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Characterization and expression of H-2I region gene products on bone marrow-derived macrophages*

Antigen-presenting macrophages (MΦ) were derived from day 7 cultures of bone marrow stem cells using L cell conditioned medium. The adherent bone marrow-derived macrophages (BMMΦ) were 100% esterase-positive, 95% positive for C3 receptors, 93% positive for Fc receptors, and 95% actively phagocytic. Indirect immunofluorescence using anti-Ia monoclonal antibodies resulted in 60% Ia-positive BMMΦ on day 7 of stem cell culture. BMMΦ could stimulate mixed lymphocyte reaction (MLR) proliferation across an I-A subregion difference, but not across I-J subregion differences. This contrasted with splenic MΦ which stimulated MLR proliferation across both an I-A and I-J subregion difference. The apparent lack of I-J subregion determinants on BMMΦ correlated with their ability to function as antigen-presenting cells. In these experiments, BMMΦ effectively reconstituted the trinitrophenyl-specific IgM plaque-forming cell (PFC) response of B cells but not the primary burro red blood cell (BRBC)-specific IgM-PFC response of MΦ-depleted spleen cells. When BMMΦ were added to BRBC-primed T and B cells, they reconstituted the secondary IgG PFC response to levels obtained using splenic MΦ. These experiments relate the differential expression of H-2I region determinants on antigen-presenting cells with their functional capacity.

1 Introduction

There is considerable evidence documenting genetic restriction of macrophage (MΦ)-T cell interactions [1–8]. In the mouse, investigations have demonstrated that these restrictions reside in the I region of the H-2 major histocompatibility complex (MHC) [5–8]. Previous experiments have examined the relationship between Ir genes and Ia antigens, and determined the role of Ia antigens in MΦ-T cell interactions during the induction of immune responses. Such experiments have used MΦ isolated from the spleen or peritoneum, and have depended on the ability to eliminate other non-MΦ cells [9–11].

These MΦ preparations can also be separated into at least two subpopulations; those expressing Ia antigens, and those not bearing such determinants. The expression of Ia determinants by a subpopulation of MΦ correlates with their capacity to present antigens to immune cells [10, 11]. While it is clear that only Ia-bearing MΦ (and not Ia-negative MΦ) present antigen, it is not known if all Ia-positive MΦ have this capacity. A homogeneous source of MΦ enriched for the expression of I subregion antigens would enhance the ability to further investigate the role of these products in the regulation of the immune response.

Using purified MΦ preparations, analysis of the H-2I region restriction for successful MΦ-T cell interaction has demonstrated that different I subregion restrictions exist for different Ir gene-controlled antigens. For example, antigen-induced T cell proliferation to the terpolymer L-Glu-L-Ala-L-Tyr (GAT) and to bovine insulin require I-A subregion MΦ-T cell compatibility. The Ir genes controlling the level of response to these antigens map to the I-A subregion. The response to these antigens can be blocked at the level of the antigen-presenting cell by alloantibodies or monoclonal antibodies reactive with products of the I-A subregion [12–16]. On the other hand, the primary *in vitro* antibody response to the multideterminant burro erythrocyte (BRBC) antigen has been found to have different restrictions. In the model, antigen-presenting MΦ and T helper (T_h) cells must be compatible at the I-J subregion [4–7]. In addition, the primary *in vitro* IgM plaque-forming cell (PFC) response is specifically blocked by treatment of antigen-presenting MΦ with alloantibodies to the I-J subregion, but not by anti-Ia antibodies specific for products of the I-A or I-E subregions [4–7].

Recent reports indicate that pure populations of MΦ can be derived from bone marrow (BM) stem cells cultured in the presence of L cell conditioned medium [14–19]. These bone marrow-derived macrophages (BMMΦ) were capable of inducing delayed-type hypersensitivity [14], presenting antigen to immune T cells [14–16, 19] and functioning as accessory cells in the generation of alloreactive cytotoxic T lymphocytes [18].

The experiments reported in this manuscript characterize the MΦ for surface receptors, enzymatic activity and examine the expression of H-2I region gene products on their membrane. BMMΦ were found to contain varying numbers of Ia-positive cells which expressed Ia determinants of both the I-A and I-E subregions, but not the I-J subregion [16]. This differential

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Abbreviations: **MHC:** Major histocompatibility complex **BMMΦ:** Bone marrow-derived macrophage(s) **GAT:** Synthetic terpolymer L-Glu^{60}-L-Ala^{40}-L-Tyr^{10} **LN:** Lymph node **PFC:** Plaque-forming cells **BRBC:** Burro red blood cells **SDS-PAGE:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis **SMΦ:** Splenic macrophages **PEC:** Peritoneal exudate cell(s) **MLR:** Mixed lymphocyte reaction **LCM:** L cell conditioned medium **PBS:** Phosphate-buffered saline **C:** Rabbit complement **FCS:** Fetal calf serum **MEM:** Minimum essential medium **IF:** Indirect immunofluorescence

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expression of I subregion products by BMMΦ provided the opportunity to compare antigen presentation by BMMΦ ($I\text{-}J^-$) and splenic macrophages (SMΦ) ($I\text{-}J^+$) in models of immune response.

2 Materials and methods

2.1 Mice

The experiments were conducted with 12- to 20-week-old mice bred at the University of Michigan. Mice in each experiment were matched for age and sex. The original breeders for this colony were obtained from Jackson Laboratories, Bar Harbor, ME, and from Dr. Donald Shreffler, Washington University, St. Louis, MO.

2.2 Antisera

Restricted anti-Ia alloantisera were prepared as previously described [7, 15, 16, 19]. Batches of serum were pooled from multiple bleedings after a series of immunizations and were tested in a dye exclusion microcytotoxic assay for appropriate anti-Ia reactivity and antibody titer. Batches of anti-Thy-1.2 antiserum were prepared by immunizing A.AKR mice with C3H thymocytes. Rabbit anti-mouse Ig antiserum was prepared by injecting rabbits with rabbit red blood cells coated with mouse anti-rabbit red cell hyperimmune antibodies.

Complement (C) for antiserum testing and experiments was obtained by cardiac puncture from 2- to 4-week-old New Zealand white rabbits. The C was screened against thymocytes and lymph node (LN) cells for natural cytotoxicity and only batches with less than 5% natural cytotoxicity were used.

2.3 Preparation of BMMΦ

The BMMΦ were derived from cultures of BM cells. Femurs of congenic mice were removed aseptically and the marrow flushed with supplemented minimum essential medium (MEM) containing 5% fetal calf serum (FCS). A single cell suspension was prepared from the marrow plugs, gravity sedimented, and cultured (1.0×10^6 nucleated cells/ml) with 10% L cell conditioned medium (LCM) containing 15% FCS. Following 7 days of incubation in 100-mm tissue culture dishes at 37 °C in 7.5% CO_2, the nonadherent cells were removed, and the remaining adherent monolayers were washed and harvested using cold EDTA treatment as described by Stern et al. [23]. The harvested adherent cells were washed by centrifugation in fresh medium and then readhered for 2 h in medium containing 10% FCS. Nonadherent cells were again decanted and the remaining monolayer was used in experiments. MΦ used in mixed lymphocyte reaction (MLR) or for antigen presentation were treated for 30 min with mitomycin C at a concentration of 40 μg/10^7 cells/ml.

2.4 Preparation of colony stimulating activity

Mouse LCM was used as a source of colony stimulating activity [24]. The L cells were grown in 10 ml of MEM supplemented with 10% FCS and fed every third day with fresh medium (50%, v/v). Following day 7, the culture medium was removed, centrifuged at $300 \times g$ for 10 min, and filter (0.22 μm) sterilized. The LCM was aliquoted and frozen until used to supplement the BM cultures.

2.5 SMΦ isolation

Adherent splenic phagocytic cells were prepared as previously described [7]. Briefly, dispersed spleen cells were seeded into plastic petri dishes at a concentration of 10^7/ml and incubated for 2.5 h. Nonadherent cells were decanted by 2 additions of fresh medium. Anti-Thy-1.2 antiserum and rabbit anti-mouse Ig antiserum were added to the dishes, and the dishes were returned to the 37 °C chamber. After a 30-min incubation with gentle rocking, the antibodies were decanted, C was added, and the incubation continued for 30 min. The MΦ were again washed by adding fresh medium and decanting. The number of adherent cells remaining in each dish ranged from 0.5×10^6 to 1.2×10^6 cells per 10^7 original spleen cells.

2.6 Phagocytic and esterase activities

Following 7 days incubation in LCM as described, the nonadherent cells were removed and the adherent BMMΦ were harvested by cold EDTA treatment. These cell fractions were then assayed for nonspecific esterase activity [25] and for their phagocytic capacity by adding latex particles (1.0 μm diameter) to the cultures for 1 h at 37 °C. A positive phagocytic cell was one which had ingested > 5 particles/cell.

2.7 Fc and C3 receptors

BMMΦ were re-adhered for 2–8 h at 37 °C on tissue culture chamber slides (Lab-Tek 4804, Miles Laboratories, Kankakee, IL) in supplemented MEM containing 5% FCS. Fc- and C3 receptor-bearing BMMΦ were determined by addition of either sheep erythrocytes opsonized with IgG(EA) (Cordis Laboratories, Miami, FL, rabbit 7S anti-SRBC) or with IgM (rabbit 19S anti-SRBC) with C5-deficient mouse serum (EAC) as outlined by Bianco et al. [26].

2.8 Indirect immunofluorescence (IF)

The BMMΦ were harvested, re-adhered on chamber slides for 2 h at 37 °C with supplemented MEM containing 5% FCS and washed (3 times) with Dulbecco's phosphate-buffered saline (PBS) to remove any nonadherent cells and serum proteins. The monolayer was fixed using 1% paraformaldehyde for 15 min at 20 °C as described by Beller et al. [27] and washed with Dulbecco's PBS. Specific alloantibodies (diluted 1 : 2) or monoclonal antibodies (50 μl neat supernatant) appropriate for the target cell were added to the monolayer. Antibodies inappropriate to the target cell were used as controls. The monolayers were incubated with antiserum for 30 min at 4 °C and washed. The bound anti-Ia antibody was then detected using fluorescein-conjugated $F(ab')_2$ rabbit anti-mouse IgG (both heavy and light chain; Cappel Laboratories, Downington, PA). The conjugate (50 μl of 250 μg/ml) was added to the antiserum-treated BMMΦ, incubated for 30 min at 4 °C, and washed; coverslips were added, and the monolayers were examined using phase contrast optics and fluorescence epi-illumination.

2.9 MLR

BMMΦ were mitomycin C-treated (40 μg/10^7 cells) for 30 min at 37°C, washed three times in MEM, and resuspended in MLR medium (RPMI 1640 supplemented with 2 mM L-glutamine, 3 mM HEPES, 50 μg/ml gentamycin, and 3% human AB serum). Responder cells were prepared from inguinal and mesenteric LN obtained from 10- to 36-week-old mice. In all experiments, mice were matched for age and sex. A single-cell suspension of LN cells was prepared, sedimented by gravity for 5 min, washed three times, and resuspended in MLR medium. Responder LN cells (4×10^5) and syngeneic or allogeneic BMMΦ were added to 96-well Costar plates and incubated for 96 h at 37°C in 5% CO_2. Cultures were performed in triplicate and pulsed 18 h before harvest with 1 μCi (= 37 kBq) [^{3}H]thymidine.

2.10 Culture conditions

Dispersed spleen cell cultures were prepared according to the method of Mishell and Dutton [28]. The cultures were established in 35-mm plastic dishes with each dish receiving a daily addition of 90 μl of enriched culture medium. Cultures were immunized with 10^7 BRBC and maintained for 5 days at 37°C in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 while rocking at 7 cycles/min.

MΦ-depleted (T-B) cells and adherent SMΦ were prepared as previously described [5, 6]. The B cell population was prepared by treating the above MΦ-depleted fraction with anti-Thy-1.2 antiserum and C. MΦ-depleted T-B cells obtained from normal mice or primed mice were added to each MΦ monolayer (10^7 cells/ml/dish), and the cultures were stimulated with 10^7 BRBC. In experiments using a thymus-independent antigen, B cells (10^7 cells/ml/dish) were added to the MΦ monolayer and stimulated with 1 μg/culture of trinitrophenylated-Ficoll.

The *in vitro* antibody response was assayed at the end of a 5-day culture period by determining the number of PFC using a modified Jerne plaque assay [29]. PFC were expressed as the mean of triplicate cultures for direct and indirect plaques. Indirect plaques were facilitated using a 1 : 25 dilution of rabbit anti-7S antiserum in the presence of anti-μ antiserum to block direct PFC.

3 Results

3.1 Characterization of BMMΦ

Initial experiments with BM cells demonstrated that the culture conditions yielded a maximum number of adherent mononuclear cells after 7 days of incubation with LCM. Prior to day 4 the adherent cell population also contained significant numbers of polymorphonuclear cells. The adherent cells were initially examined for their ability to phagocytize latex particles and for esterase enzymatic activity [25]. Latex-positive phagocytes were scored as cells with >5 ingested particles after 1 h incubation at 37°C. More than 95% of the adherent BMMΦ population was phagocytic and displayed nonspecific esterase activity (Table 1). In contrast, cells found in the nonadherent portion of the BM culture were composed of only 50% esterase-containing cells.

Table 1. Phagocytic and nonspecific esterase activity of BMMΦ[a]

Cells	Percent positive[b] cells	
	Phagocytizing[c] latex particles	Esterase activity
Nonadherent BMMΦ	NT[d]	50 (40–60)
Adherent BMMΦ	98 (87–100)	93 (93–98)
SMΦ	NT	8 (4–12)

a) BMMΦ were prepared from 7-day cultured B10.A(5R) BM cells.
b) Mean % positive from two experiments with range indicated in parentheses.
c) Enumerated cells contained >5 particles/cell.
d) Not tested.

Table 2. Determination of surface receptors on BMMΦ

Particle administered	Percent positive cells[a]	
	BMMΦ[b]	PEC[c]
E	1	10
EA(19S)C	95	95
EA(19S)	2	10
EC	11	27
EA(7S) 2000[d]	93	75
5000	83	67
10000	77	88
15000	73	39

a) Enumerated cells contained >5 particles/cell.
b) A.TL BMMΦ obtained from 7-day stem cell cultures.
c) Mineral oil-induced 3-day PEC after 2 h of adherence.
d) Reciprocal dilution of anti-IgG antiserum used for sensitization.

The adherent BMMΦ cell population was also evaluated for the presence of C3[EA(19S)C] and Fc[EA(7S)] receptors which are present on MΦ isolated from the spleen, thymus, and peritoneum. The results of these determinations are shown in Table 2. The adherent BMMΦ fraction was 95% positive for C3 receptors and 95% positive for Fc receptors. Mineral oil-induced peritoneal exudate cells (PEC) yielded similar values for both the Fc and C3 receptors. The affinity of the Fc receptor for binding Ig was higher for the BMMΦ since dilution of the sensitizing antibody resulted in fewer rosettes for PEC than BMMΦ. These results are consistent with properties attributed described by Cline et al. [30] when a soft agar method was used to establish BMMΦ.

3.2 Detection of Ia antigens on BMMΦ using IF

Adherent BMMΦ were examined for expression of Ia antigens using IF. A.TL(H-2^{tl}) BMMΦ were stained with various antisera to determine which antigens were present. The results of those experiments are shown in Table 3. Using IF, 60% of the BMMΦ stained with the monoclonal antibody 10-2.16 (anti-Ia.17). However, no A.TL BMMΦ or PEC were stained using B10.A(3R) anti-B10.A(5R) antibodies specific for I-J^k subregion specificities. The lack of staining with anti-I-J antiserum was consistent with many experiments we have performed using MΦ obtained from spleen and other lymphoid organs.

Table 3. Detection by IF of Ia antigens on A.TL BMMΦ

Antiserum	Ia antigens detected	% positive cells[a] BMMΦ	PEC[b]
Monoclonal 10-2.16	Ia.17	60 ± 2[c]	NT[d]
B10.A(3R) anti-B10.A(5R)	J^k	< 1	< 1
B10.A(5R) anti-B10.A(3R)	J^b	< 1	< 1
A.TL normal serum	None	< 1	< 1

a) Results (%) represent values obtained from > 200 cells counted; 1% cells stained with fluorescein-conjugated $F(ab')_2$ rabbit anti-mouse IgG (heavy and light chains).
b) PEC obtained 5 days after i.p. administration of mineral oil.
c) Mean ± SD of 6 determinations.
d) Not tested with 10-2.16 but when stained with A.TH anti-A.TL antiserum there were 4% stained.

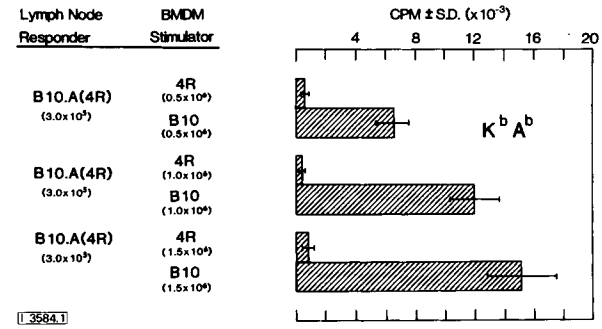

Figure 1. MLR of B10.A(4R) LN cells stimulated with mitomycin C-blocked C57B1/10 BMMΦ. Cells were cultured for 96 h and harvested following an 18-h pulse of [^{3}H]thymidine. The number of specific cells added to each culture well is noted in parenthesis. C57B1/10 and B10.A(4R) differ at both the K and the I-A subregions. The results are expressed as the mean ± SD of triplicate wells.

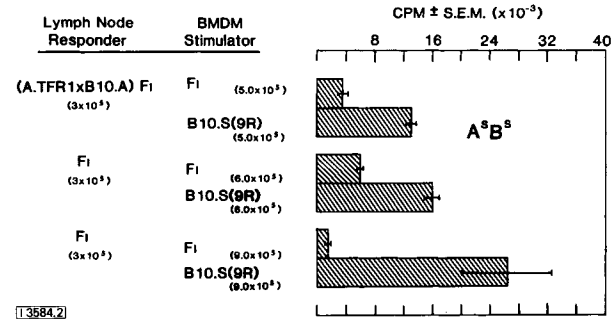

Figure 2. MLR of (A.TFR1 × B10.A)F_1 LN cells stimulated with mitomycin C-blocked B10.S(9R) BMMΦ. The responder LN cells and the BMMΦ stimulator cells differ at the I-A and I-B subregions. In other experiments, using strains B10.ASR7 and B10.BASR1, which differ only at the I-B subregion, no stimulation was observed. The results are expressed as mean ± SE of triplicate wells.

Normal mouse serum or inappropriate anti-Ia antiserum resulted in less than 1% staining of the BMMΦ. Addition of the $F(ab')_2$ rabbit anti-mouse IgG fluorescein conjugate also stained less than 1% of the BMMΦ, demonstrating a lack of surface Ig. In contrast, PEC obtained 5 days after administration of mineral oil had 4% Ia-bearing cells when stained with A.TH anti-A.TL antiserum and less than 1% when other more restricted anti-Ia antisera were used.

3.3 Detection of H-2I-expressed gene products on BMMΦ by MLR

Membrane Ia determinants coded for by genes of the I-A and I-E subregion have been routinely identified by serologic methods. Products of the I-J subregion, however, have been identified by functional assays for suppression or antigen presentation. To further evaluate the expression of I-J subregion molecules on BMMΦ, the BMMΦ were examined for their ability to stimulate a MLR across restricted H-2I subregion incompatibilities. For these experiments, BMMΦ were harvested, treated with mitomycin C, and added to immunocompetent T cells in a MLR assay. The various combinations used in the MLR assays are presented in Table 4. Differences at the I-A subregion (Figs. 1, 2) and I-E subre-

Table 4. Detection of I-J subregion gene products on splenic and BMMΦ by MLR

Responder LNC (5×10^5)	No. of stimulator MΦ × 10^{-6}[a]	cpm ± SD[b]	S.I.[c]	p value[d]
Exp. 1				
B10.A(3R)	1.5 B10.A(3R) SMΦ	586 ± 123		
	1.5 B10.A(5R) SMΦ	1804 ± 354	3.1	< 0.01
	1.5 B10.A(5R) BMMΦ	122 ± 20		
	1.5 B10.A(3R) BMMΦ	127 ± 41	1.9	NS[e]
B10.A(3R)	1.0 B10.A(3R) SMΦ	1256 ± 256		
	1.0 B10.A(5R) SMΦ	2652 ± 669	2.1	< 0.02
	1.0 B10.A(3R) BMMΦ	203 ± 42		
	1.0 B10.A(5R) BMMΦ	389 ± 193	1.9	NS
B10.A	0.5 B10.A LNC	169 ± 40		
	0.5 B10.A(5R) SMΦ	15193 ± 2931	90	< 0.001
Exp. 2				
B10.A(3R)	1.4 B10.A(3R) SMΦ	647 ± 59		
(+ 10% anti-I-J^k)[e]	1.4 B10.A(5R) SMΦ	1723 ± 571	2.7	< 0.01
(+ 10% anti-I-J^k)	1.4 B10.A(3R) SMΦ	223 ± 104		
	1.4 B10.A(5R) SMΦ	164 ± 122	0.7	NS

a) The number of each stimulator population added to 5×10^5 responder LN cells and cultured in 0.2 ml medium. Stimulator cells were pretreated with mitomycin-C 40 μg/ml.
b) DNA incorporation of [^{3}H]thymidine was determined on day 4 of the culture. Data represent mean ± SD of 3 culture wells.
c) Stimulation index = cpm of experimental MLR/cpm of syngeneic control.
d) p value determined by Student's t-test (degrees of freedom = 4). NS = not significant.
e) Serum (A.BY × B10.HTT) anti-A.TL contains antibodies reactive with I-J^k subregion determinants and was present throughout culture at a concentration of 10%.

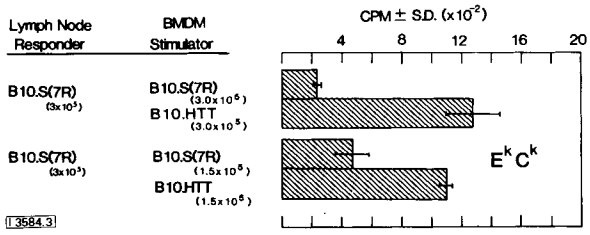

Figure 3. MLR of B10.S(7R) LN cells stimulated with mitomycin C-blocked B10.HTT BMMΦ. The responder LN cells and the BMMΦ stimulator cells differ at the I-E and I-C subregions. The results are expressed as mean ± SD of triplicate wells.

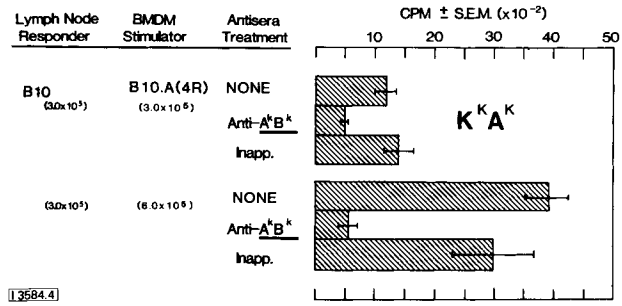

Figure 4. Blocking of a MLR with anti-Ia antibodies specific for stimulator BMMΦ. C57B1/10 responder LN cells were stimulated with K region- and I-A subregion-incompatible BMMΦ. Anti-Ia antiserum was added to culture well at a final concentration of 5% and was present during the entire culture. The antiserum (A.BY × B10.HTT)F_1 anti-A.TL is specific for Ia.1, Ia.2 and Ia.19 present on B10.A(4R) BMMΦ. The inappropriate control serum was B10.S(7R) anti-B10.HTT. Results are expressed as mean ± SE of triplicate culture wells.

gion (Fig. 3) resulted in MLR responses substantially greater than those elicited by the syngeneic controls. However, differences at the I-J subregion did not evoke a MLR greater than the syngeneic controls. Previous work in this laboratory established that a significant MLR could be generated across I-J subregion differences using SMΦ as stimulator cells (Table 4). This MLR could be blocked by pretreating the stimulator MΦ with anti-I-J antiserum (Niederhuber, J. E., unpublished observations).

To confirm that the MLR was the result of membrane determinants coded for by genes in the I subregion, specific anti-Ia antibodies were used to block stimulation (Fig. 4). K^kA^k differences were significantly blocked by the addition of appropriate anti-I-A^k antibodies. No inhibition of the MLR occurred when inappropriate anti-Ia antiserum was added.

3.4 The ability of BMMΦ to function in the *in vitro* antibody response

During their characterization, the major difference between the BMMΦ and the SMΦ appeared to be in the expression of I-J subregion-associated products. The BMMΦ were therefore tested for their ability to replace Ia^+ SMΦ in the primary *in vitro* antibody response to BRBC. Using a wide range ($0.05 \times 10^6 - 2.0 \times 10^6$) of BMMΦ cultured with 10^7 T-B cells, we were unable to reconstitute the primary antibody response to BRBC. Similar results had been reported by Watson et al.

Table 5. Effect of antigen-presenting cell number in the reconstitution of an *in vitro* antibody response to BRBC-primed T and B cells[a]

No. of MΦ × 10^{-5} added to T and B cells	Source of MΦ[b] BM IgM PFC[c]	BM IgG PFC[d]	Spleen IgM PFC	Spleen IgG PFC
None	0	0	3 ± 2	3 ± 5
1.0	154 ± 8	165 ± 56	203 ± 54	355 ± 20
5.0	1345 ± 301	623 ± 73	843 ± 254	1498 ± 250
7.5	683 ± 63	278 ± 260	1375 ± 97	1089 ± 130
10	358 ± 78	405 ± 169	2215 ± 607	5368 ± 208
20	NT[e]	NT	5368 ± 208	1100 ± 243

a) [B10.A(3R) × B10.A(5R)]F_1 mice were primed to BRBC 14 days prior to use in experiment.
b) Syngeneic MΦ were prepared from unprimed mice.
c) The anti-BRBC antibody specific responses are expressed as mean PFC ± SEM/10^6 cultured cells for 3 culture dishes.
d) Values obtained after blocking IgM PFC with anti-μ antiserum and facilitating IgG PFC with anti-IgG antiserum. Anti-μ antiserum treatment blocked IgM PFC below background levels (< 10 PFC/culture).
e) Not tested.

[31]. These primary cultures were carefully examined for viability with no evidence that the lack of response was due to the inability of BMMΦ to maintain adequate numbers of T and B cells during the culture. Furthermore, the T-B cells responded normally with SMΦ. In an effort to overcome this functional defect, the BMMΦ were first incubated with BRBC for 1, 2, 3 or 4 days and then cultured with unprimed T-B cells. This also proved ineffective in reconstituting the PFC response and led to the conclusion that the $I\text{-}J^-$ BMMΦ were functionally different from SMΦ.

Experiments were therefore designed to use BMMΦ with T-B cells primed to BRBC (Table 5). Mice were primed with 4×10^8 BRBC i.v., and 14 days later their spleens were removed and used as the source of primed T and B cells. The results of these experiments demonstrated that BMMΦ were able to reconstitute a T-dependent secondary response which was optimal when 5.0×10^5 BMMΦ were added to 10^7 BRBC primed T-B cells. This was a different response curve than observed with increasing numbers of SMΦ. When SMΦ were used to reconstitute the secondary BRBC response, the number of IgG PFC increased proportionately with the number of MΦ added. This difference was thought to be related to the percentage of Ia^+ cells present in the two different MΦ preparations. As noted earlier, differences in PFC values between BMMΦ and SMΦ reconstituted cultures were not related to the selective viability of the T-B lymphocyte fraction.

Additional experiments compared BMMΦ with SMΦ obtained from either unprimed or primed animals. The results, presented in Table 6, demonstrated that BMMΦ and SMΦ from primed or unprimed mice were equal in their ability to reconstitute the secondary IgG PFC response. These results were consistent with our previous demonstration that the I-J-bearing MΦ was required for induction of a primary, but not a secondary, *in vitro* antibody response to the BRBC antigen [4–7].

Table 6. Reconstitution of the secondary (IgG) *in vitro* antibody response to BRBC by BMMΦ[a)]

Source of T and B cells	Source of MΦ	PFC ± SD/culture[b)] IgM	IgG[c)]
Unprimed spleen	Unprimed spleen	3273 ± 775	3 ± 4
Primed spleen	Unprimed spleen	2263 ± 1088	358 ± 43
Unprimed spleen	Primed spleen	2560 ± 1007	25 ± 17
Primed spleen	Primed spleen	2506 ± 954	308 ± 87
Unprimed spleen	BMMΦ	193 ± 39	5 ± 9
Primed spleen	BMMΦ	473 ± 276	668 ± 42

a) B10.S(9R) mice were primed to BRBC 14 days prior to use in experiment.
b) Data are presented as the mean PFC ± SD/10^6 cultured cells of triplicate dishes on day 5 of culture.
c) Values obtained after blocking IgM PFC with anti-μ antiserum and facilitating IgG PFC with anti-IgG serum. Anti-μ antiserum treatment blocked IgM PFC below background levels (< 10 PFC/culture).

Table 7. Effect of antigen-presenting cell number in the reconstitution of the B cell *in vitro* antibody response to TNP-Ficoll[a)]

No. of MΦ × 10^{-5} added to 10^7 B cells	Source of MΦ BMMΦ	Spleen
1.0	105 ± 5[c)]	0
5.0	203 ± 31	197 ± 76
7.5	290 ± 25	737 ± 61
10	228 ± 20	1647 ± 196
20	263 ± 33	1402 ± 224

a) [B10.S(9R) × B10.HTT]F_1 mice were used as source of BMMΦ, SMΦ and B cells.
b) Each culture was stimulated with 1 μg/ml final concentration of TNP-Ficoll.
c) The number of direct TNP-specific PFC ± SEM/10^6 cultured cells was determined by a modified Jerne plaque assay. Sheep erythrocytes were coupled with TNP by the method of Rittenberg and Pratt [30]. The results are expressed as the mean ± SEM/10^6 cultured cells of triplicate cultures determined on day 5.

The absence of a requirement for I-J determinants for antigen presentation in a secondary IgG response suggested that a similar situation might exist for the response to a T-independent antigen such as TNP-Ficoll. In previous experiments, anti-I-J antiserum had no effect on the MΦ-B cell response to TNP-Ficoll (unpublished observations). The experiments presented in Table 7 demonstrated that BMMΦ could reconstitute the B cell response to TNP-Ficoll. Optimal TNP-specific IgM PFC occurred when 7.5×10^5 BMMΦ or 10^6 SMΦ were added to 10^7 B cells. Thus, the BMMΦ provide a pure source of large numbers of antigen-presenting cells and serve as an excellent model for examining the Ia antigen requirements for the induction and regulation of *in vitro* antibody responses.

4 Discussion

The finding that mononuclear phagocytic cells derived from BM stem cells cultured in the presence of LCM were 60% Ia-positive suggested that these cells would be efficient antigen presentors. As noted, BMMΦ also demonstrated nonspecific esterase activity, C3 and Fc receptors; all classic properties of the MΦ. What was not anticipated was the absence of demonstrable I-J subregion products on BMMΦ.

Although BMMΦ appeared to be I-J^-, they clearly expressed Ia molecules characteristic of the I-A and I-E subregions. Unfortunately, detection of membrane determinants coded for by genes of the I-J subregion cannot be accomplished by known serologic or biochemical methods. Rather, the characterization of a cell subset as I-J^+ has required the effect of restricted alloantibodies on functional assays for suppression or antigen-presentation [4–7, 32]. Evidence to conclude that the BMMΦ lacked expression of I-J subregion molecules was drawn from two functional assays. First, their ability to function as antigen-presenting cells was compared with SMΦ and second, their ability to stimulate a MLR across I-J subregion disparity was also compared to I-J^+ SMΦ.

The first evidence that BMMΦ differed from SMΦ in their Ia antigen expression was the inability of the BMMΦ to present BRBC in the primary *in vitro* antibody response. Previous experiments in this laboratory demonstrated that antibodies specific for I-J determinants could block the primary *in vitro* anti-BRBC response at the level of the MΦ [4–7]. Anti-I-J antiserum did not, however, significantly block the IgG PFC of the secondary BRBC response. As predicted, BMMΦ were perfectly capable of replacing SMΦ when used with primed T and B cells to generate BRBC-specific IgG PFC. Although levels of IgG PFC were comparable to those with SMΦ, the numbers of IgM PFC were always low. Thus, the functional capacity of the BMMΦ was as expected for an I-J^- MΦ and supported previous experiments which documented an I-J determinant requirement for the induction of a primary response to BRBC.

Other thymus-dependent antigens with Ir gene regulation mapping to I-A or I-E subregions were effectively presented by BMMΦ. For example, in experiments reported elsewhere, GAT was presented equally well by BMMΦ and SMΦ [15, 16]. The immune T cell proliferation to GAT has been shown to require T cell recognition of I-A subregion products [14, 15, 33]. This was further confirmed by the observation that antibodies directed to I-A subregion determinants could significantly inhibit the presentation of GAT or bovine insulin by BMMΦ*.

Thus, BMMΦ are morphologically and functionally identical to SMΦ except for the expression of I-J subregion determinants. Presumably, BMMΦ are also similar in these properties to MΦ from other tissue sites, but this has not been rigorously tested. Obviously, what remains to be answered is why the apparent deficiency in BMMΦ I-J subregion determinant expression. Does the I-J^- BMMΦ reflect a step in the ontogeny of the monocyte? Can the BMMΦ be induced to express I-J subregion determinants?

If this differential expression of I-J subregion molecules reflects maturation of the monocyte, then perhaps the presence of I-J subregion products on the SMΦ is due to the effect of the splenic microenvironment on MΦ differentiation. Experiments to explore this possibility are currently in pro-

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gress. It will also be important to examine other tissue sources of monocytes for evidence of I-J subregion products.

It was somewhat surprising that the BMMΦ were markedly enriched for Ia-bearing cells. MΦ obtained from spleen, peritoneum and other sources have only a minority of their cells bearing Ia determinants, depending on the cell source. It is unclear whether BMMΦ generated in this culture system are selected for the expression of Ia antigens or whether there are conditions in the system which simply drive normally Ia^- cells to be Ia^+. This latter question concerning the ability to drive Ia^- MΦ to express Ia antigens has been raised on numerous occasions in a number of laboratories and remains unresolved. It is clear from work by Scher et al. [34] that soluble mediator(s) elaborated during the interaction of antigen-stimulated T cells and MΦ can induce exudates rich in Ia^+ MΦ. Evidence seems to indicate that this is the result of recruitment of MΦ capable of Ia expression. Preliminary experiments in our laboratory indicate that the addition of fresh medium containing concanavalin A supernatant to day 7 BMMΦ cultures significantly increased the percent Ia^+ cells at days 8 and 10.

Although ideally one would like to have a cloned functionally active MΦ for study, this has proved extremely difficult. BMMΦ, however, have provided adequate numbers of a homogeneous MΦ preparation for functional assays free of contaminating non-MΦ cells. In addition, the absence of I-J subregion products provides the opportunity to compare the functional properties of $I\text{-}J^+$ and $I\text{-}J^-$ antigen-presenting cells.

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