treated with or without β-SQAG9. No significant difference in the T-cell populations was observed between the β-SQAG9 and the control groups. Surprisingly, β-SQAG9-treated rats showed a significant dose-dependent decrease in the CD62L$^+$ T-cell subset population in ALNs. These data suggest that β-SQAG9 binds to the CD62L$^+$ T-cell subset in vivo as well as in vitro, thereby affecting T-cell migration.

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360 Park Avenue South, New York, NY 10010-1710

0041-1345/05/$–see front matter
doi:10.1016/j.transproceed.2004.12.204

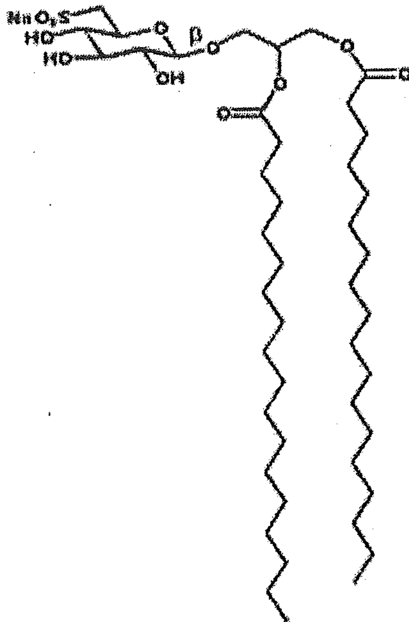

Fig 1. Chemical structure of β-SQAG9.

MATERIALS AND METHODS

Synthesis and Preparation of β-SQAG9 Liposomes

1,2-di-O-acyl-3-O-(-D-sulfoquinovosyl)-glyceride with two stearic acids (β-SQAG9) was synthesized in accordance with our previous report.[4] Its chemical structure is shown in Fig 1. β-SQAG9 liposomes were prepared using a conventional technique similar to the method described by our previous report.[5] Briefly, β-SQAG9 mixed with cholesterol at a ratio of 3:1 was dissolved in chloroform methanol H_2O = 100:10:1 in a test tube. The organic solvent mixture was removed using a rotary flash evaporator under reduced pressure. The residual organic solvent was further removed by drying overnight at room temperature in a desiccator under vacuum. The dried lipid film was hydrated with PBS (pH 7.4), followed by incubation at 64°C for 30 minutes. The test tube was then shaken vigorously on a vortex mixer. Vesicles were filtered for uniform 200-nm pore size.

MTT Assay

The MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, St Louis, Mo, USA) assay was performed using the Jurkat human T-cell lymphoma line. Cells (5×10^3 per well) were cultured in 96-well flat-bottom plates for 24 hour before glycolipids were added. Following cultivation for 48 hour, 50 μg of MTT was added to the culture medium for a 3-hour incubation. Then 4% hydrochloric acid in isopropanol added to each well was mixed with a pipette to destroy the cells. The absorbance in each well was measured using a multiwell scanning photometer (Micro ELISA MR600, Dynatech Laboratories, Alexandria, Va, USA) at a wavelength of 570 nm. Percent growth was defined as absorbance of the well with glycolipid versus absorbance of the well with the control PBS added.

Human and Rat Allogeneic MLR Assay

Peripheral blood lymphocytes (PBL) from healthy human volunteers were obtained by density centrifugation. PBL obtained from one donor as stimulator cells, namely antigen-presenting cells (APCs), were prepared by incubating 10^6 cells/mL with 250 μg/mL mitomycin C for 30 minutes. Likewise, lymphocytes from spleens of Lewis rats ($RT1^1$; Charles River Japan, Tsukuba, Japan) employed as APCs were prepared as APCs in the same manner. After treatment, these cells were washed with RPMI1640 (Sigma) supplemented with 10% fetal calf serum four times and suspended with medium at 10^6 cells/mL. PBL obtained from another donor and lymphocytes from spleens of ACI rats ($RT1^a$; CLEA Japan, Shizuoka, Japan) as responder cells were incubated on a nylon wool column (Biotest AG, Dreieich, Germany) for 1 hour at 37°C in 5% CO_2. T cells that did not bind to the nylon wool column were suspended with medium at 10^6 cells/mL. Responder T cells and APCs mixed in a 1:1 ratio with vesicles or a solution of β-SQAG9 were aliquoted into triplicate wells of 96-well round-bottom plates. After 4 days of incubation, [^{3}H]thymidine was added, followed by overnight culture. The cells were harvested with a SKALTON harvester. The radioactivity of responder T cells was measured in a TOPCOUNT-NXT microscintillation counter (Packard, Meriden, Conn, USA).

Animals and Skin Transplantation

Ten- to 15-week-old inbred male ACI and Lewis rats, weighing 200 to 250 g, were kept under pathogen-free conditions in an animal facility. Lewis rat skin was transplanted to the posterior lateral thoracic wall of ACI rats. The recipient wounds, measuring approximately 1 cm × 1 cm, were made by excision through the panniculus carnosus. Full-thickness donor skin, approximately 1 cm × 1 cm, was obtained from the Lewis rat tail. After hemostasis by electrocautery, the allografts transplanted onto the wounds were sutured with 5-0 nylon (BEAR Medic Corporation, Chiba, Japan). They were covered with bactericidal gauze and the entire chest was wrapped with an elastic bandage. Each animal was housed in a separate cage after transplantation. A control group was injected intravenously with PBS ($n = 7$). The β-SQAG9 groups ($n = 10$ or 11) were injected intravenously with β-SQAG9 0.5, 5, or 50 mg/kg of rat weight once a day for 7 days after the transplantation.

Assessment of Graft Survival

Graft biopsy and tissue processing for morphological studies were performed on day 7 after transplantation. For microscopic examination, subdivided tissue was fixed in 20% formalin, processed routinely by embedding in paraffin, and stained with hematoxylin and eosin. We used the histological criteria previously described.[6] Briefly, grafts that showed changes compatible with mild rejection, moderate rejection, or complete rejection were defined as grades 1, 2, or 3, respectively.

Flow Cytometry

Lymphocytes from peripheral blood, ALNs, and spleens of skin-allografted ACI rats were prepared by density centrifugation. These lymphocytes were stained with FITC-anti-rat CD62L mAb (HRL1, BD Pharmingen, San Diego, Calif, USA) and PE-anti-rat CD3 mAb (1F4, BD Pharmingen). The total number of lymphocytes was determined by the lymphocyte-gating method. The proportions of T cells and $CD62L^+$ T cells were determined by two-color flow cytometry.

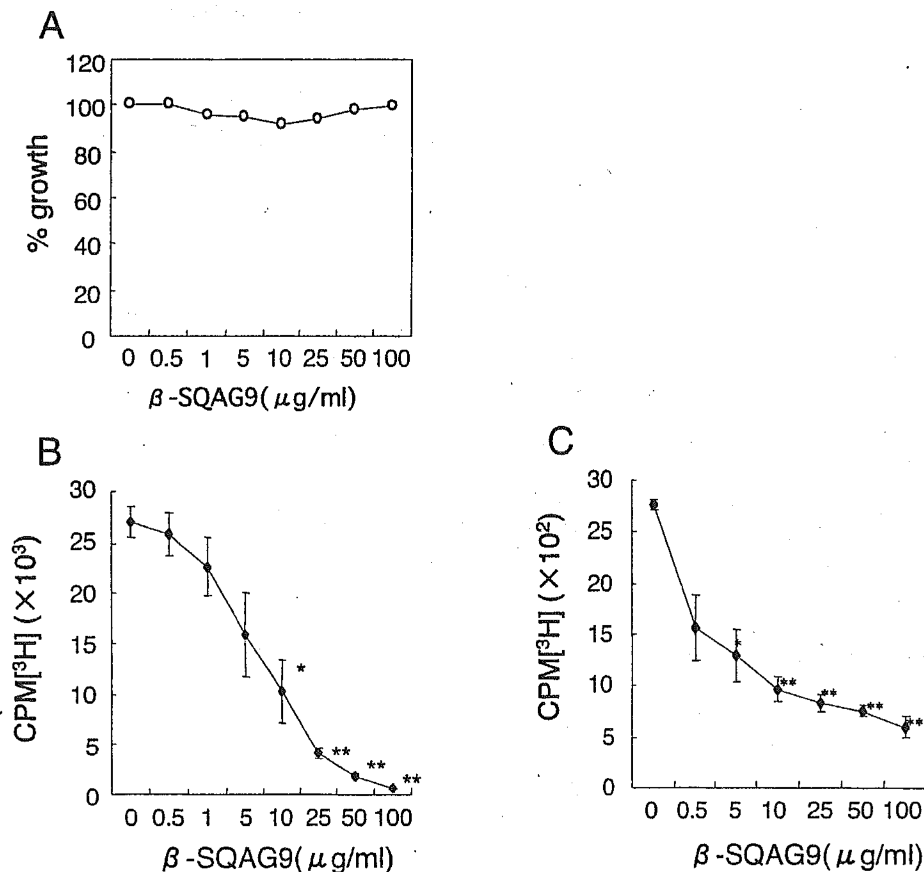

Fig 2. Cytotoxicity of β-SQAG9 and effect of β-SQAG9 liposome on human and rat allogeneic MLR. **(A)** Cytotoxic study of β-SQAG9 by MTT assay. (**B** and **C**) Allogenic MLR was done with or without β-SQAG9. Each closed circle represents the mean ± SD of triplicate determinations. Results represent one of three independent experiments. $*P < .05$, $**P < .01$.

RESULTS AND DISCUSSION

Inhibitory Effect of β-SQAG9 Liposome on Human Allogeneic MLR

We first tested whether β-SQAG9 liposomes had cytotoxic activity by an MTT assay. β-SQAG9 liposomes did not exert any cytotoxicity on human cultured cells over the tested concentration ranges (Fig 2A). Subsequently, we assessed the immunosuppressive effect of β-SQAG9 liposomes in human and rat allogeneic MLR. As shown in Figs 2B and 2C, β-SQAG9 liposomes inhibited T-cell responses by human and rat allogeneic MLR in a dose-dependent manner. These data suggested that the suppressive effects of β-SQAG9 liposomes were not responsible for T-cell damage as has been observed with β-SQDG(18:0).[1,2]

Immunosuppressive Effect of β-SQAG9 Liposomes on the Rat Skin Allograft

Subsequently, we investigated the effect on rat allograft skin survival of β-SQAG9 liposome treatment. Five of 11 (45.5%) rats treated with 50 mg/kg β-SQAG9 showed grade 1 (mild) rejection and the other six rats showed grade 2 (moderate) rejection (Table 1). Although no rats treated with 50 mg/kg of this reagent showed grade 3 (complete rejection), 2 (20.0%) of 10 rats and 5 (50.0%) of 10 rats showed grade 3 when treated with 5 mg/kg or 0.5 mg/kg agent, respectively. Thus, β-SQAG9 liposomes prolonged rat skin allograft survival in a dose-dependent manner, the effective dose for skin allograft survival was 50 mg/kg.

Table 1. Effect of β-SQAG9 Liposome on Rat Skin Allograft Survival

Histological grade	Control	β-SQAG9 (mg/kg)		
		0.5	5	50
Grade 1 (mild rejection)	0/7 (0%)	0/10 (0%)	1/10 (10.0%)	5/11 (45.5%)
Grade 2 (moderate rejection)	2/7 (28.6%)	5/10 (50.0%)	7/10 (70.0%)	6/11 (54.5%)
Grade 3 (complete rejection)	5/7 (71.4%)	5/10 (50.0%)	2/10 (20.0%)	0/11 (0%)

Table shows no. of grafts/no. of total rats in group (%).

Table 2. Population of T Cells and $CD62L^+$ T-Cell Subset in Spleen, Blood, and ALN of Skin Allografted Rats With or Without β-SQAG9 Liposome

	Control	FK506 (1 mg/kg)	β-SQAG9 (mg/kg)	
			5	50
T cell (%)				
Spleen	48.4	45.5	57.8	57.6
Axillary lymph node	83.3	85.5	81.2	82.6
Peripheral blood	64.3	65.8	73.3	76.2
$CD62L^+$ T cell subset (%)				
Spleen	29.1	28.7	40.1	36.0
Axillary lymph node	63.6	60.9	58.3*	53.9*
Peripheral blood	51.7	47.9	56.6	61.7

*$P < .05$.

Effect of β-SQAG9 Liposomes on $CD62L^+$ T Cells of Rats Receiving Skin Allograft

The mechanism of skin allograft survival was unclear. We previously showed that β-SQAG9 bound to CD62L (L-selectin) in vitro and in vivo.[3] Therefore, we investigated the $CD62L^+$ T-cell subset population in spleen, ALNs, and peripheral blood of skin allografted rats treated with or without β-SQAG9. No significant difference was observed in the T-cell population ($CD3^+$) between β-SQAG9 and the controls (Table 2). Surprisingly, the $CD62L^+$ T-cell subset population in ALNs of β-SQAG9-treated rats showed a significant dose-dependent decrease (Table 2). These data suggest that β-SQAG9 is bound to $CD62L^+$ T cells in vivo as well as in vitro and affects T-cell migration.

DISCUSSION

CD62L is expressed on naïve and central memory T cells, suggesting a critical role of immunological surveillance.[7] In particular, $CD62L^+$ markers are required for efficient recirculation and compartmentalization of naïve T cells between blood and lymph nodes.[8–10] It might be supposed that the successful skin allograft survival by β-SQAG9 may lead to $CD62L^+$ T-cell tolerance by inhibiting homing to ALN. Taken together, our current study implies that β-SQAG9 has a great deal of potential as an immunosuppressive reagent with a novel mechanism to target homing of $CD62L^+$ T cells.

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Review

A Mechanism of Antitumor Immunity Induced by Hyperthermia

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Key words : heat shock proteins, intracellular hyperthermia, magnetite, tumor immunity,

Abstract: Resent evidence is accumulating for the important role of heat shock proteins (HSPs), initiating immune responses for cancer. On the other hand, because expression of HSPs protects cells from heat-induced apoptosis, HSP expression has been considered to be a complicating factor in hyperthermia. If HSP expression induced by hyperthermia is involved in cancer immunity, novel cancer immunotherapy based on this novel concept can be developed. In such a strategy, a tumor-specific hyperthermia system would be highly advantageous, because, for conventional hyperthermia systems, it is difficult to induce necrotic cell death without damaging normal tissue because of the thermotolerance induced by HSPs. To achieve tumor-specific hyperthermia, an intracellular hyperthermia system using magnetite nanoparticles was developed. This novel hyperthermia system can induce necrotic cell death via HSP expression, which in turn induces antitumor immunity. In the present article, a mechanism of antitumor immunity caused by intracellular hyperthermia using magnetite nanoparticles is reviewed.

Introduction

"Quae medicamenta non sanat ; ferrum sanat. Quae ferrum non sanat ; ignis sanat. Quae vero ignis non sanat ; insanabilia reportari oportet. Hippocrates."

(Those diseases which medicines do not cure, the knife cures ; those which the knife cannot cure, fire cures ; and those which fire cannot cure, are to be reckoned wholly incurable.)

As Hippocrates (460-370 BC) described in his aphorism, he believed that any diseases could be cured by heating patients' body. Thus, hyperthermia is a promising approach to cancer therapy. A major technical problem with hyperthermia is the difficulty of heating the local tumor region to the intended temperature without damaging normal tissue. Conventional hyperthermia systems are designed to heat tissue to around 42.5°C to 44.0°C. However, higher temperatures can kill greater numbers of tumor cells, and in principle, tumor-specific hyperthermia can kill all kinds of tumor cells.

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Using magnetite nanoparticles, we have developed an intracellular hyperthermia system that achieves artificial local control of temperature within tumors in the human body. This hyperthermia treatment has produced unexpected biological responses, including overcoming thermotolerance due to specific heating of the tumor at high temperature, and an anti-tumor immune response induced by expression of heat shock proteins (HSPs). These results suggest that our hyperthermia system can kill not only heated tumors but also non-heated tumors, including metastatic cancer cells. We have investigated the role of HSP70, in order to elucidate the mechanism of immune induction by hyperthermia [1) 2)]. We discussed here a mechanism of the anti-cancer immune response induced by our intracellular hyperthermia using magnetite nanoparticles.

Intracellular hyperthermia using magnetite nanoparticles

Various heating methods have been applied in hyperthermia, including the use of hot water, capacitive heating and inductive heating [3-5)]. However, an inevitable technical problem with hyperthermia is the difficulty of uniformly heating only the tumor region to the required temperature without damaging normal tissue. Accordingly, some researchers have proposed the use of intracellular hyperthermia to achieve "tumor-specific hyperthermia system", and have developed submicron magnetic particles for this purpose [6) 7)]. Intracellular hyperthermia is based on the principle that a magnetic particle can generate heat by hysteresis loss under an alternating magnetic field (AMF). In 1979, Gordon *et al* first proposed the concept of intracellular hyperthermia using dextran magnetite (Fe_3O_4) nanoparticles [8)]. We have used drug delivery system (DDS) techniques to develop antibody-conjugated liposomes (immunoliposomes) containing magnetite nanoparticles (antibody-conjugated magnetoliposomes, AMLs ; Fig.1). We constructed immunoliposomes using mouse G22 monoclonal antibody (MAb) against human glioma cells [9)], mouse G250 MAb against human renal cell carcinomas [10)] and humanized MAb against human epidermal growth factor receptor-2 (Herceptin®) [11)], and demonstrated the tumor-specific targeting ability of these immunoliposomes. Also, accumulation of magnetite nanoparticles in tumor cells can be enhanced by conferring a positive surface charge to liposomes. We have developed "magnetite cationic liposomes (MCLs ; Fig.1) " with improved adsorption and accumulation properties. MCLs, which have a positive surface charge, have ten-fold higher affinity for glioma cells than neutrally charged magnetoliposomes [12)]. We demonstrated the efficacy of hyperthermia using magnetite nanoparticles in animals with several types of tumor, including B16 mouse melanoma [13)], MM46 mouse mammary carcinoma [14)], T-9 rat glioma [15)], Os515 hamster osteosarcoma [16)], VX-7 squamous cell carcinoma in rabbit tongue [17)], PLS 10 rat prostate cancer (unpublished result), and PC3 and LNCaP human prostate cancer in nude mice

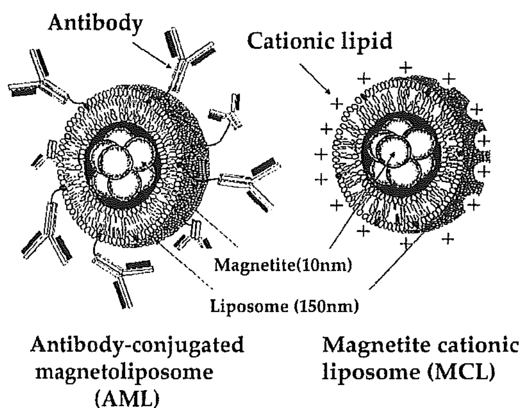

Fig. 1. Liposomal drugs containing magnetite nanoparticles for DDS.

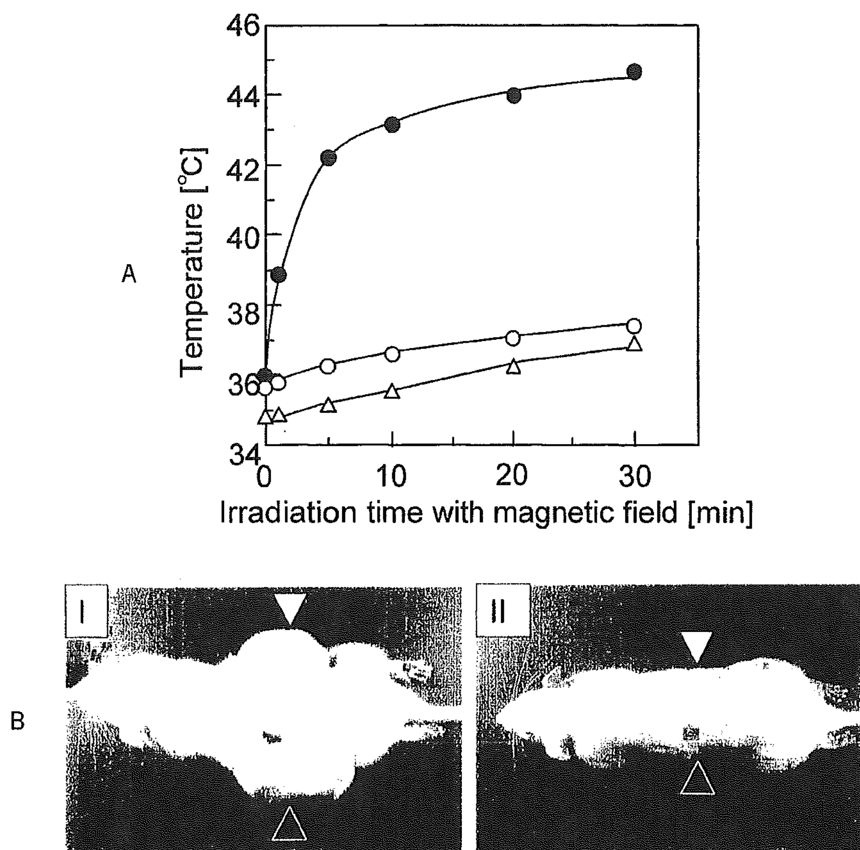

Fig. 2. Anti-cancer immune response induced by hyperthermia using magnetite nanoparticles. Rats bearing tumors on each side of the body were prepared. MCLs were injected into the left tumor only, and the rats were irradiated with an alternating magnetic field (AMF). A) Temperature of left tumor, containing MCLs (closed circles), increased specifically, whereas temperature of right tumor (open circles) and rectum (open triangles) remained below 37°C. B) The tumor-specific hyperthermia treatment induced an anti-tumor immune response, and both tumors had disappeared on the 28th day after hyperthermia treatment. *I)* Control rat without AMF irradiation ; *II)* rat with AMF irradiation. Open triangles in *B*, the side without MCLs ; closed triangle in *B*, the side with MCLs.

(unpublished results).

We observed antitumor immune response induced by hyperthermia using MCLs in an experimental T-9 rat glioma model in which a tumor was transplanted into each femur of a rat (Fig.2) [18]. Interestingly, although only one tumor was subjected to hyperthermia, the other tumor also disappeared completely. Immunohistochemical assay revealed that NK cells and CD8- and CD4-positive T cells migrated into the tumors after the hyperthermia treatment [18]. These results suggest that the therapeutic magnetite nanoparticles are potentially effective tools for hyperthermia, because in addition to killing tumor cells with heat, they induce a host immune response. As a mechanism for recognition of tumor antigens by the host immune system, we have proposed heat shock protein (HSP) -mediated antitumor immunity.

Augmentation of tumor immunogenicity by hyperthermia

HSPs, which are abundant intracellular proteins, function as molecular chaperons that control the folding and prevent the aggregation of proteins [19]. Because expression of HSPs protects cells from heat-induced apoptosis [20], HSP expression has been considered a complicating factor in hyperthermia. Because hyperthermia is a physical treatment, it is likely to have fewer side effects than chemotherapy. Consequently, an advantage of hyperthermia is the feasibility of frequent repeated treatment [14]. However, conventional hyperthermia systems involve treatment only once or twice per week, performed at an interval of more than 48 h, to prevent thermotolerance [21,22] caused by HSP expression.

On the other hand, recent reports have shown the importance of HSPs in immune reactions, including HSP70, HSP90 and glucose-regulated protein 96, and studies suggest that HSPs chaperon tumor antigens [23-25]. With regard to the mechanism of antitumor immunity induced by hyperthermia using MCLs, our findings suggest two possible mechanisms of antigen presentation via HSP70 expression during hyperthermia [1) 2)]. One possible mechanism is heat-induced augmentation of tumor immunogenicity due to presentation of antigenic peptides via MHC class I antigens of tumor cells. Srivastava *et al.* proposed the following "relay line model" for tumor antigenic peptide transfer during antigen processing and presentation by HSPs [26-28]. 1) The peptides are first bound to HSP70 or HSP90, which carry them to the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). 2) The peptides are transferred to gp96 in the lumen of the ER. 3) In the terminal step, gp96 transfers the peptides to the MHC class I -β_2 microglobulin complexes. Wells *et al.* demonstrated that stably transfected B16 melanoma cells that constitutively expressed human HSP70 exhibited significantly increased levels of MHC class I antigens on their surface [29) 30)]. We have shown that augmentation of MHC class I antigens on the tumor cell surface via HSP70 expression causes immune induction [1]. The working hypothesis is illustrated in Fig.3. In that study, HSP70 expression reached its maximum 24 h after heating, and the augmentation of MHC class I surface expression began 24 h after heating and reached its maximum 48 h after heating. The expression of other immunologic mediators, such as intracellular adhesion molecule-1 (ICAM-1) and MHC class II, did not increase. In an *in vivo* experiment, growth of T-9 cells in immunocompetent syngenic rats (F344) was significantly inhibited by hyperthermia, with augmentation of MHC class I antigen surface expression, whereas growth of T-9 cells was not inhibited in nude rats, suggesting that the effector cells were T lymphocytes. Furthermore, compared with lymphocytes from non-immunized rats or rats injected with non-heated T-9 cells, the splenic lymphocytes of rats injected with heated T-9 cells displayed specific cytotoxicity against T-9 cells. Okamoto *et al.* [31] reported that immunization of mice with heat-treated colon-26 cell extract, which was enriched in HSP70, elicited antitumor immunity against subcutaneously injected colon-26 cells. Also, similar immunization enhanced the cytotoxic activity of mouse splenic lymphocytes against heat treated colon-26 cells. Because cell extracts contained various antigens, HSP-independent antigens may be also involved in the antitumor immunity. However, these results suggest that HSP70 is an important modulator of tumor cell immunogenicity during hyperthermia, and that cytotoxic T-lymphocytes (CTLs) are the effector cells.