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原　著

ACTIVATION OF POLYMORPHONUCLEAR NEUTROPHILS BY IMMUNE COMPLEX： POSSIBLE INVOLVEMENT IN THE DEVELOPMENT OF TRANSFUSION-RELATED ACUTE LUNG INJURY

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免疫複合体による好中球の活性化：輸血関連急性肺障害への関与

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【背景及び目的】好中球は，輸血関連急性肺障害（TRALI：transfusion-related acute lung injury）を代表とする非溶血性輸血副作用の発症に関与することが示唆されている．しかしながら，好中球が関与する非溶血性輸血副作用発症の詳細な機序については，未だ明らかにされていない．種々の刺激によって活性化された好中球は，活性酸素，サイトカイン，ケモカイン等を産生し，炎症反応の継続をもたらす．本研究は免疫複合体による好中球の活性化，活性化好中球により産生される可溶性因子について，明らかにすることを目的とした．

【方法】免疫複合体により活性化された好中球による tumor necrosis factor α（TNFα），および perforin の産生を ELISA で測定した．免疫複合体と好中球との共培養上清を肺組織由来初代培養株（lung-tissue-derived primary culture cells：LT cells）に処理し，肺組織由来細胞の増殖抑制を ^{3}H-thymidine の取り込み実験にて調べた．また，免疫複合体と好中球との共培養上清の LT cells に対する細胞障害活性を ^{51}Cr-release assay にて測定した．

【結果】免疫複合体で活性化した好中球は tumor necrosis factor α（TNFα），および perforin を産生することが分かった．また，免疫複合体と好中球との共培養上清は，LT cells の増殖抑制，および細胞障害活性を有することが分かった．

【考察】免疫複合体により活性化した好中球は，炎症性，細胞傷害性因子を産生し，TRALI を代表とする非溶血性輸血副作用の発症に関与することが示唆された．

Key words：Polymorphonuclear neutrophils（PMNs），Transfusion-related acute lung injury（TRALI），Immune complex（IC），Tumor necrosis factor α（TNFα），Perforin

ABSTRACT

Background：Human polymorphonuclear neutrophils (PMNs) are suspected as a causative factor in the development of febrile nonhemolytic transfusion reactions (FNHTRs), such as transfusion-related acute lung injury (TRALI), although the mechanisms underlying FNHTRs have not yet been precisely clarified. The aim of this study was to evaluate the potency of immune complexes (ICs) to activate PMNs and to identify soluble factors produced by PMNs. Methods：We investigated the production of tumor necrosis factor α (TNFα) and perforin by PMNs on stimulation with artificial oligomerized ICs by enzyme-linked immnosorbent assay (ELISA). Furthermore, the potency of culture supernatants of PMNs that were stimulated with ICs was investigated with regard to the growth inhibitory activity against lung-tissue-derived primary cell culture (LT cells) and cytotoxic activity against LT cells by ^{3}H-thymidine uptake experiment and 4-hour ^{51}Cr release assay, respectively. Findings：PMNs activated by ICs were proved to produce TNFα and perforin. The growth of LT cells was inhibited in the presence of cell-free culture supernatants of PMNs cultivated with ICs. Moreover, cell-free culture supernatants of PMNs cultivated with ICs appeared to have cytotoxic activity against LT cells. Conclusion：These results suggest that IC-stimulated PMNs produce inflammatory and cytotoxic mediators, which are potent inducers of FNHTRs such as TRALI.

INTRODUCTION

Febrile nonhemolytic transfusion reaction (FNHTR) is occasionally observed as serious side effect of transfusion[1]. Several reports have described the roles of anti-human serum antibodies (Abs), human histocompatibility leukocyte antigen (HLA) class I and class II Abs, and polymorphonuclear neutrophil (PMN)-specific Abs in the development of some FNHTRs, such as transfusion-related acute lung injury (TRALI)[2)3)]. These Abs, which are usually detected in the blood of multiparous donors or donors who have received transfusion, may react with corresponding multivalent antigens of recipients to result in the formation of immune complexes (ICs). These ICs may in turn activate the intracellular signal transduction pathway resulting in the release of soluble factors, such as reactive oxygen species, and detrimental cytokines and chemokines. Here, we describe the potency of ICs to activate PMNs in vitro, particularly focusing on the ability of ICs to produce soluble mediators that may induce FNHTRs. The ICs used here were artificially oligomerized using a combination of IgG-rich serum with mouse anti-human IgG monoclonal antibody (mAb), IgA1-rich serum with mouse anti-human IgA mAb, and anti-PMN antigen HNA1a Ab-positive serum (whose Ig class was IgG) with anti-human IgG mAb. In this study, the ability of these ICs to induce the release of soluble media-

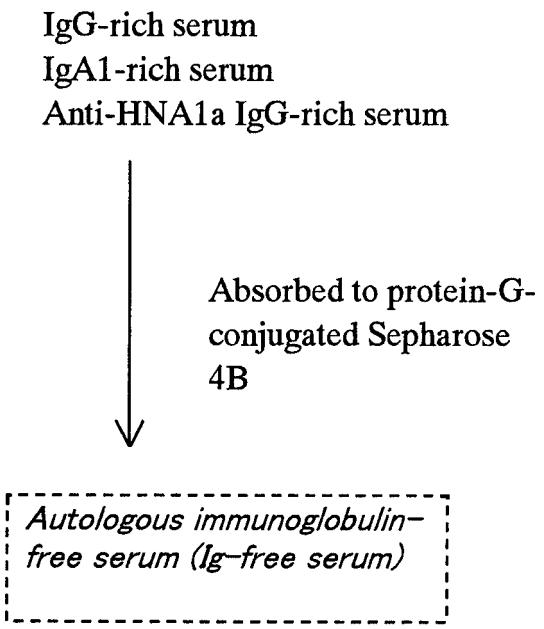

Fig. 1 Preparation of immunogloblin-free serum

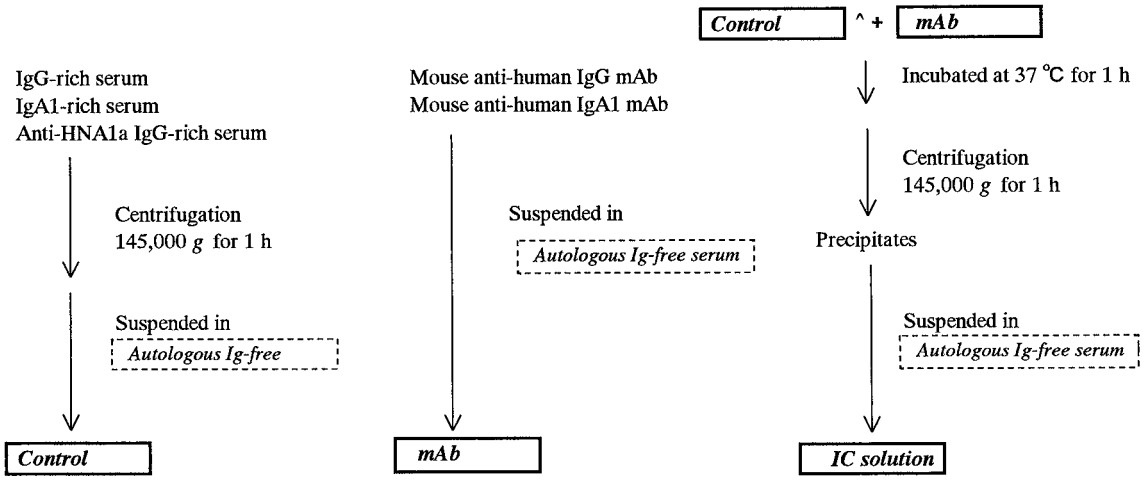

Fig. 2　Preparation of control serum, mAb solution and IC solution

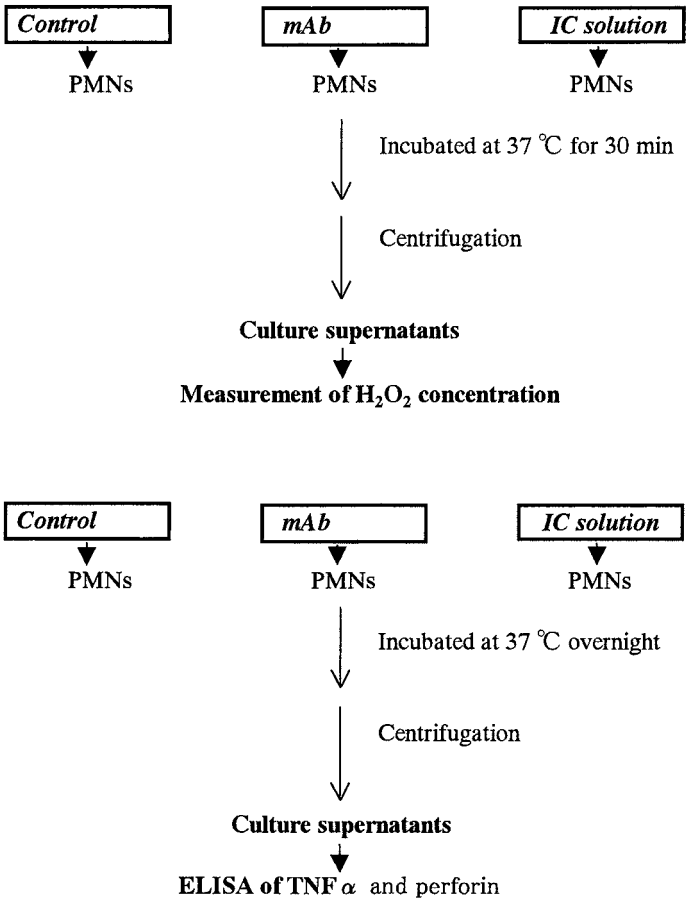

Fig. 3　Detection of H_2O_2, TFNá and perforin

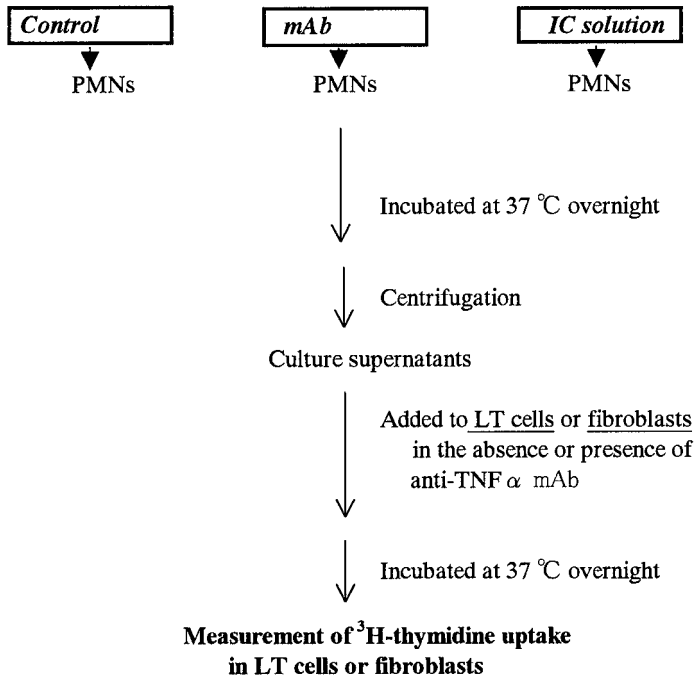

Fig. 4 Assessment of LT cells and fibroblasts growth inhibitory activity

tors by PMNs upon stimulation is investigated. Furthermore, the growth arrest and cytotoxic activities of these factors against lung-tissue-derived cell line (LT cells) are discussed.

MATERIALS and METHODS

PMN preparation

PMNs were prepared from the peripheral blood of healthy individuals by dextran sedimentation, followed by Ficoll-Paque (Amersham, Uppsala, Sweden) density gradient centrifugation and NH_4Cl lysis of contaminant red cells, as described previously[4]. The PMN purity was 99.1±2.8% (range 98.9～99.7%, n=30), as assessed by staining using a fluorescein isothiocyanate (FITC)-conjugated anti-PMN-specific monoclonal antibody (mAb) (anti-CD16b : clone ID3, Immunotech, Marseille, France) and flow cytometry (FCM) using Cytron, (Ortho Diagnostic System K.K., Tokyo, Japan). The viability of PMNs immediately after the preparation was more than 98%, as estimated by the trypan blue dye exclusion method.

Reagents and materials

Rabbit anti-human IgG mAb was purchased from Serotec (Oxford, England). FITC-conjugated goat anti-mouse (GAM) IgG F (ab')$_2$ was purchased from Dako Japan (Kyoto, Japan). Mouse anti-human IgA1 mAb was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Mouse anti-human perforin mAb (clone δG9) was obtained from Biosource International (Camarillo, CA, USA) and clone C-20 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The RPMI 1640 medium was obtained from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Protein G-conjugated Sepharose 4B (fast flow) was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). The 96-well ELISA plate, and flat-bottomed and round-bottomed culture plates used were obtained from Nalge Nunc In-

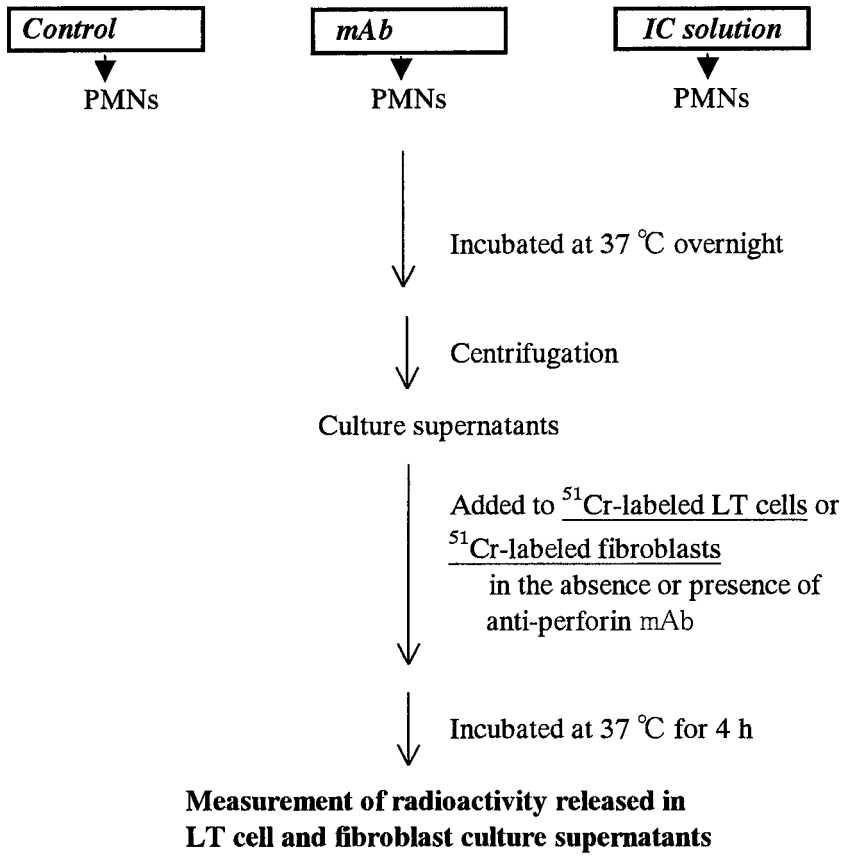

Fig. 5　Assay of cytotoxic activity

ternational (Roskilde, Denmark). O-phenilenediamine was obtained from Sigma (Missouri, St. Louis, USA). Mouse anti-human TNFα mAb (clone 6402.31) was purchased from Dako Japan. Tetramethyl-benzidine (TMB+substrate-chromogen) was obtained from Dako Japan. ^{3}H-thymidine was purchased from NEN (New England, USA). Lung tissue-derived primary culture cells (LT cells) were the kind gift of Dr. N Hidaka (Teikyo University, School of Medicine). LT cells were cultured in RPMI 1640 medium containing 10% FCS. Human primary culture of fibroblasts (fibroblasts), which were used as control of LT cells, were prepared from a small fragment of lower arm skin derived from a normal healthy individual and maintained in RPMI 1640 medium containing 10% FCS and 1 ng/m*l* of fibroblast growth factor (Biosource International). For stripping LT cells and fibroblasts from culture vessels, phosphate-buffered saline (PBS) containing 0.25% trypsin was used.

Preparation of immunogloblin-free serum

Immunoglobulin-free serum (Ig-free serum) was prepared using protein G-conjugated Sepharose 4B. In brief, 500 μ*l* of serum was mixed with 50 μ*l* of protein G-conjugated Sepharose 4B and the mixture was shaken gently at room temperature for 2 hours. The mixture was then centrifuged at 3,000 rpm for 10 min and the resulting supernatant was used as Ig-free serum. ELISA was performed according to conventional methods to confirm the depletion of Igs

Table 1 H_2O_2 production by PMNs stimulated with control serum, mAb solution or IC solutions.

Stimulus	H_2O_2 concentration Mean ± SEM (nM)
(1)Medium alone	nd
(2)Control serum	
IgG serum	6.2 ± 1.8
IgA1 serum	1.7 ± 1.2
Anti-HNA1a serum	1.0 ± 0.8
(3)mAb solution	
Anti-IgG mAb	3.4 ± 0.3
Anti-IgA1 mAb	2.3 ± 0.3
(4)IC solution	
IgG serum + anti-IgG mAb	33.9 ± 5.4
IgA1 serum + anti-IgA1 mAb	51.3 ± 9.7
Anti-HNA1a serum + anti-IgG mAb	24.9 ± 8.6

nd：not detected.
(1)：PMNs were treated with serum-free RPMI 1640 medium.
(2)：PMNs were treated with control serum.
(3)：PMNs were treated with mAb solution.
(4)：PMNs were treated with IC solutions that were oligomerized with the indicated three species of serum and mAb in combination.
Shaded data indicate significant increases in H_2O_2 production ($p < 0.01$).
Mean ± SE calculated from the results of five identical independent experiments is shown.

Table 2 TNF α production by PMNs stimulated with control serum, mAb solution or IC solution

Stimulus	TNF α concentration Mean ± SE (ng/ml)
(1)Medium alone	nd
(2)Control serum	
IgG serum	12.1 ± 0.83
IgA1 serum	8.3 ± 0.23
Anti-HNA1a serum	5.6 ± 0.32
(3)IC solution	
IgG serum + anti-IgG mAb	405.2 ± 40.1
IgA1 serum + anti-IgA1 mAb	218.0 ± 23.6
Anti-HNA1a serum + anti-IgG mAb	130.2 ± 28.1

(1)：PMNs were cultured in RPMI 1640 medium containing 10% FCS.
(2)：PMNs were cultured in control serum.
(3)：PMNs were cultured in IC solution oligomerized by the combination of indicated serum and mAb.
TNF α concentrations in the cell-free culture supernatants of PMNs cultured in the presence of (1), (2) or (3) were measured by ELISA as described in Materials and Methods.
Shaded data indicate significant increases in TNF α production compared with (1) or (2) ($p < 0.01$).
Mean ± SE calculated from the results of five identical independent experiments is shown.

(Fig. 1)[5]

IC formation

IgG-rich (32.1 mg/ml) and IgA1-rich (5.1 mg/ml) sera were obtained from the peripheral blood of normal healthy individuals. IgG and IgA1 concentrations were calculated by the conventional ELISA method[5]. Anti-HNA1a Ab-positive serum with an IgG concentration of 12.0 mg/ml was obtained from the peripheral blood of normal healthy blood donors by the granulocyte immune fluorescence test (GIFT) performed according to the ordinal method[6][7]. IgG-rich serum or anti-HNA1a Ab-positive serum was mixed with anti-human IgG mAb to obtain a final mAb concentration of 0.25 mg/100μl for each serum. IgA1-rich serum was mixed with anti-human IgA1 mAb to obtain the above specified concentration. These mixtures were incubated at 37℃ for 1 hour, followed by centrifugation at 145,000 g for 1 hour. The precipitate was suspended in 100μl of autologous Ig-free serum and the resulting suspension was used as "IC solution." The precipitate of serum alone after centrifugation at 145,000 g for 1 hour was suspended in 100μl of autologous Ig-free serum and the resulting suspension was used as "control serum." The anti-human IgG mAb and anti-human IgA1 mAb were suspended in allogeneic Ig-free serum at a concentration of 0.25 mg/100μl and the resulting suspension was used as "mAb solution"(Fig. 2).

Assessment of H_2O_2 production by PMNs

PMNs (2×10^5) were incubated in 100μl of IC solution, control serum or mAb solution at 37℃

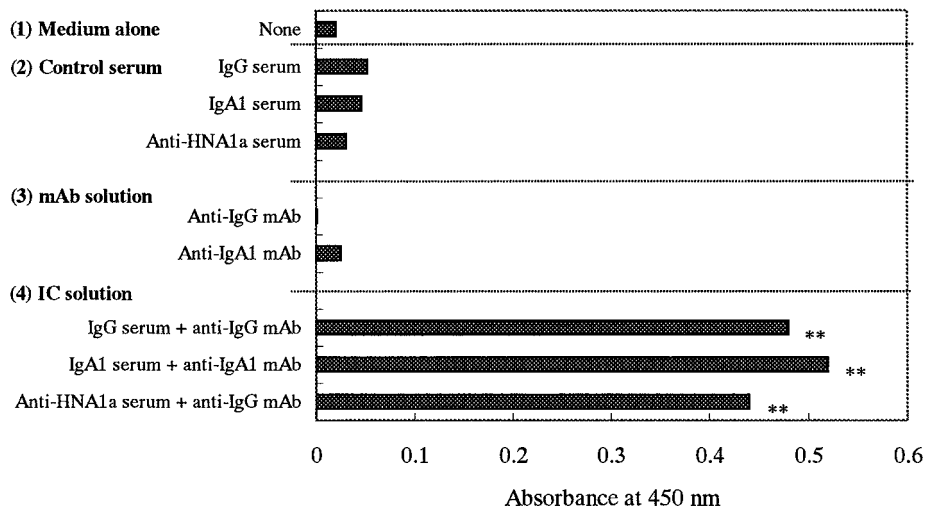

Fig. 6　Perforin production by PMNs stimulated with (1) medium alone, (2) control serum, (3) mAb solution or (4) IC solution. PMNs were cultured with control serum, mAb solution or IC solution at 37℃ overnight. Perforin in the culture supernatants was then measured by double-determinant ELISA, and absorbances obtained at 450 nm are indicated. A representative result from five identical independent experiments is shown. ** : $p < 0.01$.

for 30 min. Then, 50μl of cell-free supernatant was mixed with 50μl of *o*-phenylenediamine in citrate-phosphate buffer (pH 5.0) to obtain a final concentration of 320μl/ml in each well of a 96-well ELISA plate. The plate was then incubated at room temperature for 15 min in the dark. After incubation, 25μl of stopping solution (1N H_2SO_4) was added and absorbance at 490 nm was measured using a microplate reader (Bio-Rad Model 3550) (Fig. 3).

ELISA of TNFα and perforin

TNFα and perforin concentrations in the culture supernatant of PMNs (2×10^5) incubated in a 96-well flat-bottomed culture plate with 100μl of IC solution, control serum or mAb solution/well at 37℃ overnight were determined using an ELISA kit for TNFα according to the manufacturer's instructions (Medical and Biological Laboratories Co.,Ltd., Nagoya, Japan ; the sensitivity of this kit is 10 pg/ml) and double-determinant ELISA for perforin using anti-human perforin mAb clone C-20 as a capture Ab and clone δG9 as a detection Ab according to the ordinal method (Fig. 3)[5]

Assessment of LT cell and fibroblast growth inhibitory activity

The culture supernatants of PMNs (2×10^5) incubated in a 96-well flat-bottomed culture plate with 100μl of IC solution, control serum or mAb solution per well at 37℃ overnight were directly added to LT cells or fibroblast cells to obtain a final cell density of 5×10^4 cells/well in the same flat-bottom plate and incubated at 37℃ overnight. During the last 3 hours of incubation, 37 kBq of ^{3}H-thymidine was added to each well. The cells were then stripped from the culture vessels using trypsin and incorporated radioactivity was harvested on a "Filter mat" (Wallac, Turku, Finland) followed by treatment with "Melt-on scintillator : MeltiLex A" (Wallac) and enumerated with a scintillation counter "Microbeta TRILUX" (Wallac). In some experiments, the previously

Table 3 Cytotoxic activities of culture supernatant of PMNs cultured in control serum, mAb solution or IC solution against LT cells or fibroblasts

Culture supernatants added to LT cells or fibroblasts were obtained from PMN cultures in the presence of (1), (2), (3) or (4)	Specific lysis (%) : Mean ± SE	
	LT cells	Fibroblasts
(1) Medium	nd	nd
(2) Control serum		
IgG serum	1.8 ± 0.8	1.1 ± 0.5
IgA1 serum	4.3 ± 2.6	2.5 ± 1.2
Anti-HNA1a serum	3.6 ± 2.9	3.1 ± 1.1
(3) IC solution		
a). IgG serum + anti-IgG mAb	21.8 ± 8.9	15.6 ± 2.3
b). IgA1 serum + anti-IgA1 mAb	35.5 ± 6.6	20.3 ± 4.1
c). Anti-HNA1a serum + anti-IgG mAb	29.8 ± 6.3	19.5 ± 3.4
(4) Culture supernatants were obtained from PMN cultures in the presence of (3) and added to LT cells or fibroblasts with anti-perforin mAb		
a) + anti-perforin mAb	15.6 ± 4.2	8.9 ± 2.6
b) + anti-perforin mAb	10.9 ± 5.6	9.6 ± 3.2
c) + anti-perforin mAb	10.3 ± 3.8	8.7 ± 2.3

(1) : PMNs were cultured in RPMI 1640 medium containing 10% FCS.
(2) : PMNs were cultured in control serum.
(3) : PMNs were cultured in IC solution oligomerized by the indicated serum and mAb combinations.
(4) : Saturated concentration of anti-perforin mAb was present during the treatment of culture supernatants to LT cells or fibroblasts.
Perforin concentrations in the cell-free culture supernatants of PMNs cultured in the presence of (1), (2), (3) or (4) were measured by ELISA as described in Materials and Methods.
Lightly shaded data indicate significant increases in % specific lysis compared with (1) or (2) ($p<0.01$).
Heavily shaded data indicate significant decreases in % specific lysis compared with (3) ($p<0.01$).
Mean ± SE calculated from the results of five identical independent experiments is shown.

confirmed saturated concentration of anti-TNFα mAb was present during the culture period (Fig. 4).

Cytotoxic activity assay

One hundred microliters each of the culture supernatants of PMNs (2×10^5) incubated in IC solution, control serum or mAb solution at 37℃ overnight was added to ^{51}Cr-labeled LT cells or ^{51}Cr-labeled fibroblasts (1×10^4/well) in a 96-well round-bottomed culture plate. The plate was incubated at 37℃ for 4 hours, then centrifuged at 2,000 rpm for 10 min. The radioactivity of 50μl of cell-free culture supernatant in each well was enumerated using a gamma counter, Aloka SRC-370MA (Aloka, K.K., Tokyo, Japan). Specific lysis (%) was calculated using the following formula : {[cpm of sample]−[cpm of spontaneous release]/[cpm of total release]−[cpm of spontaneous release]}×100. Spontaneous release in the wells was assessed by adding culture medium (RPMI 1640 medium supplemented with 10% FCS) instead of culture supernatant. Total release in the wells was estimated by adding RPMI 1640 medium supplemented with 10% FCS and 0.1% Triton-X 100. In some experiments, a saturated concentration of anti-perforin mAb clone δG9 was present during the 4-hour incubation (Fig. 5).

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance with pair-wise comparison using Bonferroni's test, in which $p<0.05$ was considered significant.

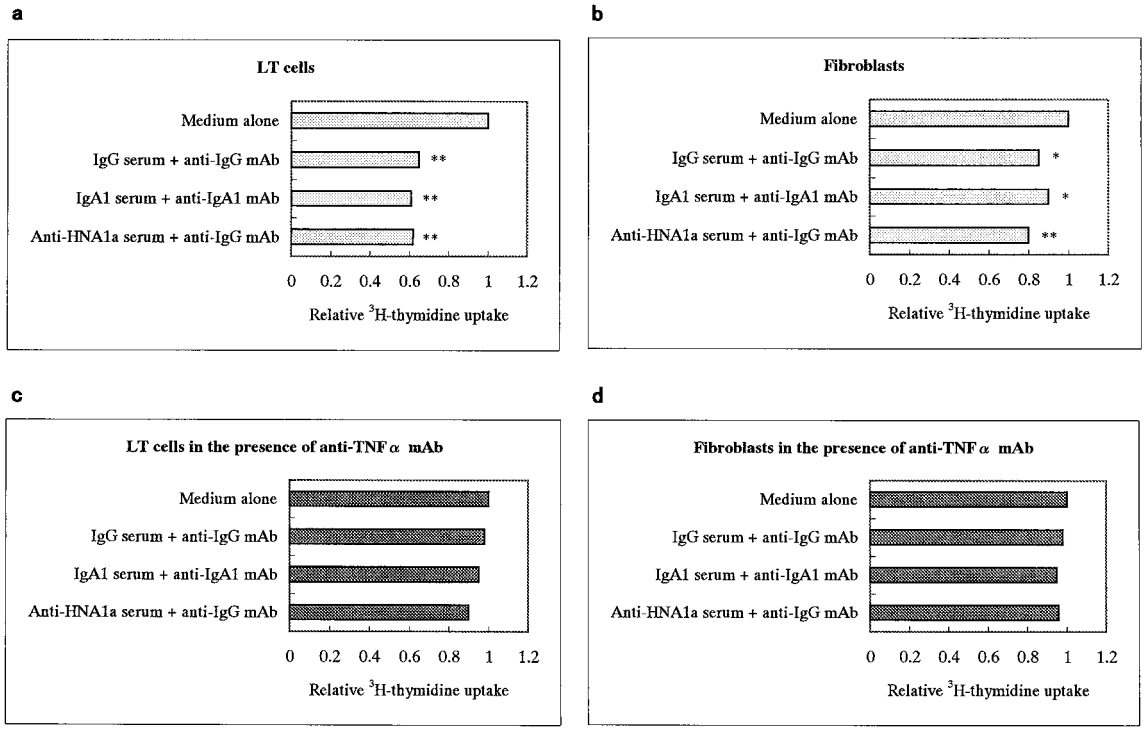

Fig. 7　Inhibition of LT cell proliferation by culture supernatants and recovery of proliferation by the addition of anti-TNFα mAb. The culture supernatants of PMNs cultured at 37℃ overnight with (1) medium alone, (2) control serum, (3) mAb solution or (4) IC solution were added to LT cells (a) or fibroblasts (b) and incubated at 37℃ overnight. Simultaneously, cultures were performed in the presence of anti-TNFα mAb during incubation at 37℃ overnight with both LT cells (c) and fibroblasts (d). During the last 3 hours of incubation, 37 kBq of ^{3}H-thymidine was added to the cultures in each well. The relative ^{3}H-thymidine incorporation (cpm) when the cpm of LT cells cultured in the medium (RPMI 1640 medium supplemented with 10% FCS) instead of the culture supernatants was scored as 1. A representative result from five identical independent experiments is shown. *：$p<0.05$, **：$p<0.01$.

RESULTS

The IC solutions used here were confirmed to trigger PMNs at an early stage on the basis that H_2O_2 production was detected after 30-min stimulation with these solutions at 37℃ but not with the control serum or mAb solution (Table 1).

One of the major inflammatory cytokines, TNFα, and a representative serine protease, perforin, were shown to be produced by PMNs upon stimulation with IC solutions (Table 2 and Fig. 6). The TNFα concentration in the culture supernatant appeared to be comparable with those in culture supernatants in peripheral blood mononuclear cells stimulated with mitogens, as previously reported[8].

Moreover, the culture supernatants of PMNs cultured with IC solutions appeared to have potencies in inducing the growth arrest and death of LT cells, which were inhibited by the addition of anti-TNFα mAb and anti-perforin mAb, re-

spectively (Table 3 and Fig. 7). Although their inhibitory and cytotoxic activities were slightly lower than those of LT cells, the culture supernatants of PMNs cultured with IC solutions also inhibited the growth of fibroblasts and showed cytotoxic activity against fibroblasts (Table 3 and Fig. 7). Thus, it is likely that the growth inhibitory and cytotoxic activities of the culture supernatants are not always restricted to lung tissue-related cells.

DISCUSSION

In this study, we detected considerable amounts of TNFα and perforin produced by PMNs stimulated with IC solutions. We therefore speculated that TNFα and perforin play major roles in the induction of lung tissue injury. Perforin is a serine protease and is significantly implicated in the apoptosis of various cells[9]. In our previous report, we confirmed that perforin is produced by PMNs cultured with various stimulants[10]. It may be speculated that perforin derived from PMNs damages tissue cells leading to the upregulation of inflammatory reactions not only in the lung tissue but also at other sites where activated PMNs accumulate. TNFα is the most common inflammatory cytokine and has been proved to be produced in the early stages of immune responses[11].

The results of the present and our previous studies consistently suggest that de novo synthesis of TNFα mRNA and perforin mRNA may be upregulated, probably involving several mRNAs of proteins, such as the macrophage inflammatory protein (MIP), macrophage-chemoattractant protein (MCP), apoptosis-related proteins (Fas, FasL, p53 and c-myc), including perforin, and antiapoptotic protein (bcl-2, bcl-xL)[12)13)].

Several previous reports have revealed the TNFα-dependent mechanisms underlying PMN recruitment in the pathogenesis of IC-induced tissue injury. Moreover, TNFα was reported to activate PMNs resulting in granulocyte polarization, superoxide anion release, and visually apparent PMN aggregation[14].

On the basis of the present observations, we suggest that TNFα and perforin production may be induced when Abs of FNHTR patients' sera form oligomerized ICs through the association of ICs with their corresponding donor-derived allergens. Likewise, ICs formed by the donor-derived Abs that were oligomerized with corresponding multivalent antigens of the recipient may activate PMNs of the recipient, donor-derived PMNs or both. In conclusion, the results of this study strongly suggest that TNFα and perforin are involved in lung microvascular endothelial cell injury and lingering inflammatory reactions in TRALI.

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