MEMOIRS OF THE FACULTY OF SCIENCE, KYOTO UNIVERSITY, SERIES OF BIOLOGY
Vol. X, pp. 1–16, March 1985

Roles of Murine Dendritic Cells and Macrophages in the Initiation of Primary Antibody Response *In Vitro*

I. Culture Conditions and Cell Preparations

By

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(Received August 27, 1984)

Abstract. 1) Culture conditions for *in vitro* anti-sheep erythrocyte antibody response were investigated. The response in 96-well flat-bottomed plate was dependent on 2-mercaptoethanol, and in order to obtain high antibody response, selection of antigen and serum lots was essential. Adjuvanticities of B cell and T cell mitogens were also examined. 2) Preparation and characterization of the following cells are described: spleen lymphoid cells devoid of accessory cell activity, splenic dendritic cells, cultured peritoneal Ia$^+$ and Ia$^-$ macrophages and peritoneal cell populations containing Ia$^+$ macrophages activated *in vivo*.

Introduction

Accessory cells (A-cells)[1] in the primary antibody response reside in the Ia-bearing (Ia$^+$) adherent cell population. As such adherent cells, there are two major categories of cells, dendritic cells (DC) and Ia$^+$ macrophages (Mϕ).

DC seem potent as A-cells. We have previously demonstrated that low dose DC manifest A-cell activity with help from Ia$^-$ Mϕ (Inaba and Muramatsu 1980, Inaba et al. 1981, Inaba et al. 1982). Furthermore, Inaba et al. (1983a, b) have demonstrated that high dose DC manifest A-cell activity without help from Mϕ.

Are Ia$^+$ Mϕ really potent as A-cells? This seems still under debate. It has been conceived that Ia$^+$ Mϕ are actually A-cells (reviewed in Unanue 1981). However, we find no conclusive evidence for this assumption, since it seems possible that A-cell and/or lymphocyte preparations employed in the investigations, which appeared to argue that Ia$^+$ Mϕ are potent as A-cells, contained Ia$^+$ non-Mϕ cells such as DC and the manifestation of A-cell activity might be due to the synergy between such Ia$^+$ non-Mϕ cells and Mϕ but not Ia$^+$ Mϕ themselves. Thus, a critical point in the investigation on A-cell activity of Ia$^+$ Mϕ is to employ cell preparations free from such Ia$^+$ non-Mϕ cells.

In Part I of this paper, we investigated the *in vitro* culture conditions suitable for the investi-

[1] Abbreviations: A-cell(s), accessory cell(s); BCG, Bacillus Calmette-Guerin; Con A, concanavalin A; DC, dendritic cell(s); EA, opsonized erythrocyte(s); LPS, lipopolysaccharide; Mϕ, macrophage(s); PEC, peritoneal exudate cells; PFC, plaque-forming cell(s); SRBC, sheep erythrocyte(s); TGC, thioglycollate medium.

gation of roles of DC and Mϕ in antibody response. In Part II, we addressed ourselves to the preparation of cell populations which are necessary for the investigations.

Materials

Mice—Inbred female mice of the following strains at the age of 2–5 months were used; C3H/HeSlc, BALB/cCrSlc, A/JSlc (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, or Institute of Animal Experiments, Kyoto University), and CBA/JSn (originally gifted by Dr. T. Sado, National Institute of Radiological Sciences, Chiba). The strain of mice routinely employed was C3H/HeSlc unless mentioned.

Medium—Hanks' balanced salt solution (HBSS; Nissui Seiyaku Co., Tokyo) was used for cell preparation and washing. For cell culture, the following mediums were used. Medium 1: Eagle's minimum essential medium containing 0.06 mg/ml kanamycin (MEM; Nissui) supplemented with 2 mM glutamine (Gln; Wako Pure Chemical Industries, Ltd., Osaka), 5 mM HEPES (Wako), and 0.05 mM 2-mercaptoethanol (2-ME; Wako). Medium 2: 1 mM sodium pyruvate and 1% MEM nonessential amino acids solution (100×concentrated; GIBCO, Grand Island, NY) was added to Medium 1. Medium 3: RPMI-1640 (Nissui) supplemented with 2 mM Gln, 10 mM HEPES, 100 U/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo) and 0.1 mg/ml streptomycin (Meiji). Medium 4: 0.05 mM 2-ME was added to Medium 3. $NaHCO_3$ was employed to adjust pH of the mediums. Culture mediums for the antibody response were as follows: in Tables 1 and 2, and Fig. 1, Medium 2 containing 5% fetal bovine serum (FBS) and 5% horse serum; in Table 3, Medium 4 containing 5% FBS; and in Tables 4–7, Medium 4 containing 10% FBS.

Antigen—SRBC stored in Alsever's solution (Shimizu Experimental Materials, Kyoto) at 4°C for 1–8 wk after aseptic bleeding were washed 3 times before use.

Results and Discussion

I. In vitro *anti-SRBC antiboody response*

Culture vessels—Various kinds of culture vessels are commercially available. In order to select suitable vessels, spleen cells of C3H/He mice were cultured in 4 kinds of plastic culture vessels (A/S Nunc, Kamstrup, Roskilde) for 4 days, and anti-SRBC antibody-secreting cells in each well were evaluated by direct plaque-forming cell (PFC) assay in agarose gel on slides (Jerne et al. 1963, Hosono and Muramatsu 1972). In the experiment shown in Table 1, 10^6, 5×10^6, and 10^7 spleen cells were cultured in flat-bottomed 96-well plate, 24-well plate, and 3.5 cm dish, respectively. Although the number of PFC per culture (per well) varied among the vessels, PFC per 10^6 plated cells were almost constant in three flat-bottomed vessels. In Fig. 1, PFC per 10^6 cells were higher in 96-well plate than in 24-well plate. So it seems profitable to use 96-well plate, since this culture vessel needed fewer cells than the others.

PFC response in 96-well round-bottomed plate was higher than that in flat-bottomed one (Table 1). Round-bottomed plate, however, seems to be inadequate for the investigation of A-cell activity, since 1) it was difficult for Mϕ to adhere on the plate uniformly, and 2) requirement of A-cells was much lower in this plate than in flat-bottomed one (data not shown), so that even

Table 1. Comparison of PFC responses in various plastic culture vessels[a]

Culture Vessel	Spleen Cells ($\times 10^{-6}$)			PFC/Culture		PFC/10^6 Plated Cells	
	/Culture	/ml	/Base area (cm^2)	Ag+	Ag−	Ag+	Ag−
96-well flat[b]	1.0	5.0	2.86	57±20	0	57±20	0
96-well round[c]	1.0	5.0	—[f]	235±54	0	235±54	0
24-well flat[d]	5.0	5.0	2.62	278±72	16±16	56±14	2±2
3.5 cm dish[e]	10.0	5.0	1.12	491±144	108±48	49±14	11±5

[a] Spleen cells of C3H mice were cultured for 4 days and anti-SRBC antibody-secreting cells were evaluated by direct PFC assay.
[b] Flat-bottomed multi-well plate. The diameter of a well is 0.67 cm.
[c] Round-bottomed multi-well plate. The diameter of a well is 0.67 cm.
[d] Flat-bottomed multi-well plate. The diameter of a well is 1.56 cm.
[e] Plastic Petri dish. The inside diameter is 3.4 cm.
[f] Plated cells concentrate into the central area of a well, since the bottom of a well is round.

lymphocyte preparations depleted of A-cells as far as possible, frequently respond to antigen without further addition of A-cells.

PFC response per culture on day 4 was higher in 24-well plate than in 96-well plate and the decrease of the response on day 5 was more gradual (Fig. 1). So it might be better to use 24-well plate if it is possible to prepare sufficient number of cells to investigate. The yield of DC per mouse, however, was very low (Table 11; 10^6 DC per 10–20 mice). So it seems very difficult to use 24-well plate in the investigation on A-cell activity of DC.

As for 3.5 cm dish, this vessel needed at least 5–10 times as many cells and much other materials as 96-well plate did. So this vessel seems to be inadequate for this study mainly for an economic reason.

Collectively thinking, 96-well flat-bottomed plate seems to be most convenient for the investigation of the roles of DC and Mϕ in the *in vitro* antibody response. Most of the following experiments were performed by using this plate.

Kinetics of anti-SRBC PFC response—In order to investigate the kinetics of PFC response, spleen cells were cultured in 24-well plates or 96-well plates for 3–5 days (Fig. 1 and Table 2).

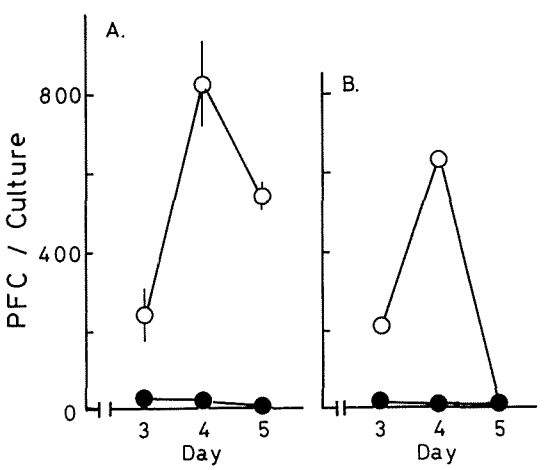

Fig. 1. Kinetics of anti-SRBC PFC response. 4×10^6 or 10^6 spleen cells were cultured in 24-well plates (A) or 96- well plates (B) respectively in the presence (○) or absence (●) of SRBC. After 3–5 days, the cells in each well were assayed for direct PFC.

Table 2. Dependency on 2-mercaptoethanol (2-ME)

Day	Spleen cells are cultured[a] with 2-ME[b]		without 2-ME	
	Ag+	Ag−	Ag+	Ag−
3	92±17	1±1	5±3	0
4	164±14	2±1	6±5	0
5	25±6	1±1	1±1	0

[a] Spleen cells were cultured in 96-well plates and assayed for direct PFC on the indicated day.
[b] 0.05 mM.

Significant PFC response was observed on day 3, and maximum response on day 4. In 96-well plates, PFC response on day 5 was completely (Fig. 1B) or almost completely (Table 2; with 2-ME) abolished. In case of 24-well plate, the level of PFC response on day 5 decreased significantly but was still high (Fig. 1A)—Thus, it seems best to assay on day 4 if it is impossible to assay at several points of culture period.

Dependency on 2-mercaptoethanol (2-ME)—In order to obtain significant PFC response in 96-well flat-bottomed plate, it was necessary to supplement the medium with 2-ME. Table 2 demonstrates the dependency on 2-ME. 10^6 spleen cells were cultured in the presence (0.05 mM) or absence of 2-ME for 3–5 days. When spleen cells were cultured in the presence of 2-ME, significant PFC response was observed. The maximum response on day 4 was 164 PFC per culture. However, when spleen cells were cultured in the absence of 2-ME, only 5, 6, and 1 PFC were detected on day 3, 4, and 5, respectively.

The necessity to select SRBC lots—In order to obtain high level of anti-SRBC PFC response in 96-well plate, it was necessary to select SRBC lots, since the immunogenicity of commercially

Table 3. Level of PFC response depends on SRBC lot[a]

Exp. 1 SRBC used in culture	SRBC used in PFC assay lot 1	lot 2	lot 3	lot 4
lot 1	144±49	ND.[b]	ND.	ND.
lot 2	ND.	305±39	〃	〃
lot 3	〃	ND.	61±13	〃
lot 4	〃	〃	ND.	533±99
Ag−	〃	37±18	〃	ND.
Exp. 2	lot 5	lot 6	lot 7	
lot 5	**390±23**	88±24	202±16	
lot 6	70±5	**77±11**	112±16	
lot 7	229±16	99±14	**261±8**	
Ag−	66±23	16±13	43±15	

[a] Spleen cells were cultured in 96-well plates for 4 days and the cells in each well were assayed individually (Exp. 1), or the cells in each group were pooled and assayed using 3 different SRBC lots (Exp. 2).
[b] Not done.

available SRBC differed among lots (Table 3). In Exp. 1, the immunogenicity of lot 4 was the highest. Lots 2 and 1 also induced considerably high level of PFC response, whereas, lot 3 induced poor PFC response. In Exp. 2, lots 5 and 7 induced high PFC response, whereas lot 6 induced low PFC response.

It was suggested from Exp. 2 of Table 3 that in PFC assay, it was better to use the lot which had been used in PFC culture. For example, when the spleen cells cultured with SRBC lot 7 were assayed using lot 7, 261 PFC per culture were detected, but when the spleen cells from the same group were assayed using lot 5 or 6, PFC decreased to 229 and 99 respectively. Lot 5 was more immunogenic than lot 7 and lot 6 was less immunogenic than lot 7, so that the decrease was not only due to the level of immunogenicity of SRBC used in PFC assay.

The necessity to select serum lots—In order to obtain high PFC response in 96-well plate, it was necessary to select PFC-supportive sera. 10^6 spleen cells in medium supplemented with 10% serum of various lots were cultured in 96-well plate for 4 days (Table 4). 98 lots of fetal bovine serum (FBS) were tested. 4 lots were strongly supportive (more than 300 PFC per culture as the mean of 2 independent experiments). 23 lots were fairly supportive (100–300 PFC), and 71 lots were non-supportive (less than 100 PFC). Table 5 showed one of the results of FBS lot test. Lot 1 was strongly supportive, lots 2, 3, 6, and 7 were fairly supportive, and the others were non-supportive. 12 lots of calf serum, 15 lots of newborn calf serum, and 11 lots of pseudo-FBS (serum of newborn calf which did not suck colostrum yet) were also examined (Table 4), but all lots were non-PFC-supportive (0–28 PFC per culture). Freshly isolated normal mouse sera were also examined. The sera were added to the culture at the concentration of 0.5–10%, but they did not induce PFC response (data not shown).

Why did some lots of FBS support PFC response and others not? PFC-potentiating factor(s) in PFC-supportive FBS might be derived not from fetal calf but from some bacteria contaminating during the preparation of FBS, since FBS may usually be prepared under non-aseptic conditions. In fact, in some cases, bacteria proliferated in FBS which was stored at 4°C for more than 1 month. This is probably because of the incomplete sterilization of contaminated FBS before shipment. Table 6 showed the enhancement of PFC response by culture supernatant of bacteria

Table 4. Summary of serum lot test[a]

FBS		98 lots
strongly supportive	4	
fairly supportive	23	
non-supportive	71	
Calf serum		12 lots
	all were non-supportive.	
Newborn calf serum		15 lots
	all were non-supportive.	
Pseudo-FBS[b]		11 lots
	all were non-supportive.	

[a] Spleen cells in medium supplemented with 10% serum were cultured for 4 days and assayed. The experiment for each lot was done twice and the mean of the results was evaluated as follows. Strongly supportive: more than 300 PFC/culture. Fairly supportive: 100–300 PFC. Non-supportive: less than 100 PFC.

[b] Precolostrum newborn calf serum.

Table 5. Dependency on FBS lot[a]

FBS lot	PFC/Culture			
	Exp. 1		Exp. 2	
	Ag+	Ag−	Ag+	Ag+
1	704±128	23±12	1015±34	25±5
2	60±12	4±2	149±39	5±1
3	97±34	3±3	208±103	9±3
4	44±18	5±3	44±9	12±11
5	52±18	0	89±21	15±8
6	119±36	9±5	100±40	4±2
7	219±38	16±6	145±42	25±12
8	13±6	1±1	24±2	5±4
9	11±8	3±3	21±16	12±5
10	0	1±1	36±30	8±4
11	13±10	13±10	11±7	1±1

[a] Spleen cells in medium supplemented with 10% FBS of 11 lots were cultured in 96-well plates for 4 days.

Table 6. Culture supernatant of bacteria isolated from PFC-supportive FBS enhances *in vitro* antibody response[a]

Serum	Bacterial culture Supernatant	PFC/Culture	
		Ag+	Ag−
FBS-con.[b]	none	1708	113
FBS-non.[c]	none	405	8
FBS-non.	10%	2585	130

[a] Spleen cells were cultured in 24-well plate in the presence or absence of bacterial culture supernatant and assayed after 4 days.

[b] FBS from which PFC-enhancing bacteria had been isolated.

[c] Non-contaminated FBS which support antibody response fairly.

which were isolated from PFC supportive FBS. 4×10^6 spleen cells in the medium supplemented with 10% FBS were cultured in 24-well plate in the presence or absence of bacterial culture supernatant. The supernatant was obtained by culturing the bacteria in Eagle's MEM at 37°C for 1 day and sterilizing the culture supernatant. The FBS lot from which PFC-enhancing bacteria had been isolated, induced 1708 PFC per culture after complete sterilization of the serum. A FBS lot in which bacteria had not proliferated, induced 405 PFC per culture, whereas, in the presence of 10% supernatant, 2585 PFC could be induced. Although this experiment did not make clear that bacterial culture supernatant could induce PFC response in the absence of FBS, it seems likely that such bacterial products are one of the factors of PFC-supportiveness of FBS.

Adjuvanticities of B cell and T cell mitogens—If mitogen is added, can SRBC-specific PFC response occur in medium containing non-PFC-supportive FBS? The answer is yes. Table 7 showed adjuvanticities of lipopolysaccharide (LPS: a B cell mitogen; Difco Laboratories, Detroit, MI) and concanavalin A (Con A: a T cell mitogen; Pharmacia Fine Chemicals, Uppsala). 10^6 spleen cells were cultured in the presence or absence of LPS and/or Con A. PFC

Table 7. Adjuvanticities of LPS and Con A[a]

Mitogen (ng/ml)	PFC/Culture					
	A. LPS		B. Con A		C. LPS+Con A	
	Ag+	Ag−	Ag+	Ag−	Ag+	Ag−
0	7±4	22±2	7±4	22±2	7±4	22±2
0.1	13±3	38±19	10±5	25±15	80±12	18±16
1	17±7	3±2	55±18	38±16	42±19	13±4
10	18±12	22±6	137±27	5±5	103±15	15±6
100	38±11	45±28	278±97	10±3	298±50	13±4
1000	392±12	22±4	370±10	78±31	not done	

[a] Spleen cells in medium supplemented with 10% non-PFC-supportive FBS were cultured in the presence or absence of lipopolysaccharide (LPS) and/or concanavalin A (Con A), and PFC assay was done after 4 days.

response without mitogen did not occur significantly, since FBS employed was non-PFC-supportive. LPS at the dose of up to 100 ng/ml seemed insufficient to induce significant PFC response. As for Con A, the minimum dose necessary to induce significant PFC response was much less than that of LPS. It seemed that 10 ng/ml of Con A was sufficient to induce significant PFC response.

Synergy between LPS and Con A was not observed significantly in the presence of 1, 10, and 100 ng/ml of both mitogens. At the dose of 0.1 ng/ml, the synergy was apparently observed. However, the synergy did not seem so significant, since the response decreased in the presence of 1 ng of both mitogens.

II. *Cell preparations and their characterization*

Spleen lymphoid cells devoid of A-cell activity—The principle of preparation of spleen lymphoid cells devoid of A-cell activity is based on the fact that A-cell activity resides mainly in adherent cell population. For this purpose, dish-adherence technique (Mosier 1967) and a method of Sephadex G-10 (Pharmacia) passage (Ly and Mishell 1974) were combined and employed. The former technique is easy to manipulate, but incomplete to deplete A-cell activity when dish-nonadherent cells are cultured in the presence of 2-ME (Unanue 1981, Chen and Hirsch 1972). The latter can not only eliminate A-cell activity efficiently, but also enrich small (or resting) lymphocytes.

In order to ensure the elimination of A-cell activity and the enrichment of small lymphocytes, only fast-filtrating cells through Sephadex G-10 columns were collected. Fig. 2 showed the accumulated yield (percentage of applied cells) in filtrate of single G-10 passage of dish-nonadherent cells. G-10-passed cells began to appear in the filtrate at about 33% column volume that corresponded to a void volume of the column. Filtrate was usually collected until to reach 60% column volume (arrow indicates the volume) and the cells filtrated thereafter were discarded.

The procedure of preparing G-10-passed cells was as follows. Spleen cells were treated with cold hemolytic Gey's solution for 2 min to eliminate erythrocytes, washed 3 times with HBSS, resuspended in Medium 1 containing 5% FBS, and incubated in plastic dishes (Falcon No. 3003) at the density of $0.7–1.0 \times 10^8$ cells per dish for 1–3 hr. Nonadherent cells were collected by pipetting and aspiration. Since care was taken not to dislodge weakly adherent cells, a con-

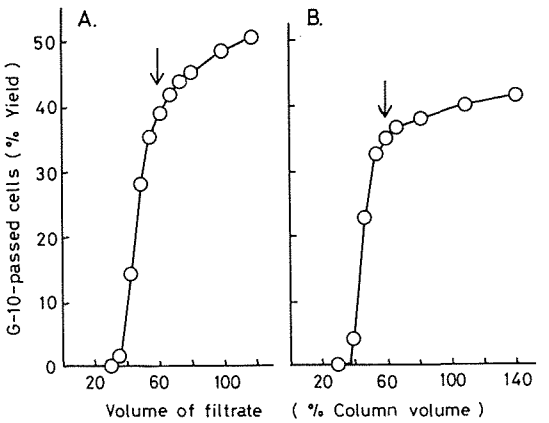

Fig. 2. Filtration of dish-nonadherent spleen cells through a Sephadex G-10 column. Spleen cells were cultured in plastic Petri dishes for 2 hr, and nonadherent cells were collected and applied to a Sephadex G-10 column of 10 ml bed-volume. 1.4×10^8 (A) or 1.7×10^8 cells in 0.9 ml medium were applied to the column and the filtrate was fractionated. Then, the cells in each fraction were counted. This figure indicates the accumulated yield of G-10-passed cells in the filtrate. Arrow indicates the yield in the filtrate which corresponds to 60% of the column-volume.

Table 8. Yield of A-cell depleted spleen cells[a]

Exp.	Percent Recovery[b]	
	Dish-nonadherent cells	G-10 twice passed[c] cells
1	88	14
2	73	21
3	83	27
4	81	17
5	79	26
6	80	24
7	77	30
8	85	31
9	84	26
10	76	22

[a] Spleen cells were cultured for 1–3 hr in plastic Petri dishes, and the nonadherent cells were collected and passed through Sephadex G-10 columns.
[b] Percentage of original spleen cells.
[c] Dish-nonadherent, G-10 twice passed spleen cells.

siderable number of lymphocytes were also left on the dishes. Dish-nonadherent cells were then passed twice through columns of Sephadex G-10, and fast-filtrating cells were collected and used as G-10-passed cells. The yields of dish-nonadherent cells and G-10-passed cells were 73–88%, and 14–31% of original spleen cells, respectively (Table 8).

In order to examine the constituents of G-10-passed cells, the cells were centrifuged to smear on slides, fixed and stained with May-Gruenwald-Giemsa stain, and examined under light microscopy ($\times 400$). Table 9 summarizes the results. 93.3–98.5% were small lymphocytes. Polymorph content varied among preparations from 0.16 to 5.4%. Although morphologically identified blastoid cells of medium size were contaminated (0.97–1.8%), Mϕ and monocytes were not detected. A small number of nonlymphoid adherent cells, however, differentiated from G-10-passed cells after the culture for 2 days or more, suggesting that depletion of precursor cells of the adherent cells was not perfect. The number of adherent cells differentiating after 4 days of

Table 9. Constituents of G-10 passed spleen cells

Exp.	Total cells counted	Percentage		
		Lymphocytes	Polymorphs[a]	The others[b]
1	957	93.3	5.4	1.3
2	1131	96.8	2.2	0.97
3	868	98.5	0.23	1.3
4	1192	97.5	0.76	1.8
5	996	95.3	2.9	1.8
6	3127	98.3	0.16	1.6

[a] Most of them were neutrophils, but some eosinophils were also detected.
[b] Morphologically identified blastoid cells of medium size. Mϕ and monocytes could not be detected.

culture was 50–500 per 10^6 G-10-passed cells. Fig. 3A and B showed the microscopic observation of G-10-passed cells ($\times 1000$).

Dendritic cells (DC): A. *Preparation*—DC were prepared according to the method of Steinman and his colleagues (Steinman et al. 1979, Nussenzweig and Steinman 1980) with minor modifications. Spleen cells suspended in dense bovine serum albumin solution ($\rho=1.082$: BSA, Fraction V; Armour Pharmaceutical Co., Kankakee, IL) were centrifuged at $1500\times g$ for 10 min, instead of $10000\times g$ for 30 min in Steinman's method. The yield and composition of cells were not significantly affected by this modification. The floating cells (low density cells: LDC) were collected, washed once, suspended in Medium 4 containing 10% FBS, and incubated in plastic dishes for 2–3 hr. After removing nonadherent cells, the adherent cells (low density adherent cells: LDAC) were irradiated with 1300 R X-ray to prevent cell proliferation, and cultured for additional 18–24 hr. The cells which became nonadherent during the culture period were collected by gentle pipetting and aspiration, and incubated in a new dish for 1–2 hr. Most of the cells still remained nonadherent, and they were collected by gentle swirling of the dish and aspiration, leaving a small number of Mϕ and some adherent DC. The cells thus prepared were used as DC.

Preparation of DC free from Mϕ greatly depends on the careful and flexible manipulation of pipetting which seems hard to mention simply. Critical points are as follows. Although DC are known to be temporarily adherent, careless washing-out of nonadherent cells will result in LDAC preparation containing either a few DC or many lymphocytes. After overnight culture of LDAC, most of the DC become nonadherent, and some Mϕ also do so which readhere on a fresh surface of a dish. It seems especially necessary to manipulate a careful pipetting at this final purification step, otherwise this step result in either a few DC or contamination of Mϕ.

B. *Characterization*—1) Contamination of blastoid cells was greatly reduced by irradiation of LDAC (C3H: 0.18–0.56%, and CBA/J: 0.65%). Table 10 showed percent blastoid cells in non-irradiated, and irradiated DC preparations. Purified DC preparations were centrifuged to smear on slides, and fixed and stained with May-Gruenwald-Giemsa stain. Blastoid cells were morphologically identified under light microscopy ($\times 400$). Blastoid cells in C3H and CBA/J preparations were greatly reduced from 2.14 to 0.35% and from 5.11 to 0.65% respectively by irradiation (Exps. 1 and 2). 2) Yield of DC preparations is summarized in Table 11 (C3H: 0.08–0.19, CBA/J: 0.10–0.17, A/J: 0.16, and BALB/c: 0.20% of original spleen cells). 3) Fc receptor-bearing (FcR^+) cells were not detected except one DC preparation of CBA/J mice.

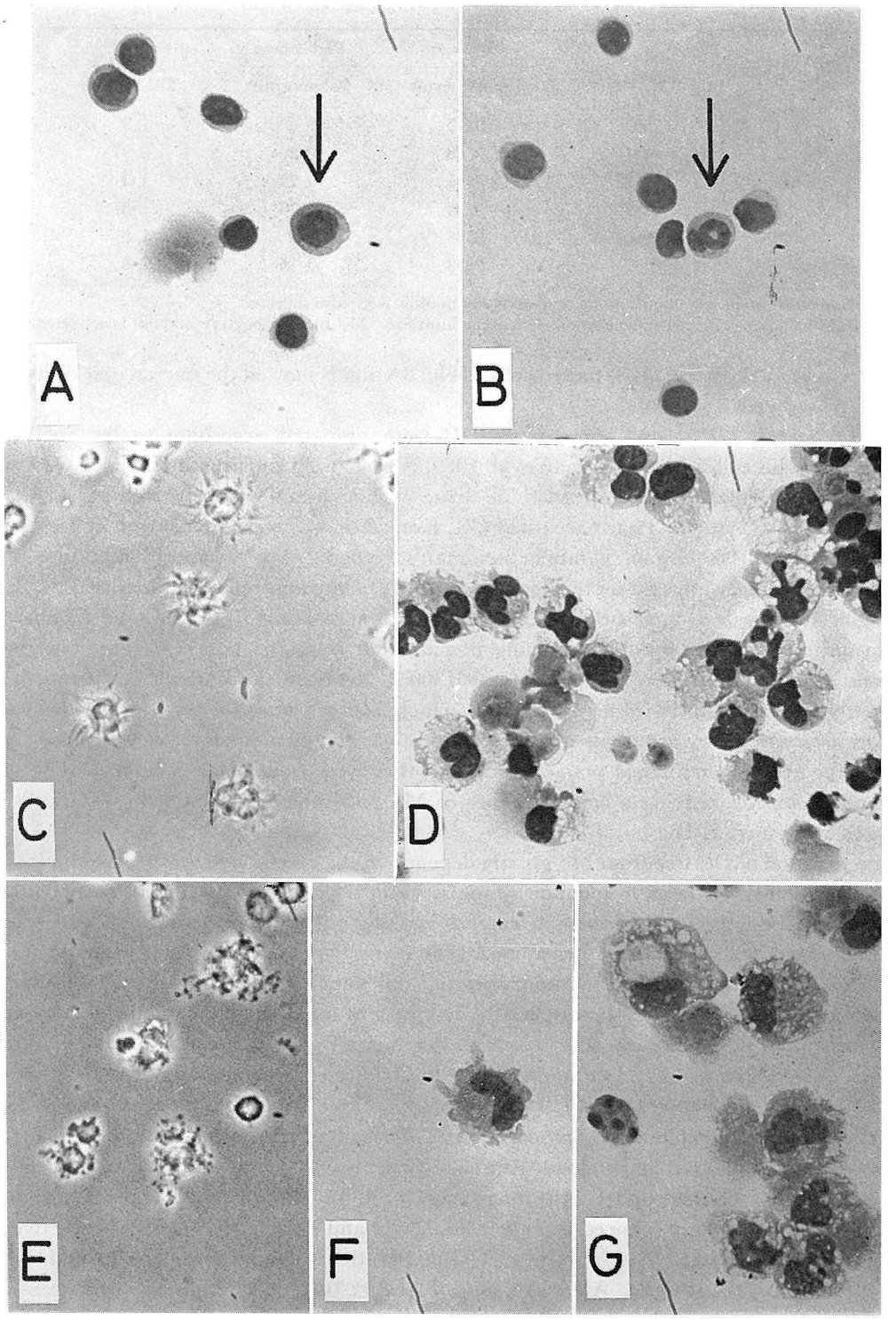
A
B
C
D
E
F
G

Table 10. Blastoid cells in DC preparations

Exp.	Strain	Non-irradiated[a]			Irradiated[b]		
		Total cell counted	Blastoid cells No.	%	Total cell counted	Blastoid cells No.	%
1	C3H	1402	30	2.14	1429	5	0.35
2	CBA/J	2329	119	5.11	1074	7	0.65
3	C3H	1706	17	1.00		not done	
4	CBA/J	1178	150	12.73		not done	
5	C3H		not done		1241	7	0.56
6	C3H		not done		1094	2	0.18

[a] Cells were not irradiated at any step of purification.
[b] Low density adherent cells were irradiated with 1300 R X-ray before overnight culture.

Table 11. Yields[a] of low density cell (LDC) and DC preparations[b]

C3H			CBA/J			A/J			BALB/c		
Exp.	LDC	DC	Exp.	LDC	DC	Exp.	LDC	DC	Exp.	LDC	DC
1	8.3	0.19	1	12.6	0.12	1	8.3	0.16	1	11.0	0.20
2	9.0	0.18	2	12.0	0.17						
3	9.4	0.15	3	9.5	0.10						
4	6.1	0.08									
5	20.0	0.14									
6	7.6	0.15									
7	8.1	0.14									

[a] Percentage of original spleen cells.
[b] Low density adherent cells were irradiated with 1300 R X-ray.

Table 12 summarizes the results. DC preparations were mixed with opsonized erythrocytes (EA) at a ratio of 1 : 30, and centrifuged at $40 \times g$. After incubation for 30 min at 4°C, the cells were suspended gently. The cells rosetted more than 3 EA were evaluated as FcR^+ cells. Although 3.9% FcR^+ cells were detected in one CBA/J preparation, FcR^+ cells were not detected in three C3H preparations, one A/J preparation, and one BALB/c preparation. 4) Ia^+ cells were 90–99% when they were examined by complement- (C-) dependent cytotoxicity test. Table 13 summarizes the results. DC preparations were mixed with either rabbit C (Low-Tox-M Rabbit Complement; Cederlane Laboratories, Ltd., Horby, Ontario) alone or anti-Ia^k serum (A. TH anti-A. TL serum; generously supplied by NIH., USA) plus C. The density of

Fig. 3. Microscopic observation of G-10-passed spleen cells (A and B), splenic DC (C and D), and Mϕ (G) and suspected DC (E and F) in 20hNA BCG-PEC. In Figs. A, B, D, F, and G, cells were centrifuged to smear on slides, and fixed and stained with May-Gruenwald-Giemsa stain (×1000). In Figs. C and E, cells were incubated at 4°C for 60 min on coverslips coated with poly-L-lysine, fixed with 2% glutaraldehyde, embedded on slides with glycerin jelly by reversing coverslips upside down, and observed by phase contrast microscopy (×1000). A: one blastoid cells of medium size (arrow), and small lymphocytes. B: one polymorph (arrow), and small lymphocytes. C: Splenic DC show typical dendritic morphology. D: In this preparation, cytoplasmic processes of DC shrink so that the cells become relatively round. E: Suspected DC in 20hNA BCG-PEC. They show typical dendritic morphology. F: A suspected DC in 20hNA PEC. It has cytoplasmic processes. G: Typical Mϕ in 20hNA PEC.

Table 12. FcR^+ cells in DC preparations

Exp.	Strain	Cell counted	FcR^+ cell[a]	Percentage
1	CBA/J	205	8	3.90
2	C3H	413	0	0
3	A/J	359	0	0
4	C3H	509	0	0
5	C3H	471	0	0
6	BALB/c	195	0	0

[a] FcR^+ cells in irradiated DC preparations were detected as EA-rosetted cells.

Table 13. Ia^+ cells in DC preparations[a]

Exp.	Strain	Viable cells after the treatment of		
		C	anti-Ia^k+C	% Ia^+
1	CBA/J	88±3	2±0	98
2	C3H	61±6	6±2	90
3	A/J	97±4	1±1	99
4	C3H	340	24	93
5	C3H	211	20	91

[a] Ia^+ cells in irradiated DC preparations were determined by complement-dependent cytotoxicity test.

cells at final was $1–3 \times 10^5$/ml, and the C and the serum were diluted to 1/18 and 1/100 at final respectively. After incubation for 1 hr at 37°C, viable cells were counted by trypan blue dye-exclusion test. Ia^+ cells in three C3H preparation, one CBA/J preparation, and one A/J preparation were 90–93, 98, and 99% respectively. 5) Microscopic observation of DC was shown in Fig. 3C and D.

CS Mϕ: A. *Con A activated spleen cell culture supernatant (Con A Sup)*—Con A Sup contains lymphokine which can induce Ia antigens on Mϕ surface. Con A Sup was prepared as follows. Spleen cells (5×10^6/ml) were cultured for 48 hr in Medium 3 containing 2% FBS and 1 μg/ml Con A. The culture supernatant was absorbed with an equal volume of Sephadex G-25 (Pharmacia) to remove free Con A, sterilized by filtration through a membrane filter (pore size: 0.22 μm; Millipore Co., Bedford, MA), and the aliquots were stored at −20°C until use.

B. *Preparation of CS Mϕ*—Peritoneal exudate cells (PEC) of mice injected intraperitoneally (i.p.) with 2 ml of thioglycollate medium (TGC, Brewer's medium; Difco) (TGC-PEC) were washed twice with HBSS and suspended in Medium 1 containing 5% FBS. The PEC were plated on flat-bottomed 96-well plates (Nunc) in graded numbers, incubated for 2–6 hr, and irradiated with 650 R X-ray to prevent the growth of fibroblasts. Nonadherent cells were carefully removed by suspending the cells with a Pasteur pipette and aspiration, and the adherent cells were cultured for 5–7 days in Medium 4 containing 10% FBS and 1 μg/ml indomethacin (Sigma Chemical Co., St. Louis, MO) in the presence or absence of 10% Con A Sup to prepare CS^+ Mϕ and CS^- Mϕ, respectively (Ia-inducing culture). CS Mϕ were irradiated with 1300 R X-ray, washed 3 times with HBSS, and cultured to assess their A-cell activity.

C. *Characterization*—1) CS Mϕ were highly purified Mϕ preparation. Since TGC-PEC

Table 14. Phagocytic cells in CS Mϕ preparations[a]

Exp.	CS+ Mϕ			CS− Mϕ		
	Cell counted	Phagocyte	%	Cell counted	Phagocyte	%
1	400	395	98.7	569	545	95.8
2	319	314	98.4	375	362	96.5

[a] TGC-PEC were cultured in the presence or absence of Con A supernatant for 5 days (Exp. 1) or 7 days (Exp. 2). The adherent cells were washed and opsonized erythrocytes (EA) were added to the culture. After incubation for 2 hr at 37°C, non-phagocytosed EA were lysed in hemolytic Gey's solution and the adherent cells were fixed. The adherent cells which ingested more than 3 EA were evaluated as phagocytes.

were irradiated before Ia-inducing culture, fibroblasts were not detected in most preparation, but in some CS+ Mϕ prepared from more than 4×10^4 TGC-PEC per well, 2% fibroblasts at most were detected. Lymphoid cells, polymorphs, and DC formed minor parts in the adherent TGC-PEC, became dead or nonadherent during Ia-inducing culture, and were finally washed out after the culture. 2) 98.4–98.7% CS+ Mϕ and 95.8–96.5% CS− Mϕ phagocytosed EA (Table 14). The number of ingested EA per Mϕ was much higher in CS+ Mϕ than in CS− Mϕ, suggesting that the former became more activated by the influence of Con A Sup (data not shown). 3) More than 90% CS+ Mϕ were Ia+, and less than 1% CS− Mϕ were Ia+. Ia antigens on the Mϕ surface were visualized by an indirect immunofluorescence technique (Naito et al. 1984). CS− Mϕ did not seem to be biologically inactive, since when they were recultured in the presence of lymphokine, they expressed Ia antigens in their surface (Naito et al. 1984). 4) Yields of CS+ Mϕ and CS− Mϕ were 50–65% and 41–50% of original TGC-PEC, respectively (Table 15). CS Mϕ in 96-well plate were fixed and stained with Giemsa stain. Adherent cells possessing a stained nucleus per cell were counted by a reverse light microscopy (X 40–100).

BCG-PEC: A. *Preparation*—PEC of the mice inoculated i.p. with 0.8 mg of lyophilized live Bacillus Calmette-Guerin (BCG; Nippon BCG Seizo Co., Ltd., Tokyo) 7–14 days previously were prepared as whole, 2hAd, 20hAd, and 20hNA PEC. Whole PEC were prepared by treating BCG-activated PEC with anti-Thy 1.2 F7D5 monoclonal antibody (Olac, 1976 Ltd., Bicester, Oxon) at 1/400 dilution per 10^7 cells plus rabbit C at 1/8–1/16 dilution for 45 min at 37°C, followed by irradiation with 1300 R X-ray. 2hAd PEC were obtained by incubation of whole PEC for 2–4 hr in flat-bottomed 96-well or 24-well plates and removal of nonadherent cells by suspending the cells with a Pasteur pipette and aspiration. In order to prepare 20hAd

Table 15. Yields of CS+ Mϕ and CS− Mϕ[a]

PEC plate	CS+ Mϕ		CS− Mϕ	
	Adherent	%	Adherent	%
2500	1485±107	59	1013±142	41
5000	2497±207	50	2058±114	41
10000	6453±339	65	4953±111	50

[a] The adherent cells after 6 days of the culture in the presence or absence of Con A supernatant.

Table 16. Constituents of BCG whole PEC[a]

Exp.	Day harvested	Cell counted	Percentage			
			Mϕ[b]	Polymorph	Lymphocyte	Mast cell
1	7	1195	51.2	2.3	46.0	0.4
2	8	1333	54.4	6.9	38.6	ND.[c]
3	11	1376	52.9	29.1	20.9	0.07
4	13	1303	65.7	17.8	16.5	ND.

[a] 0.8 mg BCG were intraperitoneally inoculated into mice. After the indicated days, PEC were harvested, treated with anti-Thy 1.2 monoclonal antibody plus C, centrifuged to smear on slides, and stained with May-Gruenwald-Giemsa stain.
[b] Suspected dendritic cells were included in this population.
[c] Not detected.

Table 17. Characterization of various BCG-PEC preparations

PEC	Yield[a]	Mϕ[b]	DC[c]	Ia$^+$ cell[d]
2h Ad	30-50	50-70	ND.	more than 95
20h Ad	20-30	60-80	ND.	more than 95
20h NA	2-4	20-40	10-20	ND.

[a] Percent yield of original PEC.
[b] Percent Mϕ of the indicated PEC. The adherent cells which ingested more than 3 EA were evaluated as Mϕ. As for 20h NA PEC, only the cells which readhered on glass coverslips were examined.
[c] Non-EA-rosetted cells which were of dendritic morphology on the coverslips coated with poly-L-lysine.
[d] Percent Ia$^+$ cells of Mϕ population.

PEC, 2hAd PEC were cultured for additional 18–22 hr, and nonadherent cells were washed out. In order to obtain 20hNA PEC, whole PEC were incubated on plastic dishes (Falcon No. 3003) at the density of $3\text{–}4\times10^6$ cells per dish for 2–4 hr, and nonadherent cells were removed by pipetting and aspiration. The adherent cells were cultured for additional 18–22 hr, and the cells dislodged by pipetting were collected as 20hNA PEC.

B. *Characterization*—**1**) Whole PEC: Table 16 summarizes the constituents of whole PEC. The PEC were centrifuged to smear on slides, fixed and stained with May-Gruenwald-Giemsa stain, and examined under light microscopy (X 400). Mϕ and suspected DC composed 51.2–65.7% of whole PEC. Polymorphs varied greatly among preparations from 2.3 to 29.1%. Lymphocytes composed 16.5–46.0% of the PEC, and in some preparation, 0.4–0.07% mast cells were detected. **2**) 2hAd PEC: Yield was 30–50% of whole PEC, and 50–70% of 2hAd PEC were Mϕ. More than 95% of the Mϕ were Ia$^+$ when they were examined by indirect immunofluorescence technique (Table 17). **3**) 20hAd PEC: Yield was 20–30% of whole PEC, and 60–80% of 20hAd PEC were Mϕ. More than 95% of the Mϕ were Ia$^+$ (Table 17). **4**) 20hNA PEC: Yield was 2–4% of whole PEC, and 20–40% of 20hNA PEC were Mϕ (Fig. 3G). 10–20% of 20hNA PEC were non-EA-rosetted cells of dendritic morphology (Table 17 and Fig. 3E and F).

III. *General discussion*

A. *Culture conditions for the* in vitro *antibody response*—It seems reasonable to employ

SRBC as antigen for the following reasons. 1) They are relatively highly immunogenic even in the primary antibody response *in vitro*. 2) Since they are particulate antigens, Mϕ may mediate the anti-SRBC antibody response. 3) It is easy to evaluate anti-SRBC PFC response.

Round-bottomed plates seem inadequate in the investigation of A-cell activity of adherent cells, and it seems profitable to use flat-bottomed 96-well plates, since these vessels need fewer cells than other flat-bottomed vessels. However, since PFC response per culture in flat-bottomed 96-well vessels is not as high as in others, it is necessary to select highly immunogenic SRBC lots. It is also necessary to select PFC-supportive FBS lots.

PFC-supportiveness of FBS may be partly due to the products of bacteria which probably contaminated during the preparation of FBS. T cell and B cell mitogens can also compensate the defectiveness of non-PFC-supportive FBS.

B. *Cell preparations and their characterization*—G-10-passed cells are practically free from Mϕ, so this preparation can be employed as lymphocyte source for the evaluation of A-cell activity of DC preparation which is also free from Mϕ. However, it appears that in some G-10-passed cell preparations, Ia^+ non-Mϕ cells may still remain in a minor part, since G-10-passed cells sometimes developed moderate PFC response even if without supplementing A-cells or in the presence of Ia^- Mϕ (see the accompanying paper).

In preparing DC, irradiation of LDAC seems useful to reduce the contamination of blastoid cells, but the irradiation also seems to reduce the final yield of DC. Thus, it might be better to eliminate such contaminating cells by treatment with specific antibody plus C, if possible.

As for a source of Ia^+ Mϕ, CS^+ Mϕ seems adequate, since they are highly purified Ia^+ Mϕ preparation free from Ia^+ non-Mϕ cells. BCG-PEC preparations will be useful, since they contain Ia^+ Mϕ activated *in vivo* after the immune response to BCG.

In the accompanying paper, we examine functional aspects of these cell preparations (Komatsubara 1985). Preliminary investigations of roles of DC and Mϕ in antibody response, and also in mixed leukocyte reaction, have been already published (Muramatsu et al. 1983).

Acknowledgment

The author is grateful to Dr. S. Muramatsu for his guidance throughout this study and manuscript preparation. Thanks are also due to all other members of his laboratory for their fruitful discussions and technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and the Naito Foundation Research Grant for 1983, to Dr. S. Muramatsu.

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