NEURAMINIDASE-TREATED MACROPHAGES STIMULATE ALLOGENIC CD8$^+$ T CELLS IN THE PRESENCE OF EXOGENOUS INTERLEUKIN 2

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It is well known that Ia$^+$ accessory cells are required for presenting antigens to T lymphocytes. This is especially true of CD4$^+$ helper T cells which recognize antigens together with Ia or class II MHC products (1, 2). There is recent evidence that Ia$^+$ dendritic cells directly stimulate CD8$^+$ cytotoxic T cells (3). Since CD4$^+$ cells were not detected in the cultures, and since neither anti-Ia nor anti-CD4 mAbs blocked dendritic cell function, it appeared that class I molecules on dendritic cells were stimulating CD8$^+$ cells directly (3). Therefore expression of Ia can serve as a marker for active APC (dendritic cells) but may not be required in the presentation of class I MHC products.

While Ia$^+$ and Ia$^-$ macrophages (MØ)[1] typically are inactive as independent presenting cells in several responses (4–7), including the response of allogeneic CD8$^+$ cells (3), MØ are able to enhance the MLR that is initiated by small numbers of dendritic cells (6). This enhancement might be due to the secretion of soluble factors from MØ, which then potentiate dendritic cell function. Higher doses of MØ suppress dendritic cell function (6, 7).

Here we describe experiments in which we try to enhance MØ presentation of alloantigens in the absence of dendritic cells. Previously we tested interferons and noted that these cytokines could up or down regulate presentation to sensitized T lymphoblasts, but did not make MØ stimulatory for resting T cells (6, 8). We now identify a combination of stimuli that are effective in the primary MLR. One is to treat MØ with neuraminidase (Nase), which enables the MØ to bind T cells; another is to add exogenous IL-2. These additions allow the MØ to stimulate class I-restricted, CD8$^+$ cells directly, but not class II-restricted, CD4$^+$ lymphocytes. The findings draw some distinctions between the mechanism of action of dendritic cells and MØ, as well as the stimulatory requirements for antigen-specific, CD8$^+$ and CD4$^+$ lymphocytes.

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[1] *Abbreviations used in this paper:* MØ, macrophages; Nase, neuraminidase; PEC, peritoneal exudate cells; SAC, spleen adherent cells; TGC, thioglycollate medium.

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Materials and Methods

Mice. Female mice were used at 7-12 wk old. C3H/HeSlc, BALB/c, (BALB/c × DBA/2)F_1 (CxD2 F1), and A/J mice were from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). A.TL and A.TH mice originally were given to us by Dr. T. Hamaoka, Osaka University (Osaka, Japan) and then bred in our facility.

Reagents. We were generously provided with murine rIFN-γ by the Research Institute for Shionogi Pharmaceutical Co. Ltd., Osaka, Japan, and with human rIL-2 by Central Research Labs, Ajinomoto Co. Inc., Kawasaki, Japan. We used the following mAbs (from American Type Culture Collection, Rockville, MD) as culture supernatants to stain or to deplete the corresponding cell populations in the presence of rabbit complement: 10.2.16 (anti-I-A^k); M5/114.15.2 (anti-I-$A^{b,d,q}$, I-$E^{d,k}$); 11-4.1 (anti-H-$2K^k$); HO-2.2 (anti-Lyt-2.2); 3.155 (anti-Lyt-2); 53-6.72 (anti-Lyt-2); GK 1.5 (anti-L3T4); HO-13-4 (anti-Thy-1.2); and J11d.2 (anti-B cell). We purchased fluoresceinated rat mAb to mouse Lyt-2, CD8, and phycoerythrin-conjugated anti-L3T4, CD4 (Becton Dickinson Immunocytometry Systems, Mountain View, CA), goat $F(ab')_2$ anti-mouse IgG + IgM, FITC-conjugate (Tago, Inc., Burlingame, CA); FITC-avidin (Vector Laboratories, Inc., Burlingame, CA); neuraminidase (type X; Sigma Chemical Co., St. Louis, MO). The culture medium, which had <1 pg/ml LPS, assessed by a chromogenic endotoxic-specific assay (Endospecy; Seikagaku Kogyo Co. Ltd., Tokyo), was RPMI 1640 (Nissui Seiyaku Co. Ltd., Tokyo) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 ug/ml), 50 μM 2-ME, 1 μg/ml indomethacin, and 10 mM Hepes.

Cells. MØ were adherent peritoneal exudate cells (PEC) from mice injected with Brewer's thioglycollate medium (TGC) (Difco Laboratories, Detroit, MI), which had 11 ng/ml LPS, 4 d previously. PEC were irradiated with 650 rad to prevent the growth of contaminating fibroblasts, and were plated into 24-well plates (A/S Nunc, Kamstrup, Roskilde, Denmark). However, the dose of 650 rad did not alter the viability, expression of Ia antigens, or MLR stimulating activity relative to unirradiated cells. After 2-3 h, nonadherent cells were removed by washing. The adherent MØ were tested outright (Table VI), but in most experiments they were cultured 4 d in 1 ml medium with or without 12.5 U/ml rIFN-γ to prepare rIFN-γ^+ and rIFN-γ^- MØ respectively. At least 80% of the former expressed Ia as shown by indirect immunofluorescence in each experiment (8). Expression of H-2K class I MHC products also was upregulated by IFN-γ (Fig. 1), and upregulation of H-2D has been reported (4).

T cells were prepared by passing a mixed suspensions of spleen and mesenteric lymph node over nylon wool and treating the nonadherent cells with mAb to Ia and J11d in the presence of rabbit complement. $CD4^+$ and $CD8^+$ T cell subsets were made by depleting Lyt-2 (HO-2.2 or 3.115) and L3T4 (GK 1.5) cells by cytotoxicity, respectively. A final step, to remove dead cells and residual accessory cells, was to sediment the populations (5-8 × 10^7) in 5 ml of 65% Percoll at 900 *g* for 25 min at 4°C.

B cells were Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) nonadherent spleen cells that were depleted for T cells by treatment with anti-Thy-1.2, L3T4, and Lyt-2 mAbs and complement. Small B cells, ~30% of the total, were obtained from the pellet fraction after centrifugation in 65% Percoll (see above).

Spleen adherent cells (SAC), a mixture of MØ and dendritic cells (9), were prepared by adhering spleen for 2 h at 2 × 10^6/ml in 16-mm wells. Typically, 3-5 × 10^4 cells adhered.

Mixed Leukocyte Reaction. MØ were cultured in 1 U/ml Nase in serum-free RPMI 1640 medium containing 10 mM Hepes for 45 min at 37°C. After washing four times with RPMI 1640/10% FCS, the MØ were X-irradiated (1,500 rad) and served as stimulators. Thus MØ were actually irradiated twice; first at 650 rad to prevent fibroblast outgrowth, and second at 1,500 rad for the MLR; but as mentioned above, the former treatment had no effect on the results. SAC and B cells were also irradiated with 1,500 rad before use in the MLR. 2 × 10^6 responder high density T cells were cultured with or without rIL-2 in 24-well plates for 82-88 h. The cultures were suspended and aliquots (0.2 ml) from each well were transferred in triplicate to 96-well flat-bottomed tissue culture plates (A/S; Nunc) to be pulsed with 0.5 μCi [^{3}H]thymidine (50 Ci/mM; ICN Radiochemicals, Irvine, CA) for 6 h. Similar results were obtained if the original macrocultures were pulsed with [^{3}H]thymidine and the aliquots were then taken for harvesting. The data represent the means of triplicate assays from duplicate wells.

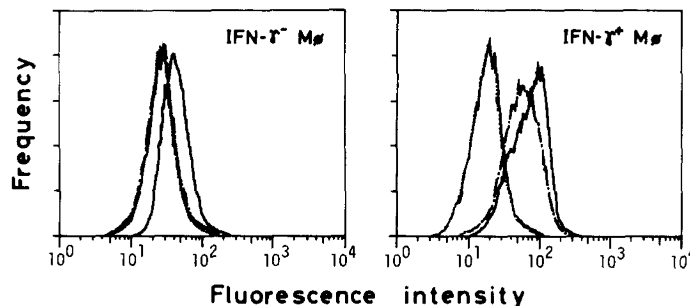

FIGURE 1. Expression of MHC class I and II antigens on the MØ cell surface. IFN-γ^- (*left*) and IFN-γ^+ (*right*) MØ from C3H/He mice were stained with anti-class I (H-2K^k; 11-4.1, *thick solid line*) or anti-class II (Ia; 10.2.16, *broken line*) mAb. Cells unstained, or stained with FITC-second antibody alone (*dotted and thin solid lines*), served as controls.

CTL Assay. P815 (H-2^d), X5563 (H-2^k), and YAC-1 (H-2^a) cell lines were labeled with $Na^{51}CrO_4$. 10^4 cells were added as targets to graded doses of effector cells in 96-well round-bottomed plates (A/S Nunc). After 5 h, the plate was centrifuged for 10 min at 400 *g* and the supernatants were collected to measure released isotope. Spontaneous release was determined in wells without effector cells, and maximal release in wells treated with 0.1 ml 0.1% Triton X-100 for 30 min before the end of the assay. Percent specific lysis was calculated as: 100× [(experimental − spontaneous release)/(maximal − spontaneous release)].

Cytofluorography. Cells were analyzed (FACStar, B-D Automated Immunochemistry, Salt Lake City, UT), before or after the MLR culture, with FITC-anti-Lyt-2 and PE-anti-L3T4. Controls were unstained cells. Dead cells were gated out after staining with ethidium bromide. Cell size was monitored by forward light scattering.

To analyze MØ in the FACS, PEC were cultured in Teflon containers for 4 d in the presence or absence of IFN-γ. After washing, the cells were incubated with 100 μg/ml human gamma globulin for 30 min at 4°C to block Fc receptors before staining. Lymphocytes were gated out on the basis of their distinct forward and side light scattering properties.

Binding of T Cells to MØ. IFN-γ treated, TGC-MØ (2 × 10^5) on 13-mm glass coverslips were treated without or with Nase and cultured with 5–6 × 10^6 T cells in 24-well plates for 2–3 h at 37°C. After washing unbound T cells, the coverslips were fixed with 1.25% paraformaldehyde for 20 min at room temperature and observed under an inverted phase contrast microscope.

Results

Effect of Nase on the Stimulatory Activity of Macrophages. We previously noted that Ia$^+$, TGC-MØ were inactive for stimulating an allogeneic MLR in bulk T cells (6). This might have been due to an excess of sialic acid on macrophage Ia antigens, as suggested previously to explain the inactivity of Ia$^+$ B cells (10, 11). Therefore, we treated rIFN-γ^+ and rIFN-γ^- MØ with Nase before use as stimulators in the primary MLR. No stimulation was observed in the C3H [H-2k] vs. BALB/c (H-2d) combination (Table I).

Exogenous IL-2 Synergizes with Nase Treatment to Induce an MLR. One defect in MØ as presenting cells may be an inability to induce the production of T cell growth factors like IL-2. Therefore, IL-2 was added to the MLR. We also monitored the effects of MØ pretreatment with rIFN-γ, which upregulates class I and II MHC products (Fig. 1), together with Nase (Table I, Fig. 2 *A*). rIL-2 itself induced T cell proliferation beginning at a dose of 300 U/ml (Fig. 2). At a lower dose, 30 U/ml, which was just maximal for this preparation in an IL-2 bioassay, IL-2 amplified the

TABLE I

Macrophages Stimulate Allogeneic, But Not Syngeneic, T Cell Proliferation in the Presence of rIL-2

Stimulator cells:			Proliferative response ([^{3}H]TdR incorporation)			
			C3H/He T cells		BALB/c T cells	
Obtained from:	Cell type	Nase treatment	– rIL-2	+ rIL-2	– rIL-2	+ rIL-2
			cpm		*cpm*	
C3H/He	IFN-γ$^-$ Mϕ	–	181	892	23	409
		+	309	284	1,215	2,293
	IFN-γ$^+$ Mϕ	–	146	1,096	57	454
		+	166	2,647	1,452	16,613
	SAC	–	1,342	ND	25,537	ND
BALB/c	IFN-γ$^-$ Mϕ	–	137	1,671	75	92
		+	1,531	2,235	85	260
	IFN-γ$^+$ Mϕ	–	494	290	56	159
		+	486	13,497	198	693
	SAC	–	16,686	ND	1,605	ND
	None		161	2,083	62	418

2.5×10^5 PEC were cultured in the presence or absence of 12.5 U/ml rIFN-γ for 4 d in 16-mm-diam wells. The IFN-γ$^-$ and IFN-γ$^+$ Mϕ were treated with Nase. SAC were ~3×10^4 SAC prepared by adhering 2×10^6 spleen cells in 16-mm wells for 2 h. 2×10^6 Ia$^-$ bulk T cells were cultured with Mϕ or SAC for 79 h in the presence or absence of 100 U/ml human rIL-2. Then, 0.2-ml aliquots were transferred into 96-well plates and pulsed with 1 μCi[^{3}H]TdR for 6 h.

MLR in the presence of MΦ, especially rIFN-γ-treated cells (Fig 2 *A*, Table I). However, the MΦ had to have been treated with Nase beforehand and only an allogeneic vs. syngeneic MLR was induced (Table I). Therefore, Nase pretreatment and exogenous IL-2 together seem to be required for a primary MLR with allogeneic, TGC-MΦ, while pretreatment with rIFN-γ further enhances the response.

In The Presence of IL-2, Nase-treated Macrophages Present Class I MHC. To determine if both class I and II MHC products could be presented by MΦ, MLRs were set up with select strains including MHC recombinants. In Exp. A, Table II, T cells from A/J mice (kkd) responded to MΦ from A.TH and CxD2F1, strains that differ at both class I and II MHC loci. A.TL (skd) T cells responded to class I, H-2K-disparate MΦ (A/J; kkd), but not class II-disparate MΦ (A.TH; ssd). In Exp. B, Table II, A/J (kkd) MΦ stimulated C3H/He (kkk) that differ at the H-2D class I locus. Exogenous IL-2 was required for these responses to class I (Table II). On the other hand mixtures of spleen dendritic cells and MΦ induced strong proliferative responses across all MHC barriers, and exogenous IL-2 was not essential (Table II). Because of prior results (3, 9), stimulation by spleen adherent cells was likely due to the dendritic cell component.

T Cell Subsets that Respond to Nase-treated Macrophages plus IL-2. It is known that CD4$^+$ (Lyt-2$^-$) and CD8$^+$ (L3T4$^-$) T cells are restricted to antigens presented on MHC class II and I products, respectively (2). Given the data that Nase-treated MΦ stimulated across a class I MHC barrier, the MLRs were performed with enriched CD4$^+$ and CD8$^+$ T cells as responders. There were prominent responses by CD8$^+$ T cells as long as the MΦ were treated with Nase (not shown) and supplemented with low doses of rIL-2 (30 U/ml; Table III). The CD4$^+$ lymphocytes did not respond to MΦ, even with IFN-γ, Nase treatment and exogenous rIL-2 (Table

TABLE II
Neuraminidase-treated Macrophages Stimulate the Proliferation of MHC Class I-incompatible T Cells in the Presence of rIL-2

Exp.	Stimulator cells: Obtained from:	Cell type	Proliferative response ([^{3}H]TdR incorporation) by bulk T cells from the indicated strain and MHC (KID)			
			A.TL (skd)		A/J (kkd)	
			− rIL-2	+ rIL-2	− rIL-2	+ rIL-2
			cpm		*cpm*	
A	A.TH	IFN-γ$^+$ Mϕ	565	2,255	387	13,697
	(ssd)	SAC	38,320	ND	20,706	ND
	A/J	IFN-γ$^+$ Mϕ	1,447	31,726	86	1,711
	(kkd)	SAC	7,595	ND	917	ND
	CXD2F$_1$	IFN-γ$^+$ Mϕ	979	12,938	866	14,938
	(ddd)	SAC	63,426	ND	55,611	ND
		None	353	1,165	47	1,135
B			C3H/He (kkk)		CXD2F$_1$ (ddd)	
			− rIL-2	+ rIL-2	− rIL-2	+ rIL-2
	A/J	IFN-γ$^+$ Mϕ	864	21,882	1,220	22,927
	(kkd)	SAC	4,570	ND	15,378	ND
	CXD2F$_1$	IFN-γ$^+$ Mϕ	1,137	17,745	118	ND
	(ddd)	SAC	16,333	ND	206	ND
		None	289	848	122	825

2 × 10^6 Ia$^-$ bulk T cells were cultured with SAC or with IFN-γ$^+$, Nase-treated Mϕ in the presence or absence of 100 U/ml rIL-2 in 16-mm-diam wells. Data are means of triplicate assays of duplicate cultures for MLRs at 80–86 h, as in Table I.

III, Fig. 2 *B*). SAC served as a positive control, inducing strong responses in the CD4$^+$ subset. The weak responses of CD4$^+$ T cells to MØ was not due to different kinetics of the MLR in this subset (Fig. 3).

Phenotype of Proliferating T Cells by Cytofluorography. During the MLR, the proliferating T cells enlarge and these blasts can be distinguished on the basis of increased forward light scattering. When MØ were treated with Nase and used to stimulate CD8$^+$ cells in the presence of IL-2, blasts were detected and these were all stained with anti-CD8 but not anti-CD4 mAb (Fig. 4). Blasts did not develop in the CD4$^+$ MLR. However, when unseparated T cells were used as the responder population, CD4$^+$ as well as CD8$^+$ blasts were noted (Fig. 4). Since purified CD4$^+$ cells did not respond to MØ, the data indicate that the presence of a CD8 response somehow potentiates the CD4 response.

Development of CTL after Stimulation with Macrophages and IL-2. Since CD8$^+$ cells are the major cytolytic subset in the primary MLR, we evaluated if C3H/He (kkk) T cells formed CTL when stimulated by IFN-γ$^+$ MØ or spleen adherent cells from class I–disparate A/J (kkd) mice. Lytic cells specific for H-2D vs. H-2K targets did develop. Nase treatment and exogenous IL-2 were required, and only allogeneic T cells formed CTL (Table IV). The response of enriched CD8$^+$ cells was greater than bulk T cells (Table IV). With exogenous IL-2, the Nase-treated MØ were as effective in inducing CTL as SAC in the absence of IL-2.

Contacts Between Nase-treated Macrophages and T Cells. When dendritic cells stimulate T cells, the two cell types remain in contact for a day or more (12, 13). Allogeneic

TABLE III

Enriched CD8+, But Not CD4+, T Cells Respond to Allogeneic Macrophages in the Presence of rIL-2

	Stimulator cells:			Proliferative response ([^{3}H]TdR incorporation)					
				Bulk T		CD8+ T		CD4+ T	
Exp.	Obtained from:	Cell type	Nase treatment	− rIL-2	+ rIL-2	− rIL-2	+ rIL-2	− rIL-2	+ rIL-2
				cpm		*cpm*		*cpm*	
A	C3H/He	IFN-γ− Mø	−	126	1,309	110	2,093	127	145
			+	175	5,012	467	9,766	123	281
		IFN-γ+ Mø	−	107	3,118	117	4,646	127	953
			+	778	12,734	615	18,336	580	982
		SAC	−	31,639	ND	29,883	ND	27,046	ND
		None		158	290	79	236	95	469
B	A/J	IFN-γ+ Mø	+	688	16,994	709	20,856	612	1,144
		SAC	−	8,857	ND	18,401	ND	1,440	ND
	C3H/He	IFN-γ+ Mø	+	54	357	45	521	95	716
		SAC	−	877	ND	534	ND	1,233	ND
		None		78	986	92	1,034	53	956

2 × 10^6 Ia− bulk, CD4+ (depleted of Lyt-2+ T cells) or CD8+ (depleted of L3T4+ T cells) T cells from C × D2F1 (H-2d, Exp. A) or C3H/He (H-2k, Exp. B) mice were cultured with stimulator cells in the presence or absence of 100 U/ml rIL-2. The proliferative response was measured as [^{3}H]TdR incorporation at 78–84 h. Each number represents the mean of triplicate assays of duplicate cultures.

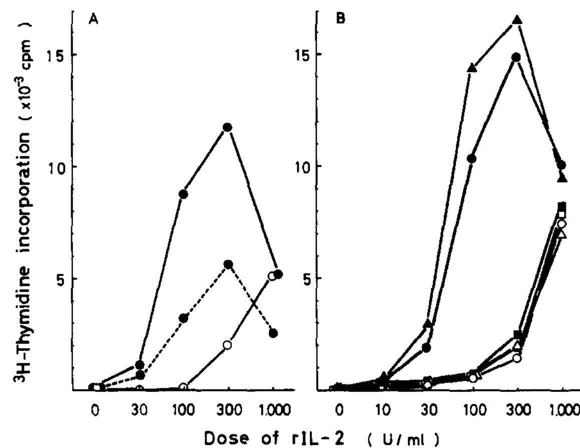

FIGURE 2. IL-2 requirement for allogeneic T cell proliferation stimulated by MØ. (*A*) Ia− bulk T cells from CxDF$_1$ mice were cultured with IFN-γ− (--●--) or IFN-γ+ (—●—) TGC-MØ from C3H/He mice in the presence of graded dose of rIL-2 for 84 h. T cells cultured with rIL-2 in the absence of MØ (○) served as control. (*B*) Ia− bulk T cells (●, ○), enriched CD4+ (depleted of Lyt-2+ T cells; ■, □) or CD8+ (depleted of L3T4+ T cells; ▲, △) from CxD2F$_1$ mice were cultured with (*closed symbols*) or without (*open symbols*) Nase-treated, IFN-γ+ TGC-MØ from C3H/He mice in the presence of graded doses of rIL-2 for 85 h. The proliferative response was expressed as [^{3}H]thymidine incorporation during the last 6 h of culture. Each point represents the mean of triplicate assays of duplicate cultures. rIL-2 alone induced significant T cell responses at high doses.

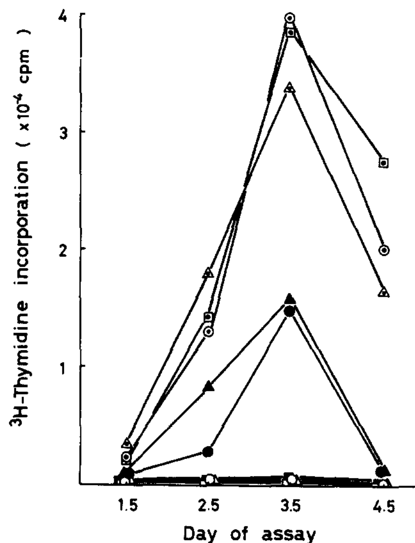

FIGURE 3. Kinetics of the proliferative response of T cell subsets to Nase-treated, IFN-γ^+ MØ in the presence of rIL-2 vs. SAC. 2×10^6 Ia$^-$ bulk (○, ●, ○), enriched CD8$^+$ (△, ▲, △), or CD4$^+$ (□, ■, □) T cells were cultured with Nase-treated, IFN-γ^+ MØ in the presence (*closed symbols*) or absence (*open symbols*) of 100 U/ml rIL-2, or SAC (*symbols with dots*). Each symbol represents the mean of triplicate assays.

MØ do not bind T cells (3, 13), but we wondered if Nase-treated cells did. This proved to be the case (Fig. 5). Both CD4$^+$ and CD8$^+$ T cells bound to Nase-treated MØ. If the nonadherent T cells were removed, some of the attached CD8$^+$ T cells later proliferated, but the CD4$^+$ did not (9,091 vs. 385 cpm, respectively).

Nase and Exogenous IL-2 Effects on MLR Stimulation by Fresh MØ. 2-h adherent, TGC-elicited PEC were used to stimulate the MLR in bulk T cells and in enriched CD4$^+$ and CD8$^+$ subsets. In some cases, the PEC were treated with anti-Ia and complement to remove a subset of 3–8% Ia$^+$ PEC. Very weak MLRs were observed with all populations of T cells, and Ia$^+$ cell depletion abolished the weak CD4$^+$ MLR (Table V). However, if either Nase treatment or exogenous rIL-2 was included, an MLR occurred, but primarily in the CD8$^+$ subset (Table V). When the same MØ were cultured for 4 d, it was again necessary to expose them to IFN-γ, treat them with Nase, and add IL-2 to observe sizable MLRs (Table V).

Effect of Nase and Exogenous IL-2 on B Cell–stimulated MLRs. Like MØ, small B cells are inactive as MLR stimulators (10, 13–15). To determine if B cells could be rendered immunogenic in a manner comparable to MØ, the lymphocytes were treated with Nase and added to allogeneic CD8$^+$ or CD4$^+$ T cells plus or minus exogenous IL-2. The results were comparable to the MØ findings. Both Nase and IL-2 were required to observe an allogeneic MLR with foreign B cells, and only the CD8$^+$ subset responded directly (Table VI).

Discussion

A considerable amount of literature has shown that lymphoid dendritic cells (isolated from lymphoid organs, blood, and lymph) actively stimulate an MLR. In the mouse it has been shown that class I and II MHC products are presented (9) and that purified CD4$^+$ and CD8$^+$ subsets respond (3). In contrast, most populations of MØ and B lymphocytes, when depleted of dendritic cells, are weak stimulator

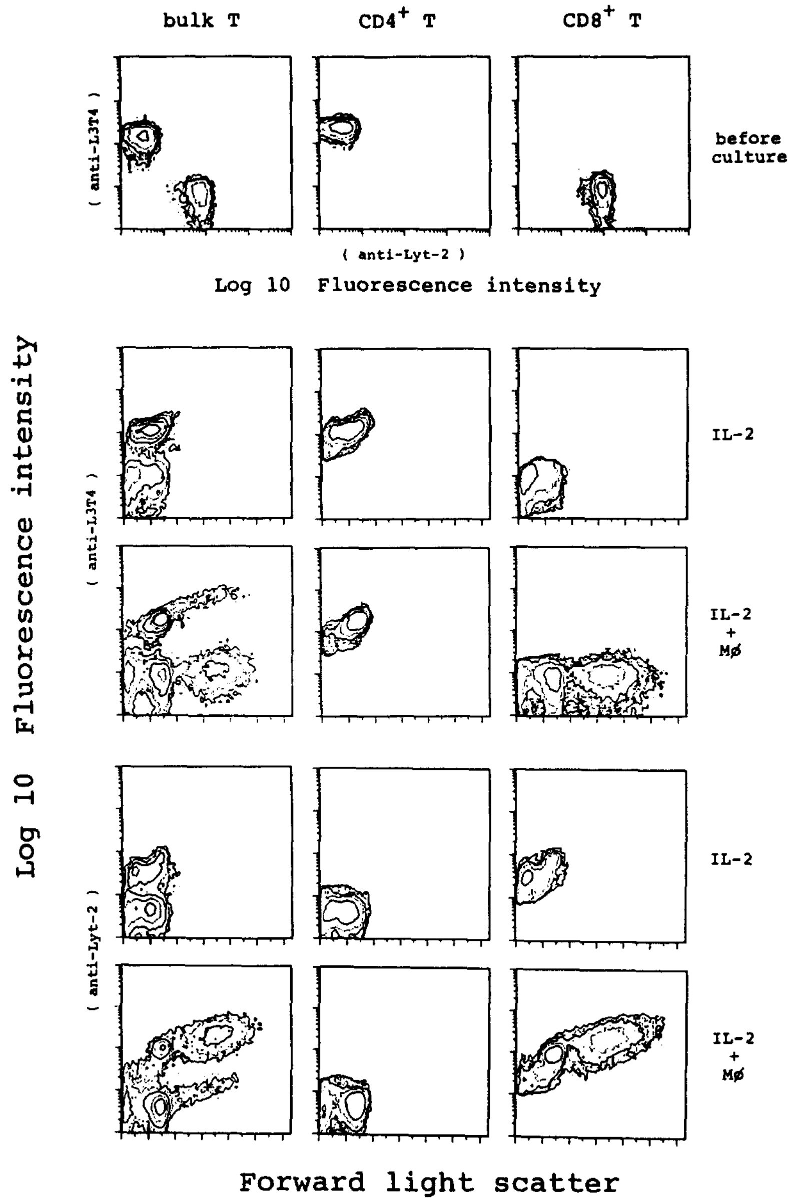

FIGURE 4. Blastogenesis of CD8+ T cells stimulated by Nase-treated, IFN-γ+ MØ in the presence of exogenous rIL-2. CD8+, CD4+, and bulk cells from CxD2F1 mice were stained with anti-CD8 or anti-CD4 mAbs before culture (*top panels*). The subsets were cultured with 100 U/ml rIL-2 without or with Nase-treated MØ from C3H mice for 85 h. At the end of culture, cells were collected, washed, stained with anti-CD8 and anti-CD4 antibodies, and analyzed by FACStar. Cell size was monitored by forward light scattering.

TABLE IV
Neuraminidase-treated, H-2D Disparate Macrophages Stimulate the Generation of CTL in the Presence of rIL-2

Stimulator cells: Obtained from:	Cells	rIL-2 in culture	Targets	Cytolysis (percent specifiic ^{51}Cr release) Bulk T cells 30:1	10:1	3:1	CD8^{+} T cells 30:1	10:1	3:1
A/J	IFN-γ^{+} Mø	–	P815	–0.3	0.2	1.8	7.6	–1.8	1.4
		+	P815	59.5	35.0	18.8	65.8	44.9	31.0
		+	X5563	–0.7	0.5	0.7	5.1	–0.4	0.8
	SAC	–	P815	54.8	29.3	18.1	65.1	44.6	28.7
C3H/He	IFN-γ^{+} Mø	–	P815	1.7	2.1	0.4	2.2	3.0	1.8
		+	P815	1.1	–0.2	0.0	3.7	1.4	0.4
		+	X5563	–0.9	1.3	1.0	5.4	1.2	1.7
	SAC	–	P815	2.4	3.3	3.0	2.2	1.4	1.6
	None	–	P815	0.6	1.5	1.6	1.8	1.2	0.2
		+	P815	1.7	2.0	1.8	0.5	2.1	2.0
		+	X5563	0.0	–0.8	0.9	–2.9	1.9	–0.8

2×10^6 Ia$^-$ bulk or CD8$^+$ T cells from C3H/He mice (kkk) were cultured with H-2D-disparate (A/J, kkd) or syngeneic C3H/He, SAC, or Nase-treated, IFN-γ$^+$ Mø in the presence or absence of 100 U/ml rIL-2 for 85 h in 16-mm-diam wells. At the end of culture, cells were collected, washed, and mixed with ^{52}Cr-labeled target cells in 96-well round-bottomed plates for the cytolytic assay. Maximum and spontaneous release of ^{51}Cr were 5,414 cpm and 879 cpm in P815 (H-2d), and 15,230 cpm and 4,633 cpm in X5563 (H-2k), respectively. Cytolytic activity against YAC-1 target cells never exceeded that against P815 (not shown).

cells across either MHC barrier and with either T cell subset as responders (3, 9). The lack of stimulation by MØ does not seem to be due to a suppressive activity. While MØ can suppress the MLR that is induced by dendritic cells, we have shown that appropriate doses of MØ actually enhance the MLR at very low doses of dendritic cells (6). Also, MØ present both class I (3) and II (8, 13) MHC products to sensitized CD8$^+$ and CD4$^+$ lymphoblasts. Here we have succeeded in making MØ directly immunogenic, that is in the apparent absence of dendritic cells. The variables that were studied were pretreatment of the MØ with rIFN-γ and with Nase, and the supplementation f the MLR with exogenous IL-2.

Lymphokines, especially rIFN-γ, have a marked effect on the expression of class II MHC products on inflammatory MØ. When cultured in the absence of lymphokine, the levels of Ia become undetectable on most cells, while in its presence, Ia is found on most MØ (4, 16) (Fig. 1). Lymphokines also upregulate class I MHC products, although the latter are expressed constitutively (4, 17) (Fig. 1). In our studies, rIFN-γ$^+$ MØ were more active as stimulators, but IFN-γ$^-$ cells had some activity (Tables I, III, and IV). Our experiments do not permit us to determine if the effect of rIFN-γ is due to a greater expression of class I products or to some other variable, such as production of an amplifying cytokine.

MØ treatment with Nase was essential for observing stimulation in the MLR (Tables I and III; Fig. 2). In contrast, no enhancement of MLR was observed when responder T cells or stimulator dendritic cells were treated with Nase (not shown). Addition of the Nase inhibitor (2,3-dehydro-2-deoxy-N-acethy-neuraminic acid, 2 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) during the treatment of MØ with Nase abrogated its enhancing effect on the MLR, while incuba-

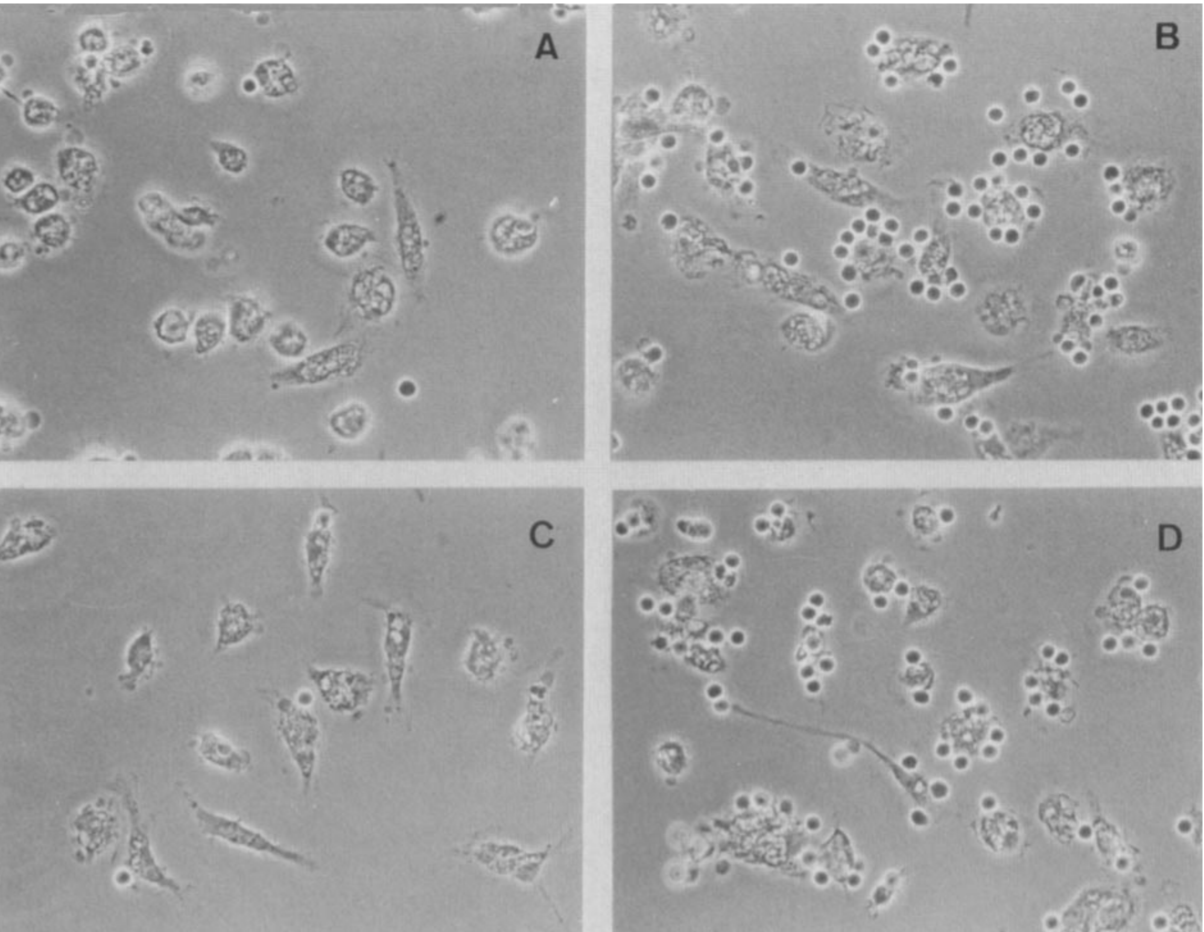

FIGURE 5. Neuaminidase treatment enables MØ to bind T cells. Allogeneic (C3H/H3; *top*) or syngeneic ($CxD2F_1$; *bottom*) IFN-γ^+ TGC-MØ on 13-mm cover slips were treated with (*B, D*) or without (*A, C*) Nase and incubated with 6×10^6 $CD8^+$ cells in 16-mm wells for 2 h. After washing off unbound cells, cells on cover slips were fixed with 1.25% paraformaldehyde for 20 min at room temperature, and observed under the inverted microscope. Nase-treated MØ bound $CD4^+$ (not shown) as well as $CD8^+$ T cells (*B, D*), whereas untreated MØ did not bind T cells (*A, C*).

TABLE V
Stimulatory Activity of Fresh TGC-Mø on Different T Cell Subsets

Stimulators		Proliferative response ([^{3}H]TdR incorporation)					
		Bulk T		CD8$^+$ T		CD4$^+$ T	
Cell preparation	Nase treatment	- rIL-2	+ rIL-2	- rIL-2	+ rIL-2	- rIL-2	+ rIL-2
		cpm		*cpm*		*cpm*	
2 h TGC-Mø (Ia$^-$)	-	2,436	10,734	3,344	17,996	461	554
	+	15,323	20,112	21,197	25,468	754	2,203
2 h TGC-Mø (untreated)	-	3,211	11,558	4,587	18,335	2,477	7,391
	+	14,678	19,635	19,233	26,221	4,890	10,232
IFN-γ$^+$ Mø	-	312	3,349	148	4,412	177	871
	+	345	12,034	296	13,001	333	1,202
IFN-γ$^-$ Mø	-	412	2,238	326	2,946	288	496
	+	349	4,344	277	6,291	391	1,269
SAC	-	32,387	NT	36,547	NT	31,875	NT
None		216	847	148	810	169	946

High density populations of Ia$^-$ bulk, CD8$^+$, and CD4$^+$ T cells were prepared from CXD2F$_1$ mice. Mø were adherent cells of PEC from C3H/He mice injected with thioglycollate medium 4 d previously. To remove Ia$^+$ cells, PEC were treated with mAbs and complement before adhering. 2 × 10^5 Mø, adjusted by morphology under the microscope, were plated into 24-well plates (16-mm wells). After washing off nonadherent cells, 2-h TBC-Mø were tested as stimulators, or replicates were cultured for 4 d in the presence or absence of rIFN-γ before use as stimulators. Proliferation was monitored by [^{3}H]TdR incorporation between 79 and 85 h of culture.

tion of Nase-treated MØ with Nase-inhibitor did not abolish stimulatory activity (not shown). Therefore, it seems unlikely that the effects we observed were due to Nase carryover.

The use of Nase was suggested by the work of Cowing and colleagues, who found that Nase enhanced stimulation by enriched populations of B cells (10, 11). They suggested that Nase acted by reducing the amount of sialic acid on class II MHC products, but it is possible that Nase modification of other molecules allows the presenting cell and T cell to contact each other (Fig. 5). Native B cells and MØ do not seem to form such contacts (3, 13, 18). It has been reasoned that one of the rate-

TABLE VI
Neuraminidase-treated, High Density B Cells Stimulate Allogeneic CD8$^+$ T Cells in the Presence of rIL-2

Stimulators			Proliferative response ([^{3}H]TdR incorporation)					
			Bulk T		CD8$^+$		CD4$^+$	
Cell type	Number	Nase treatments	- rIL-2	+ rIL-2	- rIL-2	+ rIL-2	- rIL-2	+ rIL-2
B cells	2 × 10^6	-	253	1,765	59	7,034	368	1,033
		+	197	17,775	157	20,327	1,531	4,518
	6.7 × 10^5	-	ND	ND	48	3,685	59	549
		+	ND	ND	75	11,421	321	1,158
SAC	3 × 10^4	-	ND	ND	34,739	ND	38,637	ND
	None		246	464	78	336	94	218

2 × 10^6 responder T cells of CXD2F$_1$ mice were cultured with high density B cells from C3H/He mice. The B cells were high density, T-depleted, Sephadex G10 nonadherent lymphocytes. After 79 h of culture, aliquots of cells were transferred to 96-well plates and pulsed with [^{3}H]TdR for 6 h. Each number represents the mean of triplicate assays of duplicate cultures.

limiting steps in the MLR is the capacity of the allogeneic stimulator cell to form stable conjugates with the responding T cell (13, 19). To date, lymphoid dendritic cells are the principal cell type that can form such clusters in antigen-dependent primary responses.

Clustering, however, does not seem sufficient for immunogenicity. Nase-treated MØ and small B cells still required exogenous IL-2. By inference, then, one of the distinguishing features of dendritic cells and MØ as APC is that dendritic cells seem capable of inducing the endogenous production of essential growth factors.

Together, the combined treatment with IFN-γ, Nase, and IL-2 only renders the MØ immunogenic for the $CD8^+$ subset. Resting $CD4^+$ T cells do not respond class II-disparate MØ (Table III), whereas the number of enlarged blasts in the $CD8^+$ MLR was considerable (Fig. 4). This might indicate that $CD8^+$ cells differ in the presenting cell requirements for the appearance of the IL-2-responsive state. In contrast, dendritic cells actively stimulate a state of IL-2 responsiveness in $CD4^+$ lymphocytes (13).

Although we were unable to triggr the $CD4^+$ subset directly with MØ, our studies provide preliminary information that it may be possible to do so. Specificially, we noted the development of $CD4^+$ T blasts when the responding population contained both $CD8^+$ and $CD4^+$ T cells (Fig. 4). One possibility is that MØ synthesize amplifying factors like IL-1 or IL-6, but that these factors are only released after the development of $CD8^+$ CTL (Table IV), thus liberating cell-associated activating factors for use by other MØ.

Summary

Prior work has shown that purified, resident, and inflammatory peritoneal macrophages are weak stimulators of the allogeneic MLR. We have identified conditions whereby thioglycollate-elicited macrophages become stimulatory, but primarily for the $CD8^+$ T cell subset. The conditions were to treat the macrophages with neuraminidase and to supplement the MLR with rIL-2. These treatments together led to proliferative and cytotoxic responses by isolated $CD8^+$ but not $CD4^+$ T cells. Likewise when MHC-congenic strains were evaluated, an MLR was observed across isolated class I but not class II MHC barriers. Pretreatment of the macrophages with IFN-γ further enhanced expression of class I MHC products and stimulatory activity, but did not seem essential. While these treatments did not render macrophages stimulatory for an MLR in purified $CD4^+$ cells, blastogenesis of $CD4^+$ cells was observed when the MLR involved bulk T cells. Small allogeneic B lymphocytes behaved similarly to macrophages, in that pretreatment with neuraminidase and supplementation with rIL-2 rendered B cells stimulatory for allogeneic, enriched, $CD8^+$, but not $CD4^+$, T cells. Spleen adherent cells, which are mixtures of macrophages and dendritic cells, stimulated both $CD4^+$ and $CD8^+$ T cells, and neither neuraminidase nor exogenous IL-2 was required. We think that these data suggest that most macrophages and small B cells lack three important functions of dendritic cells: a T cell-binding function that can be remedied by neuraminidase treatment, a T cell growth factor-inducing function that can be bypassed with exogenous IL-2, and an IL-2 responsiveness function that is required by $CD4^+$ lymphocytes.

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References

1. Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Havran, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch. 1983. Characterization of the murine antigenic determinant, designated L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.* 74:29.
2. Swain, S. L. 1983. T cell subsets and the recognition of MHC class. *Immunol. Rev.* 74:129.
3. Inaba, K., J. W. Young, and R. M. Steinman. 1987. Direct activation of CD8+ cytotoxic T lymphocytes by dendritic cells. *J. Exp. Med.* 166:182.
4. Steinman, R. M., N. Nogueira, M. D. Witmer, J. D. Tydings, and I. S. Mellman. 1980. Lymphokine enhances the expression and synthesis of Ia antigen on cultured mouse peritoneal macrophages. *J. Exp. Med.* 152:1248.
5. Van Voorhis, W. C., J. Valinsky, E. Hoffman, J. Luban, L. S. Hair, and R. M. Steinman. 1983. The relative efficacy of human monocytes and dendritic cells as accessory cells for T cell replication. *J. Exp. Med.* 158:174.
6. Naito, K., S. Komatsubara, J. Kawai, K. Mori, and S. Muramatsu. 1984. Role of macrophages as modulators but not as stimulators in primary mixed leukocyte reaction. *Cell. Immunol.* 88:361.
7. Komatsubara, S., Y. Hirayama, K. Inaba, K. Naito, K. Yoshida, J. Kawai, and S. Muramatsu. 1985. Role of macrophages as modulators but not as autonomous accessory cells in primary antibody response. *Cell. Immunol.* 95:288.
8. Inaba, K., M. Kitaura, T. Kato, Y. Watanabe, Y. Kawade, and S. Muramatsu. 1986. Contrasting effect of α/β- and γ-interferons on expression of macrophage Ia antigens. *J. Exp. Med.* 163:1030.
9. Steinman, R. M., B. Gutchinov, M. D. Witmer, and M. C. Nussenzweig. 1983. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* 157:613.
10. Cowing, C., and J. N. Chapdelaine. 1983. T cells discriminate between Ia antigens expressed on allogeneic accessory cells and B cells: a potential function for carbohydrate side chain on Ia molecules. *Proc. Natl. Acad. Sci. USA.* 80:6000.
11. Frohman, M., and C. Cowing. 1985. Presentation of antigen by B cells: functional dependence on radiation dose, interleukins, cellular activation, and differential glycosylation. *J. Immunol.* 134:2269.
12. Inaba, K., M. D. Witmer, and R. M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells, during primary antibody responses in vitro. *J. Exp. Med.* 160:858.
13. Inaba, K., and R. M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717.
14. Inaba, K., S. Koide, and R. M. Steinman. 1985. Properties of memory T lymphocytes isolated from the mixed leukocyte reaction. *Proc. Natl. Acad. Sci. USA.* 82:7686.
15. Krieger, J. I., R. W. Chesnut, and H. M. Grey. 1986. Capacity of B cells to function as stimulators of a primary mixed leukocyte reaction. *J. Immunol.* 137:3117.

16. Steeg, P. S., R. N. Moore, and J. J. Oppenheim. 1980. Regulation of murine macrophage Ia-antigen expression by products of activated spleen cells. *J. Exp. Med.* 152:1734.
17. Wong, G. H. W., I. Clark-Lewis, L. Mckimm-Breschkin, A. W. Harris, and J. W. Schrader. 1983. Interferon-γ induces enhanced expression of Ia and H-2 antigens on B lymphoid, macrophages, and myeloid cell lines. *J. Immunol.* 131:788.
18. Flechner, E., P. Freudenthal, G. Kaplan, and R. M. Steinman. 1988. Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed leukocyte reaction. *Cell. Immunol.* 111:183.
19. Inaba, K., G. Schuler, M. D. Witmer, J. Valinsky, B. Atassi, and R. M. Steinman. 1986. The immunologic properties of purified Langerhans cells: distinct requirements for the stimulation of unprimed and sensitized T lymphocytes. *J. Exp. Med.* 164:605.