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Nonspecific hemolytic effector of activated macrophages as activation marker of allograft rejection

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Abstract. The aim was to assess a nonspecific hemolytic effector of activated monocytes/macrophages, designated spontaneous plaque-forming cell (SPFC), as an activation marker in allograft rejection. An in vitro study on the immunologic characteristics of SPFC monocytes in man and an in vivo study in Lewis rats as to the monitoring of SPFC generation of allograft infiltrating cells with or without immunosuppression were conducted. Hemolysis of SPFC was mediated by CR3 adhesion molecules, detected by Mo-1 and OKM10 monoclonal antibodies. Hemolysis of SPFC was nonspecific, and nonrosette-forming T cells with autologous erythrocytes (non-ARFC-T) acted as suppressor T cells inhibiting SPFC-hemolysis against autologous erythrocytes. A 6-day course of immunosuppression with a daily dose of cyclosporin A (CyA) 10 mg/kg and of FK506 1 mg/kg suppressed the SPFC generation to the level of syngeneic control. In contrast, peak SPFC generation coincided with rejection, and the degree of SPFC generation reflected the grade of histoincompatibility. The present findings suggested that SPFC-activated monocytes/macrophages may be one of the activation markers in allograft rejection and lead to a new concept of graft rejection and self or nonself discrimination mediated by nonspecific, hemolytic SPFC effectors and suppressor T cells inhibiting autoreactivity.

Key words: Activated monocytes/macrophages – Hemolysis – Complement receptor 3 (CR3) of adhesion molecules – Allograft rejection – Self or non-self discrimination

As immunologic activation markers of allograft rejection, interleukin 2 (IL-2) receptor expression [4, 14, 21], major histocompatibility complex (MHC) expression [5], release of cytokines such as interferon-γ or TNF-α/β from activated T cells [15, 3], and activated macrophages were studied in rats and man [4, 6, 16, 17]. We found a new effector of activated monocytes or macrophages in man [7–11] and designated it spontaneous plaque-forming cell, SPFC, because SPFC undergoes hemolysis nonspecifically and without the addition of exogenous complement. The involvement of SPFC in human renal allograft rejection [10] and the suppression of SPFC generation by various immunosuppressive agents in normal human subjects [11] were reported.

The activated macrophage is one of the activation markers in allograft rejection in rats and man [4, 6, 16, 17]. However, since the definite immunologic understanding of cytolysis by activated macrophages is obscure, any strategy for the suppression of activated macrophages is not immediately forthcoming. We have detected SPFC in rats [12, 18]. Human SPFC is reproducible in allogeneic stimulation in vitro [9] and may be an objective tool in studying immunologic characteristics of activated monocytes or macrophages in vitro or in vivo.

To assess the role of the SPFC of activated monocytes/macrophages in allograft rejection, two kinds of experiments were performed. In one, the in vitro immunologic characteristics of the SPFC-monocyte effector of activated monocytes/macrophages in man was studied, and in the other, in vivo SPFC generation in rat allograft rejection with or without immunosuppression was attempted. These studies have demonstrated that a new concept of self or nonself discrimination can be proposed by understanding the immunologic characteristics of the SPFC effector and that SPFC might be one of the activation markers in allograft rejection.

Materials and methods

The spontaneous plaque-forming cell assay was described previously [9, 11]. Autologous or allogeneic target erythrocytes in a monolayer and effector cells, approximately $5–10 \times 10^3$, were mixed and incubated at 37 °C for 3 h in a microplate using serum-free Hanks' solution for the SPFC assay, and then the number of hemolytic plaques

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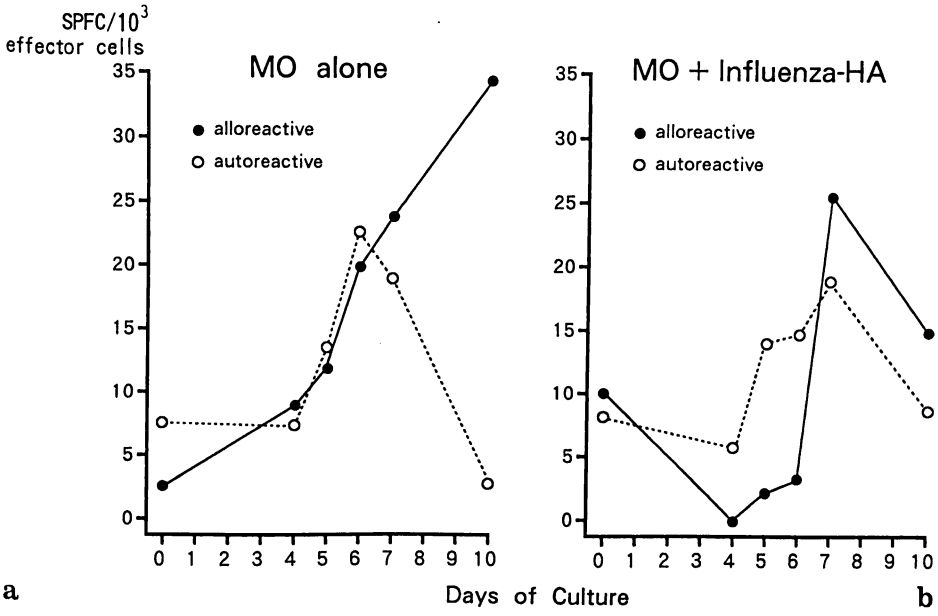

Fig. 1. Kinetics of spontaneous plaque-forming cells (SPFC) generation, in vitro, of monocytes *(MO)* enriched alone (with antigen) and monocytes enriched with influenza hemagglutinin *(HA)*, containing 14 chick cell hemagglutinins. Enriched monocytes of normal subjects, suspended in RPMI1640 supplemented with 20% AB serum, were cultured, and the number of SPFC generated in cultured monocytes were determined against autologous and allogeneic erythrocytes

(SPFC) was determined under an inverted microscope. The number of SPFC per 10^6 effector cells was calculated.

Monoclonal antibody (mAb) Mo-1(904) (IgG1), directed against the α-chain (CD11b) of CR3 [13], was a gift of Dr. J. Griffin (Dana-Farber Cancer Center, Boston, Mass.); mAb OKM10 (IgG2b), directed against the α-chain (CD11b) [23], was a gift of Dr. P. Rao (Ortho Pharmaceutical, Raritan, N.J.); and mAb 3G8 (IgG1), directed against the low avidity Fc receptor of neutrophils (FcRIII, CD16) [1] was purchased from Medarex. The $F(ab')_2$ of mAB Mo-1 was prepared by Dr. T. Kinoshita (Osaka University Medical School, Suita, Osaka, Japan).

Rats and skin transplantation. The H-1 incompatible combination of ACI and Lewis and the H-1 compatible combination of F344 and Lewis were selected. A 9-week-old male Lewis rat was the recipient of the allograft skin transplantation, and a male ACI or F344 rat of the same age was the donor. The procedure of skin transplantation was described previously [18].

Immunosuppressive agents. Cyclosporin was a gift of Sandoz Pharmaceutical (Basel, Switzerland), FK506 was a gift of Fujisawa Pharmaceutical (Osaka, Japan), and prednisolone was a gift of Shionogi & Co. (Osaka, Japan). Immunosuppressive agents were administered subcutaneously for 6 days from day 0 to day 5 after skin transplantation. Each agent was mixed with arabic gum and saline to a final concentration of 5%.

Cell preparation. Peripheral blood mononuclear cells (PBMC) of normal human subjects or of rats were separated by Ficoll-Conray (d = 1.078) gradient centrifugation. Human enriched monocytes (MO) and T lymphocytes were fractionated by Percoll discontinuous gradient centrifugation. Rosette-forming T cells with autologous erythrocytes (ARFC-T) or MO cells were fractionated by the method of Tomonari et al. [20]. Allograft infiltrating cells were separated by the enzymatic methods described previously [18].

Results

I. Immunologic characteristics of the SPFC-monocyte effector of activated monocytes/macrophages in man

To evaluate the SPFC-monocyte effectors of activated monocytes or macrophages involved in allograft rejection, the mechanism of nonspecific hemolysis of SPFC-effectors was investigated. Secondly, since nonspecific hemolysis of SPFC is also autoreactive, the mechanism of self or nonself discrimination preventing hemolysis against autologous erythrocytes in SPFC-effector limbs was studied. Thirdly, T-cell-dependent and T-cell-independent SPFC generation in vitro was demonstrated.

Table 1. SPFC generation in vitro was regulated in a T-lymphocyte-dependent or T-lymphocyte-independent manner. Autorosette-forming cells of T cells (ARFC-T) as helper cells and non-ARFC-T as suppressor cells acted on SPFC generation. SPFC effectors of monocytes were generated without participation of T lymphocytes, and enrichment of ARFC in the monocyte culture resulted in loss of SPFC generation

		SPFC generation per 10^6 cultured cells	
		Autoreactive	Alloreactive
T-cell-dependent SPFC generation	Coculture of monocytes and T cells for 7 days (ratio of MO to T = 1:5)		
	MO + unfractionated T cells	2333	1417
	MO + ARFCT cells	11467	16513
	MO + non-ARFC-T cells	333	500
T-cell-independent SPFC generation	Culture of monocytes, fractionated or not, for 7 days		
	MO alone	10500	4333
	ARFC-MO alone	0	0
	non-ARFC-MO alone	11000	21500

Table 2. Suppressor activity (%) of nonautorosette-forming T cells (non-ARFC-T) against autoreactive hemolysis of SPFC. Autoreactive SPFC were selectively inhibited by non-ARFC-T

Nonspecific SPFC effector	Ratio of cultured MO as effector to non-ARFC-T as suppressor cells	
	1:1	5:1
Autoreactive SPFC	100.0%	92.0%
Alloreactive SPFC	100.0%	−8.3%

T-cell-dependent or -independent SPFC generation in vitro. The SPFC are generated in vitro in 6–7 days of culture with or without antigen stimulation, and only monocytes are differentiated into SPFC effectors of activated monocytes or macrophages (Fig. 1). On the 7th day of culture, the peak of SPFC generation, which showed nonspecific hemolysis, was found in monocytes cultured with influenza virus or without antigen. On day 10 of culture, the SPFC generated in monocytes cultured alone showed spontaneously diminished autoreactivity and augmented alloreactivity, while the SPFC generated in monocytes cultured with influenza virus showed a different reactivity profile except for the same peak of nonspecific reactivity. The reactivity found in the present study suggested the presence of regulating cells among the monocytes.

As shown in Table 1, SPFC were generated by different regulating cells, i.e., rosette-forming T cells with autologous erythrocytes (ARFC-T) as helper cells or non-ARFC-T as suppressor cells which regulate SPFC generation, and monocytes themselves, independently of T cells. A fraction of ARFC receptor-bearing monocytes resulted in the loss of SPFC generation, which suggests that ARFC receptor-bearing monocytes might be suppressor monocytes. The SPFC showed nonspecific hemolysis against autologous and allogeneic target erythrocytes.

Nonautorosette-forming T lymphocytes inhibiting autoreactive SPFC-hemolysis. Erythrocytes have the function of clearing pathogens, and a nonspecific SPFC effector against erythrocytes is useful for clearing erythrocytes with pathogens. Meanwhile, any persisting autoreactivity of SPFC might be harmful to autologous tissues such as vascular endothelium bearing erythrocyte antigens. In Table 2, the suppressor activity of nonauto-rosette-forming T cells (non-ARFC-T) against autoreactive SPFC is shown. Since one non-ARFC-T inhibited five SPFC effector cells, it was as effective as suppressor T cells. Figure 1 suggests that suppressor monocytes might participate in preventing the autoreactivity of SPFC.

Complement-receptor 3 of adhesion molecules involved in nonspecific hemolysis of SPFC-monocyte effector. CR3-adhesion molecules (CD11b/CD18) bind iC3b [23], zymosan, lipopolysaccharide (LPS) [22], and erythrocytes [19] as ligands. CR3 expresses two different epitopes [24], one that binds LPS or erythrocytes and is recognized by Mo-1 mAb and the other that recognizes iC3b and other proteinaceous ligands and is recognized by OKM10 mAb. We determined whether the mAb of Mo-1, Mo-1-F(ab')$_2$ or OKM10 was able to inhibit hemolysis of SPFC in the SPFC assay (Table 3). The numbers of SPFC against autologous and allogeneic erythrocytes as target were suppressed by the Mo-1 and OKM10 mAb, respectively. The F(ab')$_2$-Mo-1 mAb also suppressed the hemolysis of SPFC. The inhibition of hemolysis of SPFC effectors generated in allogeneic stimulation was greater than that generated without antigen stimulation. The results confirmed that the epitope of CR3 recognized by the Mo-1 mAb binds directly to the erythrocyte, and the binding results in hemolysis by H_2O_2 released from activated SPFC-monocyte effectors.

II. Allograft rejection and SPFC response in rats

An SPFC was also demonstrated in PBMC and infiltrating cells of rejecting allograft, and not in the spleen, of rats. To assess the involvement of SPFC-monocyte effectors in allograft rejection, monitoring of the SPFC generation in the infiltrating cells of transplanted allograft skin with or without immunosuppression was performed.

Concomitant SPFC generation with skin allograft rejection and with histoincompatibility. The mean allograft survival times of the H-1 incompatible ACI-Lewis combination and the H-1 compatible F344-Lewis combination were 7.67 ± 0.82 and 10.50 ± 3.27 days, respectively. The kinetics of SPFC generation after skin transplantation is shown in Fig. 2. In the ACI-Lewis combination, the peak number of SPFC of infiltrating cells, separated from rejecting allograft skin, was 23 850 ± 1340 × 10^6 on day 6 after transplantation, and the day of peak SPFC generation coincided with the onset of acute irreversible rejection. In the F344-Lewis combination, the peak SPFC response was seen on days 6 and 10. Since two of the three allografts harvested on day 6 were rejected and all skin allografts studied on day 10 were rejected, it was likely that the pattern of SPFC generation was biphasic. The peak number of SPFC on day 10 was 11 606 ± 4235 × 10^6, and there was a significant difference in the peak number of SPFC between the ACI-Lewis and F344-Lewis combinations ($P < 0.05$, Student's t-test). As to the SPFC response of PBMC, the peak SPFC generation (3893 ± 1273 SPFC × 10^6) of PBMC in the ACI-Lewis combination was seen on day 6 and that (13 440 ± 3484 × 10^6) of

Table 3. CR3 (complement receptor 3, CD11b/CD18) involved in hemolysis of SPFC-monocyte effectors in man. Monoclonal antibodies Mo-1, Mo-1-F(ab')$_2$, or OKM10 at various dilutions were added and incubated for SPFC assay. The 50% suppression level of the number of SPFC per 10^6 effectors was expressed as IC 50 (ng/ml). As effector cells, an allogeneic PBMC mixture cultured for 7 days or antigen-unprimed cultured PBMC were used

Monoclonal antibody against		IC 50 (ng/ml) Suppression of SPFC by monoclonal antibody	
CR3	Ligand	Allo-MLC primed effector	Unprimed effector
Mo-1	LPS/RBC	43	167
MO-1-F(ab')$_2$		21	167
OKM 10	iC3b	84	167
3G8	FcRIII	> 333	333

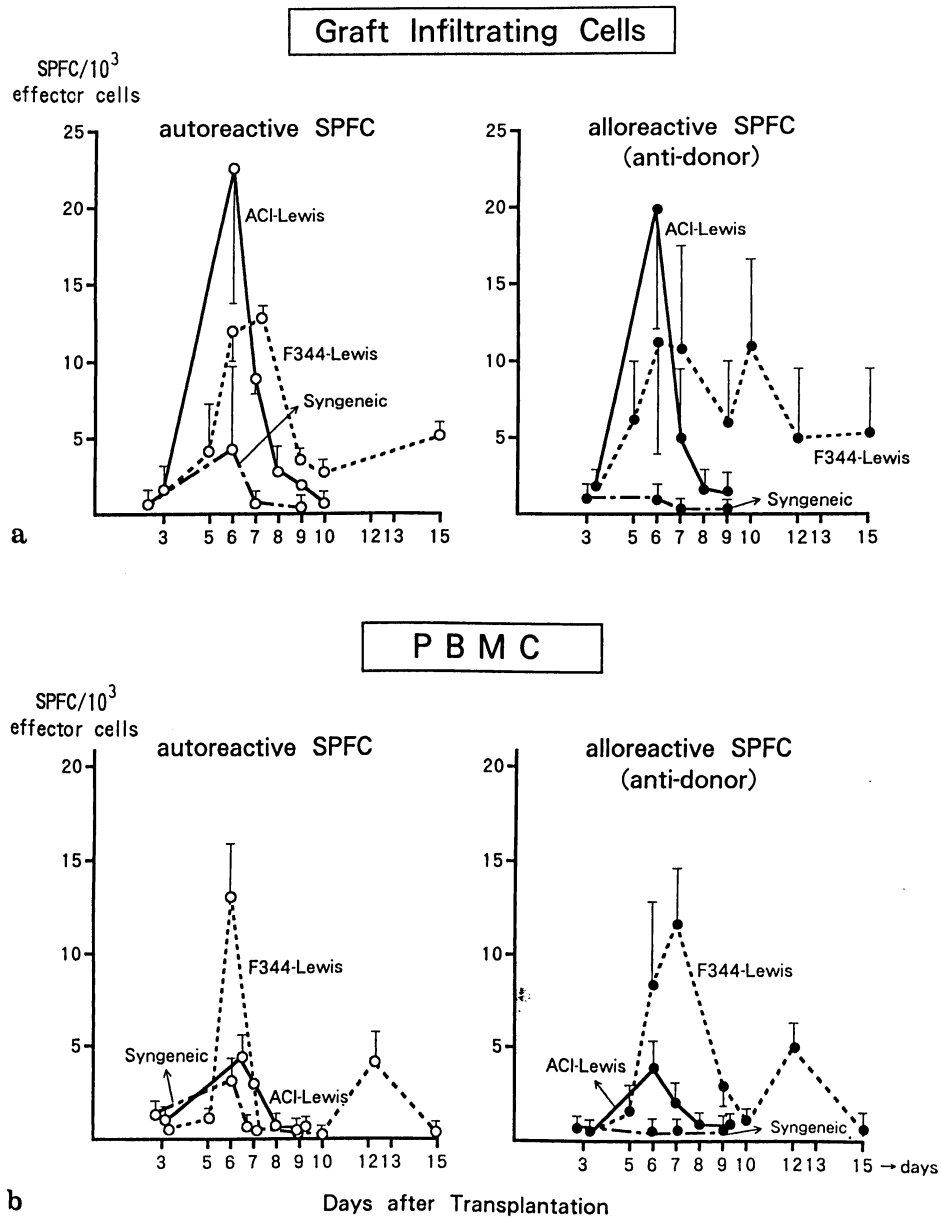

Fig. 2. Kinetics of SPFC generation, in vivo of graft infiltrating cells or PBMC of recipient Lewis rats receiving ACI H-1 incompatible (ACI-Lewis ——), F344 H-1 compatible (F344-Lewis -----), or syngeneic (Lewis-Lewis · · · · ·) skin transplantation. Autoreactive and alloreactive SPFC effectors of graft infiltrating cells and PBMC are shown

PBMC in the F344-Lewis combination was also found on day 6. In the syngeneic Lewis combination, the mean number of SPFC ($3190 \pm 4294 \times 10^6$) was observed on day 6, though the number of SPFC was smaller than that in both allogeneic combinations. Therefore, the extent of SPFC generation of allograft cellular infiltration following allograft transplantation reflected the grade of histoincompatibility.

Like the SPFC in man, the SPFC found in rats also showed nonspecific hemolysis against autologous and allogeneic RBC. In heart transplantation in the ACI-Lewis combination, the same tendency was observed between peak SPFC generation on day 5 and onset of acute rejection.

Suppression of SPFC generation by administration of immunosuppressive agents. A 6-day course of subcutaneous administration of immunosuppressive agents, cyclosporine 10 mg/kg daily, FK506 1 mg/kg daily, or prednisolone 10 mg/kg daily, was given, and the SPFC generation of allograft cellular infiltrating cells was monitored. Both cyclosporine and FK506 were able to prolong allograft skin survival by more than 21 days in the ACI-Lewis combination. Prednisolone prolonged allograft skin survival by fewer than 3 days, both compared with untreated cases. Figure 3 shows that the SPFC generation of infiltrating cells harvested on day 6 was completely suppressed by cyclosporine and FK506 to the level of a syngeneic, untreated transplantation. Meanwhile, prednisolone showed a weak inhibitory effect on SPFC generation in graft infiltrating cells and in PBMC. The suppression of SPFC generation in PBMC following allograft skin transplantation coincided with that of graft infiltrating cells. These results suggested that potent immunosuppressants which inhibit T-cell activation, such as cyclosporine and FK506, but not prednisolone, were able to suppress the SPFC generation ability of graft cellular infiltrating cells as well as of PBMC.

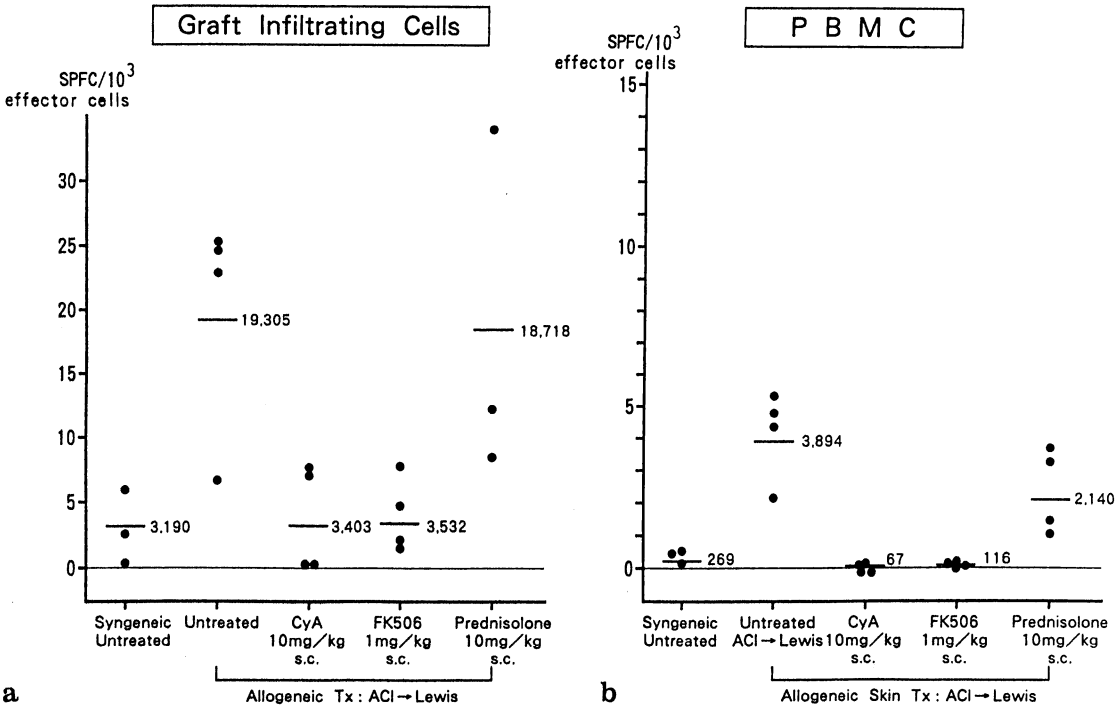

Fig. 3. Effect of immunosuppressive drugs on SPFC generation in ACI-Lewis skin allograft transplantation. Cyclosporine *(CyA)*, FK506, or prednisolone, respectively, was administered for a 6-day course of subcutaneous treatment on days 0–5 after transplantation. On day 6, the number of SPFC generated per 10^6 of graft infiltrating cells or PBMC was determined

Discussion

Activated macrophages were reported as one of the activation markers of allograft rejection in man and in rats. SPFC represent a unique effector of activated monocytes or macrophages in that nonspecific hemolysis by SPFC is mediated by the direct recognition of erythrocyte antigens or iC3b on tissues via an epitope of the CR3-adhesion molecule. The immunologic mechanism of graft destruction by activated macrophages might be explained by the concept of a network for the regulation of SPFC generation. In the effector phase of activated macrophages, SPFC recognize antigenic determinants of erythrocytes on the vascular endothelium via an epitope of CR3 expressed on the SPFC with or without local complement activation on the target endothelial tissues. Though the SPFC undergo hemolysis, nonspecifically releasing H_2O_2, suppressor T lymphocytes (detected as non-ARFC-T) or suppressor monocytes might inhibit autoreactive SPFC effectors. If the determinants of erythrocytes expressed on the allograft which are recognized by CR3 molecules are close to those of self-erythrocytes, acceptance of allograft tissues might be easily induced by the participation of suppressor cells. According to the present concept of self or nonself discrimination on the effector level as described by nonspecific SPFC effector and suppressor cells inhibiting autoreactivity, the mechanism of allograft rejection or acceptance might be clarified.

Furthermore, the SPFC have the heterogenous function of effector cells, because they express different epitopes of CR3 for iC3b and erythrocytes. Local complement activation on target tissue, either in the absence or in the presence of antibody, forms iC3b, and SPFC binds to target tissues via CR3 molecules to iC3b and erythrocytes on targets and finally undergoes hemolysis or induces cytolysis. Since the SPFC express a Fc receptor, binding of the SPFC with antibody across the target tissue might induce phagocytosis or cytolysis.

The SPFC generation is regulated in a T-lymphocyte-dependent or T-lymphocyte-independent manner in man. As to the regulation of SPFC generation in rats, the present findings revealed that the degree of SPFC generation reflected the grade of histoincompatibility, which induces T-cell activation to various degrees, and that potent immunosuppressants which inhibit T-cell activation suppressed SPFC generation of allograft infiltration. It is suggested that SPFC generation is also regulated by T cells in rats. Our previous study [12] as to the effect of FK506 on SPFC generation in rats also demonstrated that a dose of FK506 of less than 0.3 mg/kg daily suppressed graft infiltration of the SPFC but failed to suppress SPFC generation of the PBMC. Also, tumor necrosis factor (TNF)-β [2], released from activated T cells and promoting the accumulation of inflammatory cells in inflammatory sites, might be involved in the regulation of SPFC generation of graft infiltrating cells. Therefore, nonspecific SPFC-monocytes might be one of the activation markers of allograft rejection.

References

1. Fleit HB, Wright SD, Unkeless JC (1982) Human neutrophile Fcr receptor distribution and structure. Proc Natl Acad Sci USA 79: 3275–3279

2. Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA (1985) Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. 82: 8667–8671
3. Halloran PF, Cockfield SM, Madrenas J (1989) The mediators of inflammation (interleukin 1, interferon-r, and tumor necrosis factor) and their relevance to rejection. Transplant Proc 21: 26–30
4. Hancock WW, Lord RH, Colby AJ, Diamanstein T, Rickles FR, Diskstora C, Hogg NZ, Tilney NL (1987) Identification of IL-2R T cells and macrophages within rejecting rat cardiac allografts, and comparison of the effects of treatment with anti-IL2R monoclonal antibody or cyclosporine. J Immunol 138: 164–170
5. Harris HW, Gill TJ III (1986) Expression of class I transplantation antigens. Transplantation 42: 109–117
6. Häyry P, Willebrand E von, Parthenais E, Nemlander A, Soots A, Lautenschlager I, Alfordy P, Renkonen R (1984) Immunol Rev 77: 85–142
7. Ishibashi M, Ichikawa Y, Sagawa S, Takaha M, Sonoda T, Inou K (1984) Immunologic studies of red cell antigens in the renal transplant patient. Transplant Proc 16: 1509–1511
8. Ishibashi M, Ichikawa Y, Takaha M, Sonoda T (1985) Monocyte-mediated hemolytic response in renal transplant patients. Transplant Proc 17: 2644–2647
9. Ishibashi M, Kokado Y, Takahara S, Ichikawa Y, Sonoda T (1987) Cellular immune response agaist human red blood cell antigens and renal allograft rejection. Transplant Proc 19: 4511–4515
10. Ishibashi M, Kokado Y, Takahara S, Ichikawa Y, Sonoda T (1988) Novel effector monocyte against human RBC antigens invading rejected renal allograft. Transplant Proc 20: 285–288
11. Ishibashi M, Jiang H, Kokado Y, Takahara S, Sonoda T (1989) Immunopharmacologic effect of immunosuppressive agents explored by a new effector-monocyte generation assay. Transplant Proc 21: 1854–1858
12. Ishibashi M, Moutabarrik A, Kameoka H, Takano Y, Jiang H, Kokado Y, Takahara S, Sonoda T (1991) Effect of FK506 on generation of activated macrophages invading rejected skin allografts in rats: discrepancy of in vitro versus in vivo effect of FK506. Transplant Proc (in press)
13. Letvin NL, Todd RR III, Palley LS, Schlossman SF, Griffin JD (1983) Conservation of myeloid surface antigens on primate granulocytes. Blood 61: 408–410
14. Lord RHH, Padberg WM, Hancock WW, Kupiec-Weglinski JW, Tilney NL (1989) Correlation of macrophage and NK cell numbers with "activation markers" in rat cardiac allografts. Transplant Proc 21: 449–450
15. Lowley RP, Magghesco DM, Blackburn JH (1985) Immune mechanisms in organ allograft rejection. IV. Delayed-type hypersensitivity. Immunol Rev 77: 167–184
16. Macoherson GC, Christmas SE (1984) The role of the macrophage in cardiac allograft rejection in the rat. Immunol Rev 77: 143–166
17. Mason DW, Dallman MJ, Arthur RP, Morris PJ (1984) Mechanisms of allograft rejection: the roles of cytotoxic T-cells and delayed-type hypersensitivity. Immunol Rev 77: 167–184
18. Moutabarrik A, Ishibashi M, Kameoka H, Takahara S, Kokado Y, Jiang H, Takano Y, Sonoda T (1992) Monocyte hemolytic activity as an immunologic indicator of allograft rejection in rat skin transplantation. Transplant Proc (in press)
19. Ross GD, Cain JA, Lachman PJ (1985) Membrane complement receptor type three (CR3) has lectin-like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. J Immunol 134: 3307–3315
20. Tomonari K, Wakisaka A, Aizawa M (1980) Self recognition by autologous mixed lymphocyte reaction-primed cells. J Immunol 125: 1596–1602
21. Uchiyama T, Broder S, Waldmann TA (1981) A monoclonal antibody (anti-Tac) reactivity with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells. J Immunol 126: 1393–1397
22. Wright SD, Jong MTC (1986) Adhesion-promoting receptors on human macrophages recognize *Escherichia coli* by binding to lipopolysaccharide. J Exp Med 164: 1876–1888
23. Wright SD, Rao PE, Van Voorhis WC, Craigmyle LC, Iida MA, Talle MA, Westberg EF, Goldstein G, Silverstein SC (1983) Identification of the C3bi receptor on human monocytes and macrophages by using monoclonal antibodies. Proc Natl Acad Sci USA 80: 5699–5703
24. Wright SD, Levin SM, Jong MTC, Chad Z, Kabbash LG (1989) CR3 (CD11b/CD18) expresses one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. J Exp Med 169: 175–183