EARLY DEVELOPMENT OF TWO HELPER T CELL SUBSETS FOR DELAYED HYPERSENSITIVITY AFTER BONE MARROW TRANSPLANTATION

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ABSTRACT

Murine delayed hypersensitivity (DTH) was induced by the cooperation of two subsets of T cells. One of these subsets (T_{prod}) produces the antigen-specific DTH augmentation factor (DAF). The other (T_{acc}) receives DAF which is needed for the induction of DTH by antigen stimulation. These two types of T cells require the presence of the thymus only for a short term in their differentiation. In this paper, bone marrow cells were transferred to irradiated syngeneic mice and the rapid recovery of the functions of T_{prod} and T_{acc} were investigated suggesting a low degree of thymus-dependency in their development. When the reconstituted mice were immunized 2 weeks after bone marrow transplantation, the degree of delayed footpad reaction elicited 1 week later recovered to the same level as that in immunized normal mice. The functions of both T_{prod} and T_{acc} also recovered to the normal levels within such a period. The recovery of T_{acc} appeared to occur about 1 week earlier than that of T_{prod} . Furthermore, DAF was fractionated from the sera of mice immunized with chicken erythrocytes (CRBC) by DEAE chromatography and tested for binding to solubilized CRBC antigens. The DAF molecule had a molecular weight of 150-170 K as shown by blotting analysis.

Delayed hypersensitivity (DTH) is positively regulated by helper T cells when T cells recognize protein antigens in association with class II MHC determinants on antigen-presenting cells. These helper T cells express Lyt1 and L3T4 antigens but not Lyt2 antigen. Recently

several papers have shown that murine helper T cells can be divided into two subsets which are distinguished by their patterns of lymphokine secretion (17, 2, 3) or some functional criteria (28, 27, 7, 16, 11). In previous studies, we have demonstrated that helper T cells are ontogenically heterogeneous and there are differences in thymus-dependency in their development or differentiation (25, 24, 8-10, 20). In fact, DTH to xenogeneic erythrocytes and protection against an infection of *Listeria monocytogenes* were induced at normal levels in mice thymectomized neonatally within 1 day after birth, while antigen-specific helper, suppressor and killer activities or tuberculin-

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Abbreviations: DAF, delayed hypersensitivity augmentation factor; DTH, delayed hypersensitivity; T_{prod} , DAF producer T cell; T_{acc} , DAF acceptor T cell; CRBC, chicken erythrocytes; HPLC, high performance liquid chromatography

type DTH did not appear. These T cells in the thymectomized mice may require little proliferation to express their functions because such T cells weakly respond to mitogenic lectins (25, 20) and are resistant to treatment with irradiation (21, 12, 29) and cyclophosphamide (25, 24, 26, 6). Furthermore, DTH to xenogeneic erythrocytes and protective immunity to an infection of *Listeria monocytogenes* developed earlier than the other T cell responses in the mice irradiated and reconstituted with syngeneic bone marrow cells (23).

In addition, murine DTH is controlled by a cooperation of two subsets of T cells which is mediated by DAF (31, 32, 6, 19, 18, 33). One T cell subset, provisionally termed producer T cell (T_{prod}), secretes DAF and the other T cell subset ($L3T4^+$, $Lyt2^-$) called acceptor T cell (T_{acc}), receives DAF with a restriction of immunoglobulin heavy chain locus-linked gene and is needed for the induction of DTH after antigen stimulation (34). In this system, DAF allow the expansion of antigen-specific T cells, by a mechanism not involving the proliferation of the antigen-specific T cell clones. Thus immune competent T cells capable of inducing DTH are quickly recruited after antigen stimulation. We have tentatively termed the immune response associated with such T cells as 'the primitive T cell response'.

This study investigates the periods of reconstitutions of T_{prod} and T_{acc} involved in primitive T cell response after syngeneic bone marrow transplantation. T_{prod} was reconstituted within 3 weeks after the transplantation and the function of T_{acc} was restored within 2 weeks. The DAF molecule had a molecular weight of 150–170 K.

MATERIALS AND METHODS

Mice

Female C3H/He mice at 7 to 9 weeks of age were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and kept for a week before use.

Antigen

Chicken erythrocytes (CRBC) were obtained by a cardiac puncture from normal hens and washed three times with phosphate-buffered saline (PBS). To prepare ^{125}I -labeled mem-

brane antigens, CRBC was suspended in a hypotonic solution of 10 mM phosphate buffer (pH 7.2) with 10 $\mu\text{g}/\text{ml}$ DNase I (Sigma, St. Louis, MO) at 37°C for 30 min and centrifuged at 3,000 rpm for 5 min. The precipitate was washed three times with PBS and labeled with 500 μCi of Na^{125}I (Amersham, Buckinghamshire, England) using Iodogen (Pierce Chemical, Rockford, IL) (13). The preparation was resuspended in 10 mM phosphate buffer with 1% Nonidet P-40 (Sigma) in order to solubilize the protein antigens of the CRBC membranes and then separated from free iodine on Sephadex G-25 column. The radioactive fractions were pooled and then used as a probe in binding analysis to detect DAF molecules.

Bone Marrow Transplantation

Bone marrow cells from C3H/He mice were routinely treated with Thy1.2 antibody (F7D5, Serotec, Oxford, England) and complement (Cedarlane, Ontario, Canada). After washing three times with RPMI 1640 medium, the bone marrow cells (5×10^6) were transferred to syngeneic mice which had been lethally irradiated (950 rads) 24 h beforehand.

Induction of DTH and Preparation of Immune Serum

Mice were intraperitoneally treated with cyclophosphamide (Shionogi, Osaka, Japan; 50–100 mg/kg) 2 days previously or left untreated and immunized subcutaneously with 1×10^8 CRBC emulsified with complete Freund's adjuvant (Difco Lab., Detroit, MI) supplemented with 5 mg/ml heat-killed *Mycobacterium tuberculosis* Aoyama B. Seven days after immunization, 2.5×10^7 CRBC in saline were injected into the left hind footpad of each mouse. Twenty-four hours later, the degrees of footpad swelling were measured and expressed as the difference in the thickness between the left and right footpad. Further, blood was taken afterwards and used as the source of immune serum.

Assay for the Activity of DAF by Delayed Footpad Reaction

DAF activity was evaluated by the degree of

delayed footpad reaction. Mice were intraperitoneally injected with cyclophosphamide (50–100 mg/kg) 4 days beforehand or left untreated and then given intravenously 1 ml of immune serum or purified fractions. Six hours later, the mice were immunized with 2.5×10^6 CRBC in saline into the right hind footpad. For measuring the appearance of T_{acc} , 2.5×10^7 CRBC in saline were injected to immunize firmly the recipients. Three days after the immunization, delayed footpad reaction was elicited by an injection of 2.5×10^7 CRBC in saline into the left hind footpad and the footpad swelling was measured 24 h later. The results are shown as the mean $\pm$ the standard deviation of the five mice.

Assay for the Appearance of DAF Producer T Cells (T_{prod})

To observe the appearance of T_{prod} , DTH was induced in the reconstituted mice 1, 2 or 3 weeks after bone marrow transplantation. In the cyclophosphamide-treated group, mice were intraperitoneally injected with cyclophosphamide (50 mg/kg) 1, 2 or 3 weeks after the transplantation and immunized with CRBC in complete Freund's adjuvant 2 days later. In cyclophosphamide-nontreated group, mice were first immunized with CRBC in complete Freund's adjuvant at the same time as the cyclophosphamide-treated groups tested (9, 16 or 23 days after bone marrow transplantation). Both groups were tested for delayed footpad reaction 1 week later. After the measurement of the footpad swelling to determine the recovery of DTH in the reconstituted mice, the sera were prepared and DAF activity in the sera was measured. For convenience, the groups used here and elsewhere at 1, 2 or 3 weeks after bone marrow transplantation were described as a 1-week group, a 2-week group and a 3-week group.

Assay for the Appearance of DAF Acceptor T Cells (T_{acc})

To determine the appearance of T_{acc} , the assay for DAF activity was performed using an immune serum with DAF activity and the reconstituted mice as recipients. In the cyclophosphamide-treated group, the recipients were intraperitoneally injected with cyclo-

phosphamide (50 mg/kg) 1, 2 or 3 weeks after bone marrow transplantation. These mice were transferred with immune serum 4 days later and immunized with CRBC 6 h after the serum transfer. In the cyclophosphamide-nontreated group, mice were transferred with immune serum and immunized with CRBC at the same time as cyclophosphamide-treated groups (actually 11, 18 or 25 days after bone marrow transplantation). Both groups were tested for delayed footpad reaction 3 days after immunization.

Purification of DAF from Immune Serum

The immune serum was treated with 50% (v/v) saturated ammonium sulfate solution and centrifuged at 7,500 rpm for 20 min to collect the precipitate. After repeating two cycles of this procedure, the preparation dissolved in 10 mM phosphate buffer (pH 7.2) was applied to a DEAE high performance liquid chromatography (HPLC) column and eluted by a linear gradient of NaCl concentration (0–1 M) at a flow rate of 1 ml/min. The 2.5 min fractions were then tested for DAF activity.

Identification of DAF

DAF was identified by a modified Western blotting method using ^{125}I -labeled CRBC antigens. Briefly, the fractions containing DAF activity semi-purified by DEAE-HPLC column were electrophoresed on a 7.5% polyacrylamide gel at 10 mA for 6–9 h and sequentially transferred to nitrocellulose membrane (Schleicher and Schuell, F.R.G.) in electrode buffer (Tris: 5 mM, glycine: 38.4 mM, pH 8.3) at 30 V overnight. The membrane was incubated with ^{125}I -labeled CRBC antigens in 10 mM phosphate buffer (pH 7.2) containing 3% bovine serum albumin (Sigma) and 1% polyoxyethylene(20) sorbitan monolaurate (Tween 20; Wako Pure Chemical, Osaka, Japan) at room temperature for 2 h. After incubation, the membrane was washed with 10 mM phosphate buffer containing 1% Tween 20, dried up and then exposed at -70°C to X-ray film using an intensifying screen.

Statistics

The statistical significance of the data was

Fig. 1 Recovery of delayed footpad reaction after bone marrow transplantation. Reconstituted C3H mice with syngeneic bone marrow cells (BMT mice) were immunized with 1×10^8 CRBC in complete Freund's adjuvant 1, 2 or 3 weeks after bone marrow transplantation. Seven days after immunization, these mice were injected into the hind footpad with 2.5×10^7 CRBC in saline. One day later, the footpad swelling was measured. Groups that were immunized with CRBC in complete Freund's adjuvant 1, 2 or 3 weeks after bone marrow transplantation were respectively expressed as the 1-week group, 2-week group and 3-week group, respectively.

determined by Student's *t* test. A *P* value of less than 0.05 was taken as significant.

RESULTS

Appearance of Delayed Hypersensitivity Induced by CRBC after Bone Marrow Transplantation

First, the time for the recovery of delayed footpad reaction in the reconstituted hosts was determined. C3H/He mice irradiated and transplanted with syngeneic bone marrow cells were immunized with CRBC in complete Freund's adjuvant 1, 2 or 3 weeks after bone marrow transplantation. Seven days after the immunization, the mice were injected with CRBC into the footpad and the specific swelling of the footpad was measured. As shown in Fig. 1, the 1-week group did not show positive delayed footpad reaction. However, the 2- and 3-week groups showed a positive reaction comparable to that of immunized normal control.

Recovery of DAF Producer T Cells (T_{prod}) after Bone Marrow Transplantation

We have observed that DAF occurs in the

serum of normal mice following immunization. In this study, the period of recovery of T_{prod} activity was determined by measuring DAF activity in the serum of the reconstituted mice. Sera prepared from the 1-week group with or without cyclophosphamide treatment showed virtually no DAF activity (Table 1). On the other hand, sera prepared from the 2- and 3-week groups showed DAF activity and the activity in the serum of the 3-week group with cyclophosphamide treatment was as high as that in the serum of immunized normal mice.

Recovery of DAF Acceptor T Cells (T_{acc}) after Bone Marrow Transplantation

We previously showed that DAF required T_{acc} to induce DTH (18). T_{acc} , as well as T_{prod} , showed a low degree of thymus-dependency (6) and was phenotypically $L3T4^+$ and $Lyt2^-$ (34). In this experiment, we observed the recovery of T_{acc} activity after bone marrow transplantation. As shown in Table 2, T_{acc} activity was already detectable in the 1-week group. The data showed T_{acc} activity quickly recovered and the recovery of T_{prod} may require about 1 week longer than that of T_{acc} .

Table 1 *Recovery of T_{prod} after Bone Marrow Transplantation*

Serum donor ^a	Footpad swelling ($\times 0.1$ mm)	
	Experiment 1	Experiment 2
Non-transferred control	0.4 $\pm$ 0.6	0.6 $\pm$ 0.3
CRBC-immunized normal mice	4.6 $\pm$ 0.9 ^e	2.7 $\pm$ 1.1 ^c
CRBC-immunized BMT mice ^b		
1-week group	1.5 $\pm$ 1.3	0.5 $\pm$ 0.4
2-week group	2.1 $\pm$ 0.8 ^c	1.7 $\pm$ 0.1 ^d
3-week group	4.5 $\pm$ 0.8 ^e	—

^aReconstituted C3H mice (BMT mice) were treated (Experiment 1) or not treated with cyclophosphamide (Experiment 2) 1, 2 or 3 weeks after irradiation and bone marrow transplantation. These mice were immunized with CRBC in complete Freund's adjuvant 2 days after treatment and injected for elicitation with CRBC in saline 7 days after immunization. Sera were obtained 24 h after the elicitation to examine DAF activity. ^bGroups were designated as described in Materials and Methods. ^cSignificantly different from non-transferred control, $P < 0.05$. ^d $P < 0.005$. ^e $P < 0.001$

Table 2 *Recovery of T_{acc} after Bone Marrow Transplantation^a*

Experiment	Transferred serum	Footpad swelling ($\times 0.1$ mm)		
		1-week group ^b	2-week group	3-week group
1	Nontreated control	0.6 $\pm$ 0.6	0.9 $\pm$ 0.6	1.3 $\pm$ 0.9
	CRBC-immunized	1.7 $\pm$ 0.4 ^f	2.0 $\pm$ 0.4 ^c	2.8 $\pm$ 1.0 ^d
2	Nontreated control	0.1 $\pm$ 0.1	0.7 $\pm$ 0.4	—
	CRBC-immunized	1.3 $\pm$ 0.2 ^e	2.8 $\pm$ 0.7 ^c	—

^aC3H mice were treated (Experiment 1) or not treated with cyclophosphamide (Experiment 2) 1, 2 or 3 weeks after irradiation and bone marrow transplantation. These mice were transferred with immune serum 4 days after the treatment and immunized with 2.5×10^7 CRBC in saline 6 h after the serum transfer. CRBC in saline were injected into a hind footpad for elicitation 3 days after the immunization and footpad swelling was measured 24 h after the elicitation. ^bGroups were designated as described in Materials and Methods. ^cSignificantly different from normal-serum transferred group, $P < 0.05$. ^d $P < 0.01$. ^e $P < 0.005$. ^f $P < 0.001$

Purification and Identification of DAF Molecule

The following experiment describes the partial purification of DAF molecules. Precipitates from immune serum by 50% saturation of ammonium sulfate were first fractionated by DEAE-HPLC column and individual fractions were assayed for DAF activity. Fig. 2 shows that DAF activity from the immune serum was eluted at 0.1–0.2 M NaCl. We then identified the DAF molecules by a modified Western blotting method using ¹²⁵I-labeled CRBC antigens as a probe. The positive fractions for DAF activity separated by DEAE-HPLC column were analyzed by polyacryl-

amide gel electrophoresis and autoradiography. As shown in Fig. 3, a molecule at 150–170 K reacted with CRBC antigens. No other bands reacted with the probe. It was observed that proteins in normal serum and in the serum immunized with sheep erythrocytes did not react with the CRBC antigens (data not shown).

DISCUSSION

In this study, delayed footpad reaction became detectable when the transplanted mice were immunized with CRBC 2 weeks after bone marrow transplantation. The results show that both T_{prod} and T_{acc} recover rapidly after the

Fig. 2 Purification of DAF molecule from CRBC-immune serum. A: Serum from mice immunized with CRBC was applied to a DEAE-HPLC column and eluted by a linear gradient of NaCl concentration (0.8 M/h) at a flow rate of 1 ml/min. B: The 2.5 min fractions were assayed for DAF activity by delayed footpad reaction (DFR) as described in Materials and Methods.

transplantation, though the time course of their recovery was not identical. At 1–3 weeks, the spleens in mice transplanted with bone marrow cells contained a small proportion of Thy-1⁺ cells (below 2.5%) (23). However, T cell subsets capable of inducing DTH existed in the spleen because DTH was produced by the footpad transfer of the sensitized spleen cells from these mice together with the antigens. Moreover, the splenic T cells hardly expressed any β chain messenger RNA of T cell antigen receptor but significantly expressed γ chain messages (23). There is a possibility that T_{prod} and T_{acc} may use TcR- γ as their antigen receptors.

Recently, Mosmann *et al.* (17) have demonstrated that helper T cell clones could be subdivided into at least two subtypes: TH1 and TH2. They showed that TH1 clone produced interleukin 2 and interferon γ , and generated DTH and cytotoxic T cells but TH2 clone produced interleukin 4 and interleukin 5, and augmented antibody secretion. We have demonstrated that the subsets of helper T cells were different in the thymus-dependency of their differentiation. Helper T cells for DTH showed a low degree of thymus-

Fig. 3 Identification of DAF molecule. Fractions containing DAF activity from the DEAE-HPLC column were electrophoresed on polyacrylamide gel under non-reducing condition and subjected to modified Western blotting method using ¹²⁵I-labeled CRBC antigens as a probe. A: Coomassie blue-stained pattern of the gel. B: The arrow indicates the reactive band to ¹²⁵I-labeled CRBC antigens.

dependency, but those for antibody production showed a high degree. Furthermore, Kurt-Jones *et al.* (14) have indicated that TH1 clones proliferate only in response to interleukin 2 and their growth is not affected by interleukin 1. We and Tucker and Bretscher (30) have observed that the function of helper T cells for DTH is resistant to treatment with irradiation or cyclophosphamide. From these results, T_{prod} and T_{acc} in primitive T cell response might require either less proliferation, or none at all, to exert the function. Although this phenomena may apparently reflect the intrinsic properties of such T cells, we have not definitively established this fact as yet, and that is a subject of further investigation.

From previous studies, the mechanism whereby DAF augments delayed footpad reaction may be by binding to non-sensitized T_{acc} and causing them to acquire antigen specificity. The alternative view is that DAF passively favours an increase of antigen specific T cell clones. DAF had an antigen binding activity and was shown to bind the specific antigen which was used for *in vivo* immunization. In this experiment, DAF was separated from the

immune serum by using DEAE-HPLC column and identified by a modified Western blotting method using the binding ability of DAF to the specific antigen. As shown in Fig. 3, only molecules of 150–170 K reacted with CRBC antigens. The data suggested this molecule was either DAF or the antigen binding part of DAF. It was unlikely to be immunoglobulins because the serum of mice immunized with CRBC contained only a small amount of CRBC specific immunoglobulins (6) and the DAF activity was not absorbed by anti-mouse Ig column (32, 18, 33).

Some investigators demonstrated antigen-specific or antigen-binding T cell helper factors in delayed type (4, 5, 15) or immediate type hypersensitivity (22, 1). However, there are some differences from DAF with respect to molecular weights, expression mechanisms or inductive conditions of each factor. To elucidate the precise mechanism of primitive T cell response, we have to characterize DAF molecule biochemically and genetically, and establish T cell clones or T cell hybridomas reflecting T_{prod} and T_{acc} .

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