Flow Cytometric Analysis and Immunohistochemistry of Delayed-Type Hypersensitivity Responses in Mice Immunized with Rat Hepatocytes

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ABSTRACT. Delayed-type hypersensitivity (DTH) responses to rat hepatocytes (HCs) in mice were investigated by flow cytometric analysis and immunohistochemistry with monoclonal antibodies directed against murine class II, CD4, and CD8 antigens. Mice were immunized subcutaneously (s.c.) with 10^6 rat HCs (referred to as s.c.-immunized mice), and control mice were injected s.c. with sterile Hanks' solution (non-immunized mice). Four days later, 10^5 rat HCs were injected into the footpad of s.c.-immunized mice and non-immunized mice. The DTH response in s.c.-immunized mice significantly increased after challenge when compared to that in non-immunized mice. The numbers of class II^+, $CD4^+$, and $CD8^+$ cells in the footpad, and $CD4^+$ and $CD8^+$ cells in the inguinal lymph node of s.c.-immunized mice significantly increased during the DTH response. An increase in the number of $CD4^+$ cells in the footpad of s.c.-immunized mice after challenge was more significant than that of non-immunized mice. The number of $CD4^+$ cells increased more markedly in the footpad of s.c.-immunized mice as compared to that of $CD8^+$ cells. Furthermore, immunohistochemical studies of the footpad of s.c.-immunized mice showed more severe infiltration of $CD4^+$ cells rather than $CD8^+$ cells at the injection site of rat HCs. These results suggest that the DTH response in the footpad of mice immunized with rat HCs is associated with severe infiltration of $CD4^+$ cells.—KEY WORDS: DTH response, flow cytometric analysis, immunohistochemistry, xenohepatocyte.

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Transplantation with isolated hepatocytes (HCs) is one of potential therapies for patients with acute fulminant hepatic failure. In fact, transplantation with syngeneic, allogeneic, or xenogeneic HCs has been studied in rats with acute hepatic failure [1, 4]. In these three combinations for the transplantation, xenogeneic transplantation is one of great importance in solving problems in the lack of donor grafts such as the liver, kidney, and heart [1].

The effect of xenogeneic HCs transplantation on toxin (D-galactosamine)-induced acute hepatic failure in rats has been reported by Makaowka *et al.* [8]. The survival rates in rats intraperitoneally (i.p.) injected with rabbit HCs were maintained for 2 days after transplantation, and followed by a gradual decrease to approximately 70% within 3 days. The survival rates in rats i.p. transplanted with porcine HCs also decreased gradually until 2 days after transplantation [8]. These results suggest a rapid rejection of transplanted rabbit HCs. The immune response to xenogeneic HCs has been shown to be associated with an increase in antibody-dependent cellular cytotoxicity in rats i.p. transplanted with xenogeneic HCs [8]. Allogeneic murine HCs expressing major histocompatibility (MHC) class I^+ and II^- were also shown to induce the enhanced activities of cytotoxic T lymphocyte (CTL) with the need for antigen-presentation of macrophages [3]. But, immunohistochemical fluctuations in cell populations including $CD4^+$ and $CD8^+$ cells involved in the immune response to xenogeneic HCs have not been fully understood.

Therefore, in this study, changes in MHC class I^+ and class II^+, $CD4^+$, and $CD8^+$ cells in the footpad, lymph node and spleen of mice were investigated by flow cytometric analysis and immunohistochemistry to define the cellular events of these populations in the delayed-type hypersensitivity (DTH) response to rat HCs.

MATERIALS AND METHODS

Animals: Female Fisher 344 rats (9–12 weeks old) were used as donors and female Balb/c ByJ mice (6–8 weeks old) as recipients. All animals were purchased from Clea Japan Inc. (Tokyo, Japan).

Preparation of HCs: HCs from rats and mice were isolated by a slight modification of the Seglen's perfusion technique [13], as described previously [14]. Briefly, isolated HCs were prepared by a perfusion technique with calcium-free salt solution containing ethylene glycol-O,O'-bis-2-aminoethyl-N'-N, N', N'-tetraacetic acid and with Hanks' solution (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.05% collagenase (Wako Pure Chemical Industries Ltd., Tokyo, Japan). The isolated HCs were obtained by density gradient separation with discontinuous Percoll® (specific gravity; approximately 1.06, Pharmacia, Upsalla, Sweden), as described previously [14]. The viability of the resulting cells was over 90%, when determined with 0.16% trypan blue in saline. The rate of contamination of non-parenchymal cells in 200 cells in the smear stained with hematoxylin and eosin was less than 1%.

Immunization and challenge with HCs for DTH: A total of 10^6 rat HCs suspended in 0.1 m*l* of Hanks' solution were transplanted into the dorsal subcutaneous (s.c.)

tissue of the mouse (referred to as s.c.-immunized mice). Mice were injected with 0.1 m*l* of Hanks' solution into the s.c. dorsal tissue (referred to as non-immunized mice). Four days later, all mice were challenged in the right hind footpad with 10^5 rat HCs and in the left hind footpad with 10^5 syngeneic mouse HCs as a negative control.

Measurement of DTH: Footpad swelling was determined with a dial thickness gauge (Ozaki Engineering, Tokyo, Japan) at challenge and at 12, 24, and 48 hr after challenge. The results were expressed as the difference between right footpad and left footpad, as described previously [14].

Monoclonal antibodies (mAbs): MAbs used in this study were FITC or biotin conjugated anti-murine class I murine IgG (Meiji Milk Product Co., Ltd., Tokyo, Japan), anti-murine class II murine IgG, anti-murine CD4 rat IgG and anti-murine CD8 rat IgG (PharMingen, CA, U.S.A.).

Flow cytometric analysis of class II^+, $CD4^+$, and $CD8^+$ cells in the inguinal lymph node and spleen of mice challenged with rat HCs: The right inguinal lymph node and spleen from s.c.-immunized mice or non-immunized mice were removed. These tissues were teased with two slide glasses, suspended in Hanks' solution containing 1% fetal calf serum (FCS), and then filtered through 220 μm nylon mesh. After centrifugation at 400 ×g for 5 min, the cell pellet was treated with ammonium chloride-Tris buffer for 5 min to hemolyze contaminated red blood cells. The cells were then washed three times with Hanks' solution. The resulting cells were suspended to the desired concentration in Hanks' solution containing 1% FCS. Lymph node cells or spleen cells (1×10^6) were incubated with 10 fold-diluted murine normal serum or FITC labeled antibody for 40 min on ice. Cells without the antibody were used as controls. After incubation, the resulting cells were washed three times with Hanks' solution. Subsequently, these pellets were incubated with 0.01 M phosphate buffered saline (PBS) containing 1% paraformaldehyde for 10 min on ice, washed three times with Hanks' solution, and then suspended in Hanks' solution containing 1% FCS at 4°C. They were analyzed by a flow cytometer (EPICS-Profile II; Coulter, Inc., Hialeah, FL, U.S.A.), as described previously [5].

Immunohistochemical analysis of MHC class I^+, II^+, $CD4^+$, and $CD8^+$ cells in the footpad, lymph node, and spleen of mice injected with rat HCs: The footpad, inguinal lymph node, and spleen were taken at challenge, and 12, 24, and 48 hr after challenge with rat HCs for immunohistochemical examination. These fresh tissues were embedded in OCT compound (Miles, IN, U.S.A.), and frozen immediately in liquid nitrogen. The frozen thin sections were fixed in acetone at 4°C for 5 min, and rehydrated in PBS. These sections were first covered with 10 fold-diluted normal murine or rat serum in PBS for 30 min to block the non-specific reaction of labeled antibodies. The sections were then incubated successively for 30 min with the biotinylated mAb for 30 min with 0.3% H_2O_2 in methanol to inhibit the endogenous peroxidase reaction, and then for 30 min with the avidin-biotin peroxidase complex (Vector Lab. Inc., CA, U.S.A.), as described previously [6]. In addition, footpad tissue sections were stained with 3, 3'-diaminobenzidine (DAB) (0.2 mg/m*l*) and 0.01% H_2O_2 in PBS. Lymph node and spleen sections were stained with DAB containing 0.025% $CoCl_2$, 0.025% $NiSO_4$ $(NH_4)_2SO_4$ and 0.01% H_2O_2 in PBS. The sections were then counterstained with aqueous methyl green. Control sections were incubated with PBS or biotinylated murine IgG substituted for mAbs. The class II^+, $CD4^+$, and $CD8^+$ populations in the footpad were counted as follows: Positive cells in one section were counted under the microscope at × 400 magnification, and the number of positive cells which infiltrated into the s.c. tissue of the footpad was expressed as the number of cells per 10 fields.

Statistical analysis: The results were expressed as the mean ± standard deviation (SD), and differences among the various parameters were analyzed by two-factorfactorial ANOVA, repeated measure ANOVA, and Scheffé's F. $P<0.05$ was considered significant.

RESULTS

Kinetics of DTH responses in mice immunized with rat HCs: The footpad thickness in s.c.-immunized mice significantly increased 12, 24, and 48 hr after challenge with 10^5 rat HCs, as compared to that in non-immunized mice (Fig. 1, A and B). The maximal DTH response was shown at 24 hr after challenge.

Changes in the numbers of class II^+, $CD4^+$, and $CD8^+$ cells in the footpad, inguinal lymph node, and spleen: The numbers of class II^+, $CD4^+$, and $CD8^+$ cells in the tissue sections of the footpad of s.c.-immunized mice and non-immunized mice were measured under the microscope at challenge, and 12, 24, and 48 hr after challenge with 10^5 rat HCs. The relative proportion of these three cell populations in the inguinal lymph node and spleen was measured with a flow cytometer. These three cell populations in s.c.-immunized mice were significantly greater than those in non-immunized mice. The footpad area of s.c.-immunized mice and non-immunized mice showed a significant increase in the numbers of class II^+ and $CD4^+$ up to 48 hr after challenge, as compared to those at challenge (Fig. 1, C and D). In the footpad of s.c.-immunized mice, the number of $CD8^+$ cells also significantly increased at 24 and 48 hr after challenge, but no apparent change in $CD8^+$ cells was observed in non-immunized mice. The number of $CD4^+$ cells in the inguinal lymph node of s.c.-immunized mice and non-immunized mice increased significantly ($P<0.05$ and $P<0.002$, respectively) at 12 hr after challenge than that at challenge (Fig. 1, E and F). The number of $CD8^+$ cells in the inguinal lymph node of s.c.-immunized mice increased significantly ($P<0.05$) at 24 hr after challenge, when compared to that at challenge. However, there was not any significant change in the numbers of $CD8^+$ and class II^+ cells in the lymph node of non-immunized mice or the

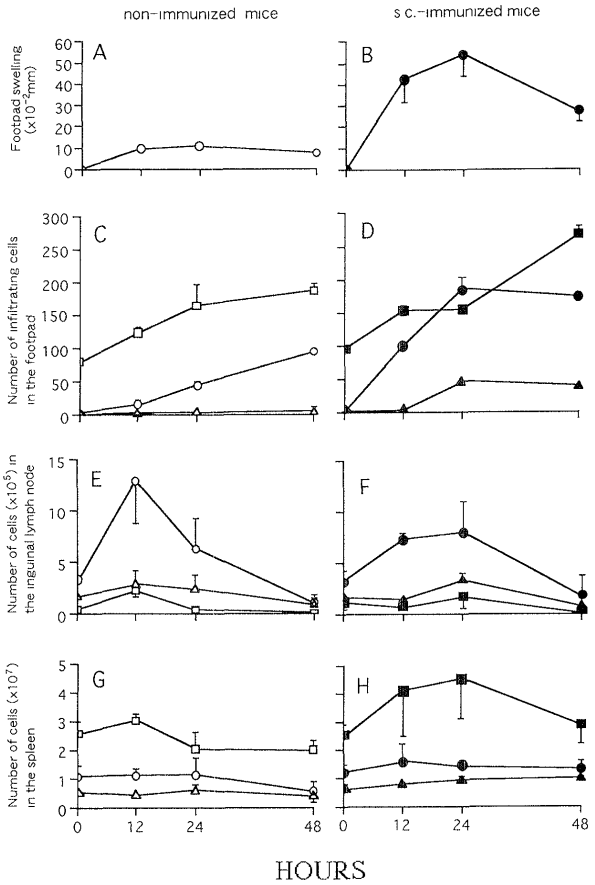

Fig. 1. Changes in the thickness of the footpad (A and B) and the numbers of class II^+ (square), $CD4^+$ (circle), and $CD8^+$ (triangle) cells in the footpad (C and D), inguinal lymph node (E and F) and spleen (G and H) in non-immunized mice (open symbols) and s.c.-immunized mice (closed symbols) challenged with 10^5 rat HCs. Each point represents the mean value for 3 to 5 mice. Vertical bars represent SD.

number of class II^+ cells in the lymph node of s.c.-immunized mice after challenge. No apparent significant change in the numbers of class II^+, $CD4^+$, and $CD8^+$ cells in the spleen in both groups was observed (Fig. 1, G and H).

Immunohistochemical findings in the footpad, lymph node, and spleen in s.c.-immunized mice and non-immunized mice: A lot of class II^+ cells morphologically similar to macrophages in both groups appeared around capillary vessels near the injection site of rat HCs at 24 hr after challenge (Fig. 2, A and B), and were also scattered in the s.c. tissue at 48 hr after challenge. A few $CD4^+$ cells were observed in the s.c. tissue of the footpad in s.c.-immunized and non-immunized mice at challenge. At 12 hr after challenge, they appeared around capillary vessels in the footpad injected with rat HCs in both groups. At 24 and 48 hr after challenge, $CD4^+$ cells scattered throughout the s.c. tissue of the footpad in both groups were observed (Fig. 2, C and D). Few $CD8^+$ cells were found in the s.c. tissue of the footpad of both groups at challenge and 12 hr after challenge. These cells gradually appeared around capillary vessels near the injection site of the footpad in both groups 24 hr after challenge (Fig. 2, E and F), and were also scattered throughout the s.c. tissue at 48 hr. Almost all of these cells in the footpad of both groups were class I^+. There was no immunohistochemically apparent change in the distribution of class I^+ and II^+, $CD4^+$, and $CD8^+$ cells in the lymph node and spleen of both groups. Almost all of these cells were also class I^+.

DISCUSSION

The results of the present study are summarized as follows: (1) the DTH response in mice immunized s.c. with rat HCs reached a peak 24 hr after challenge, (2) an increase in the number of $CD4^+$ cells in the footpad and inguinal lymph node of s.c.-immunized mice and non-immunized mice challenged with rat HCs was more significant than that of $CD8^+$ cells, (3) in the footpad and inguinal lymph node of s.c.-immunized mice, the number of $CD8^+$ cells significiantly increased 24 hr after challenge. These results therefore suggest that rat HCs strongly induce an increase in the number of $CD4^+$ cells in the s.c. tissue of the footpad and inguinal lymph node rather than that of $CD8^+$ cells.

Grafted allogeneic cells expressing both MHC class I and II antigens have been shown to stimulate $CD4^+$ helper T cells with and without association with host MHC antigens on the surface of antigen presenting cells such as macrophages and B cells [15]. The stimulated helper T cells aid the proliferation and differentiation of precursor T-DTH cells to effector T-DTH cells [9]. Moreover, helper T cells mediate the destruction of grafted cells in two ways: Helper T cells help CTL destroy grafted cells, and helper T cells release lymphokines, which stimulate macrophages to destroy grafted cells [15]. Allogeneic HCs expressing MHC class I antigens but not class II antigens have been reported to fail to induce proliferation of spleen cells in mixed lymphocyte-hepatocyte cultures, but to be able to induce CTL activity in the presence of macrophages as antigen presenting cells [4]. On the basis of these findings, allogenic MHC class I antigens have been suggested to be internalized and processed by macrophages to directly stimulate CTL without helper T cells. In the present study, xenogeneic rat HCs showed the ability to induce the DTH response in mice, and a significant increase in the number of $CD4^+$ cells rather than $CD8^+$ cells in the footpad and inguinal lymph node of s.c.-immunized mice. These results suggest that rat HC MHC class I antigens re-expressed on macrophages stimulate helper T cells to induce the DTH response. Similar findings have been shown in xenogeneic monkey skin graft rejection in mice [11]. With respect to the immune response to xenogeneic MHC class I antigens, porcine MHC class I antigens expressing in the skin of transgeneic mice have been shown to be rejected by $CD4^+$

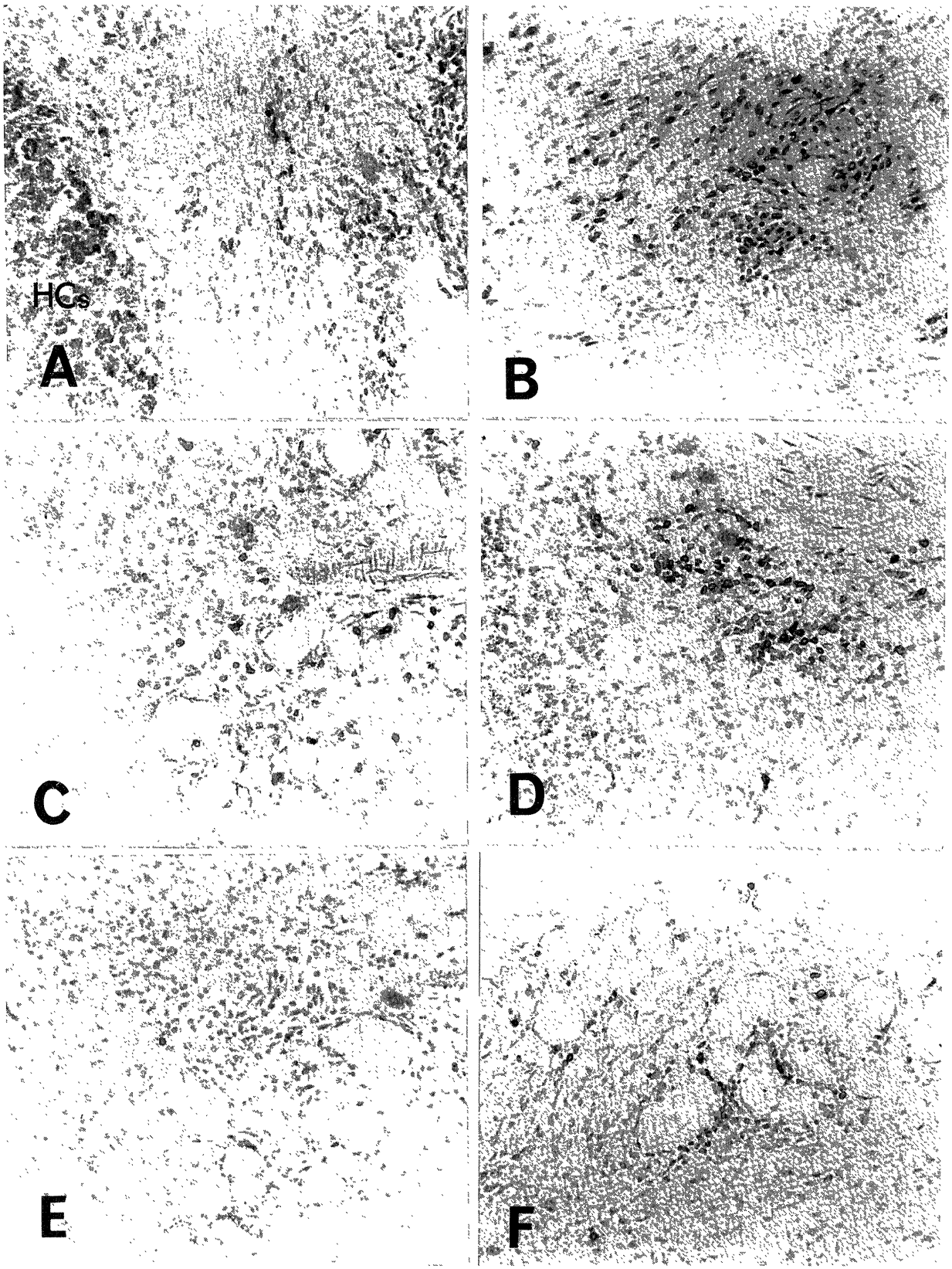

Fig. 2. Immunohistochemistry of the footpad with anti-MHC class II (A and B), CD4 (C and D), and CD8 (E and F) mAbs 24 hr after challenge with rat HCs (magnification, × 66). Rat HCs (HCs in Fig. 2A) injected into the footpad, showing nonspecific reaction to DAB, are observed.

cells but not by $CD8^+$ cells [2], suggesting that $CD4^+$ cells play a major role rather than $CD8^+$ cells. But, an increase in the number of $CD4^+$ cells in the footpad and lymph node of s.c.-immunized mice challenged with rat HCs would not be dependent on the properties of only MHC class I antigens of rat HCs. Our preliminary studies showed that the DTH response in mice immunized with F344 rat ($RT1^l$) HCs 4 days before challenge was markedly induced 24 hr after challenge not only with Lewis rat ($RT1^l$) HCs but also with ACI ($RT1^a$) HCs, suggesting its induction independent of the haplotype of class I antigens (data not shown). Our results therefore suggest that xenogeneic rat MHC class I antigens, minor histocompatibility antigens, and/or other antigenic substances on/in rat HCs induce more active infiltration of $CD4^+$ cells rather than $CD8^+$ cells into the injection site of antigens and the inguinal lymph node in mice.

It remains obscure whether the increase in the number of $CD8^+$ cells is associated with the enhancement of the functions of CTLs and/or suppressor T cells inhibiting the $CD4^+$ cells. To identify $CD8^+$ cells with these different functions, the former is detected by measuring ^{51}Cr release from ^{51}Cr labeled target cells *in vitro* [12], and the latter is detected by adoptive transfer of tolerant splenic T cells to naive recipients [9]. To define the significance of the increase in the number of $CD8^+$ cells, further studies to evaluate the activities of a $CD8^+$ cell population during the DTH response in s.c.-immunized mice are needed.

The spleen has been reported to be more suitable for the survival of transplanted HCs than the kidney, liver or abdominal cavity [7]. Our preliminary studies showed that syngeneic HCs grafted intrasplenically still remained 520 days after transplantation, whereas few intact syngeneic HCs were present in the s.c. tissue of the footpad of mice 12 hr after injection of syngeneic HCs. These results suggest that the s.c. tissue of the footpad may be unsuitable for grafted HCs. In the present study, few rat HCs remained in the s.c. tissue of the footpad in s.c.-immunized mice and non-immunized mice injected with murine and rat HCs. Non-specific immune responses mediated by polymorphoneuclear cells such as neutrophils and mononuclear cells including macrophages, might also be involved in the rejection of the grafted murine and rat HCs, since their infiltration was shown around murine and rat HCs injected into the footpad. In s.c.-immunized mice, CTL, especially MHC-restricted CTL responsible for major cytotoxicity, might also be involved in the destruction of xenogeneically transplanted rat HCs [10].

In summary, our results suggest that more severe infiltration of $CD4^+$ cells rather than $CD8^+$ cells into the s.c. tissue of the footpad is involved in the DTH response in mice immunized s.c. with rat HCs. More detailed studies on the relation between the changes in the numbers and functions of $CD4^+$ and $CD8^+$ cells are needed to understand the cellular events during the DTH response in mice immunized with xenogeneic HCs.

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