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A Role for Ia Antigens in Thymocyte Binding by Macrophages[1]

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To investigate the membrane structures involved in cellular interactions between thymocytes and macrophages, the relative ability of different murine macrophage populations to spontaneously bind thymocytes was compared. Macrophages derived from the spleen or thymus bound three to four times the number of thymocytes than macrophages from peripheral blood, peritoneum, or bone marrow. This reflects differences both in the number of macrophages binding thymocytes and in the number of thymocytes bound per macrophage. The extent of binding seems to positively correlate with the number of Ia-positive macrophages contained in these populations, as based on previously published values. This was confirmed by showing that elimination of splenic Ia-positive macrophages with anti-Ia and complement treatment dramatically reduced thymocyte binding. In addition, mouse peritoneal washout macrophages incubated for several days with supernatant fluid from concanavalin A-stimulated spleen cells, which induce Ia-antigen expression, exhibited a marked increase in the number of macrophages that bound thymocytes and the number of thymocytes bound per macrophage. To determine if Ia antigens were directly involved in binding, spleen, thymus, or Ia-induced peritoneal macrophages were treated with a monoclonal anti-Ia antibody prior to the addition of thymocytes. Treatment with anti-Ia reduced binding by around 50%, whereas treatment with anti-H-2D antibody had no effect. Monoclonal anti-I-A and anti-I-E antibody treatments of macrophages both inhibited thymocyte binding to similar extents, and treatment of macrophages with both reagents together reduced thymocyte binding by 80%. These results indicate that thymocyte binding is in part dependent on macrophage Ia expression.

INTRODUCTION

Several theories have been proposed which state that self–nonself discrimination and Ir-gene selection occur in the thymus during T-cell maturation (1–3). In substantiation of this, recent reports have indicated that genetic restrictions of T-cell reactivity may be determined by the haplotype of the thymus (4–8). These restrictions for I-region regulation appear to be manifest in mature T-cell–macrophage interactions for both alloreactive and antigen-specific responses. Previous reports have also shown that immature thymocytes interact specifically with macrophages, and it was speculated that the physical binding between these two cell types initiated thymocyte maturation

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(9–12). Some of the features of the binding of thymocytes to macrophages are that the binding is rapid and reversible, that the macrophage structures involved in binding are trypsin sensitive, that the macrophages must be viable, and that those thymocytes which are released from macrophages do not readily rebind. More importantly, it was found that this binding was species specific, but was not strain specific (9, 11, 12). The fact that thymocyte binding is species specific suggests that the cellular structures involved discriminate self from nonself to some extent, and for this reason may be involved in selection for functional reactivity by mature T cells. In this report we have examined the ability of different macrophage populations to bind thymocytes and found that binding seems to correlate with Ia expression by macrophages. In addition, thymocyte binding is specifically inhibited by treating macrophages with anti-Ia antibody.[4] These observations suggest that Ia antigens play several roles in T-cell interactions with macrophages in antigen-independent as well as antigen-dependent collaborations.

MATERIALS AND METHODS

Animals. A/J (H-2^a), C57BL/6 (H-2^b), B.C8 (H-2^b), CBA/N (H-2^k), and CBA/J (H-2^k) mice were bred and maintained at The University of Michigan Medical School or purchased from The Jackson Laboratories, Bar Harbor, Maine, and used between 6 and 8 weeks of age.

Thymocyte binding. Adherent cells were obtained by overnight culture of cell suspensions from spleen ($20–25 \times 10^6$), thymus ($20–30 \times 10^6$), resident peritoneal washout (0.5×10^6), bone marrow ($2–4 \times 10^6$), or peripheral blood (1×10^6) in 35-mm tissue culture dishes (Falcon, Cockeysville, Md.). Cultures were maintained in RPMI 1640 medium (KC Biological, Lenexa, Kans.) containing L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (50 μg/ml), 2-mercaptoethanol (5×10^{-5} *M*), and 10% heat-inactivated (56°C, 30 min) fetal calf serum (KC Biological) and cultured at 37°C in 5% CO_2 in air. Following overnight culture nonadherent cells were removed by vigorous washing with Hanks' balanced salt solution (HBSS, KC Biological). The purified adherent cells ($1–5 \times 10^5$ per dish) showed no adherent lymphocytes or debris after washing and were used immediately for thymocyte binding. Adherent cells obtained in this manner were ≥70% macrophages on the basis of ingestion of latex beads and morphology and will therefore be referred to as macrophages, although contaminating adherent cell types such as epithelial cells are present. Previous studies have shown that thymocytes bind only to macrophages (9–12). Thymocytes (2×10^6) were added in 1 ml of HBSS or RPMI 1640 without serum to dishes containing adherent macrophages and cultured for 30 min at 37°C for binding to occur. At the end of this incubation period, nonadherent thymocytes were removed by gentle washing with HBSS and the adherent cells fixed by the addition of 1% glutaraldehyde in physiologic phosphate-buffered saline. Thymocyte binding to adherent macrophages was quantitated by visual examination using an inverted phase microscope. A minimum of 250 macrophages in five different areas of each culture was examined and the results were normalized to the number of thymocytes bound per 100 macrophages. All values represent the average number of bound thymocytes from triplicate or

[4] Abbreviations used: HBSS, Hanks' balanced salt solution; Ia, I-region-associated antigens; MHC, major histocompatibility complex; Mϕ, macrophages.

duplicate cultures. Each experiment utilized macrophages from a single mouse to avoid different variables influencing binding, such as age, etc.

Macrophage treatments. In some experiments macrophages were incubated for 2 hr at 37°C with 0.5 ml of supernatant fluids containing monoclonal antibody 10.2.16 (anti-Ia.17, obtained from Dr. Susan Cullen, Washington University School of Medicine, St. Louis, Mo.), 34.4.20 (anti-H-$2D^d$, a generous gift from Dr. David Sachs, NIH, to Dr. Roderick Nairn, The University of Michigan Medical School), or 14.4.4 (anti-I-E^k, obtained from Dr. John Niederhuber, The University of Michigan Medical School). Unbound antibody was removed by washing three or four times with HBSS prior to the addition of thymocytes for binding. In other experiments, adherent peritoneal washout macrophages were incubated for 3 days with medium containing 5% of a supernatant fluid from a 24-hr culture of A/J mouse spleen cells (10×10^6/ml) stimulated with concanavalin A (5 μg/ml, Con A, Sigma Chemical Co., St. Louis, Mo.), or medium containing 5 μg/ml Con A (Control Sup fluid) and 5 m*M* α-methyl-D-mannoside (Sigma Chemical Co.). In some experiments cultures with Con A or Control Sup also included 0.5 ml of 10.2.16 or 34.4.20 supernatant fluid. All cultures were washed thoroughly with HBSS prior to the addition of thymocytes for binding.

For antibody and complement treatment, CBA/J overnight adherent spleen cells were incubated for 45 min with a 1:10 dilution of A.TH anti-A.TL serum (anti-Ia^k, a generous gift from Dr. John Niederhuber) at 4°C. After removal of the antibody the cultures were incubated for 45 min at 37°C with or without a 1:10 dilution of fresh rabbit serum that had been screened for low toxicity as a source of complement. The cultures were then washed four times with HBSS and cultured for an additional 6 hr at 37°C in complete medium to allow recovery from the anti-Ia treatment, then washed again prior to adding thymocytes for binding.

RESULTS

In initial experiments thymocyte binding to different macrophage populations was examined to determine if all macrophages exhibit similar structures for binding. As shown in Table 1, macrophages from the thymus and spleen showed a much greater ability to bind thymocytes than macrophages derived from peripheral blood, peritoneal

TABLE 1

Thymocyte Binding to Different Populations of Macrophages

	Thymocytes bound/100 Mϕ[a]		
Macrophage origin	Expt 1	Expt 2	Expt 3
Thymus	342	239	ND
Spleen	338	176	332
Bone marrow	116	76	217
Peritoneal washout	70	23	57
Peripheral blood	52	48	69

[a] Macrophages were obtained by overnight adherence of cell suspensions from A/J (Experiments 1 and 3) or B.C8 (Experiment 2) mice and syngeneic thymocyte binding performed as described under Materials and Methods. Each experiment represents binding to macrophages derived from a single mouse.

[b] Not determined.

cavity, or bone marrow. The number of thymic or splenic macrophages which bound thymocytes was 70 to 90%, whereas only 20 to 30% of macrophages from peripheral blood, resident peritoneal washout, or bone marrow showed binding (Table 2). In addition, quantitation of the number of thymocytes bound per macrophage showed that spleen macrophages had an even distribution from 1–2 to >6 thymocytes bound per macrophage, whereas most peritoneal macrophages had only 1–2 bound thymocytes (Table 3). In contrast, those bone marrow adherent cells showing binding generally had >6 bound thymocytes. Bone marrow cells with this type of binding did not show typical macrophage morphology and were large multinuclear cells. The binding by these cells also did not show species specificity since guinea pig thymocytes were bound to the same extent as mouse thymocytes (data not shown).

The results described above clearly indicate that not all macrophage populations have an equal capacity to bind thymocytes. In addition, the relative binding seemed to be correlated with the previously reported extent of Ia expression for different macrophage populations from A/J and CBA/J mice (13–15). Thus, those populations with the highest number of Ia-positive cells bound thymocytes better than those populations containing relatively few Ia-positive cells. To determine if macrophage Ia was directly involved in thymocyte binding, we determined the effects of treating macrophages with anti-Ia antibody prior to addition of thymocytes. As shown in Table 4, pretreatment of thymic or splenic macrophages from CBA or A/J mice with anti-I-A^k inhibited their binding capacity by 50 to 70%. This inhibition seemed to be specific since no reduction was noted with macrophages from B.C8 (H-2^b) mice, and since an anti-H-$2D^d$ antibody directed against the same A/J macrophage as the anti-Ia caused little or no reduction in binding. It should be pointed out that the thymocyte haplotype makes no difference in binding and that anti-I-A^k inhibits binding to CBA and A/J macrophages by both syngeneic and allogeneic thymocytes (Table 2, Experiments 3 and 4). It should also be noted that thymocytes and macrophages from different animals show different degrees of binding, so that each experiment is performed with macrophages and thymocytes from an individual animal to provide internal consistency; the extent of binding from experiment-to-experiment cannot therefore be accurately compared. The reduction in thymocyte binding by anti-Ia-treated macrophages was reflected by both a decrease in the number of macrophages binding thymocytes and a reduction in the number of thymocytes bound per macrophage (Table 5). To confirm that Ia-positive macrophages were the primary cell

TABLE 2

Numbers of Macrophages in Different Populations That Bind Thymocytes

Macrophage[a]	Thymocytes bound/100 Mϕ	% Macrophages ≥1 thymocyte bound
Thymus	342	77
Spleen	338	77
Peritoneum	70	29
Blood	52	28
Bone marrow	116	34

[a] Macrophages from a single A/J mouse were obtained as before and the binding by syngeneic thymocytes performed as described under Materials and Methods. Similar results were obtained in two additional experiments.

TABLE 3

The Extent of Thymocyte Binding by Different Macrophage Populations

Macrophage[a]	Thymocytes bound/Mϕ				
	0	1–2	3–4	5–6	>6
Spleen	23	16	28	16	18
Bone marrow	66	2	3	5	24
Peritoneum	71	21	7	1	<1

[a] Macrophages were obtained from a single A/J mouse and the binding by syngeneic thymocytes performed as described under Materials and Methods. A minimum of 250 macrophages from each of duplicate cultures was examined and the average number of thymocytes bound normalized for 100 macrophages. Similar results were obtained in two additional experiments.

type involved in binding, adherent spleen macrophages were treated with anti-Ia serum and complement to eliminate most Ia-positive cells, then allowed to recover from the anti-Ia treatment by further incubation before thymocyte binding. As shown in Table 6, anti-Ia and C′ treatment reduced macrophage binding of thymocytes by

TABLE 4

Inhibition of Thymocyte Binding by Macrophage Treatment with Anti-Ia Antibody

Expt	Thymocyte strain	Macrophage strain	Macrophage origin	Monoclonal antibody	Thymocytes bound/100 Mϕ[a]
1	CBA/N	CBA/N	Thymus	None	172
				Anti-I-A^k	47
			Spleen	None	198
				Anti-I-A^k	79
2	A/J	A/J	Thymus	None	142
				Anti-I-A^k	54
				Anti-H-$2D^d$	129
	B.C8	B.C8	Thymus	None	304
				Anti-I-A^k	339
3	B.C8	B.C8	Spleen	None	626
				Anti-I-A^k	695
	CBA/J	B.C8		None	558
				Anti-I-A^k	537
	CBA/J	CBA/J	Spleen	None	379
				Anti-I-A^k	218
	B.C8	CBA/J		None	659
				Anti-I-A^k	343
4	A/J	A/J	Spleen	None	165
				Anti-I-A^k	86
	C57BL/6	A/J		None	290
				Anti-I-A^k	97

[a] Adherent splenic or thymic macrophages from the indicated mouse strains were treated for 2 hr with anti-Ia or anti-H-2D antibody prior to thymocyte binding, as described under Materials and Methods.

TABLE 5

Extent of Thymocyte Binding by Anti-Ia-Treated Macrophages

Monoclonal antibody	Thymocytes bound/Mϕ[a]				
	0	1–2	3–4	5–6	>6
None	22	24	27	14	13
Anti-I-A^k	52	32	12	3	<1

[a] CBA/J splenic macrophages were treated with control supernatant fluid or anti-I-A^k (10.2.16) and the binding by syngeneic thymocytes performed as described under Materials and Methods. A minimum of 250 macrophages from each of duplicate cultures was examined and the average number of thymocytes bound normalized for 100 macrophages.

80%, suggesting that most of those macrophages that bind thymocytes express Ia antigens.

The results presented above suggest that not only is thymocyte binding correlated with the number of Ia-positive macrophages, but that the binding event itself seems to be in part associated with macrophage Ia antigens themselves. To examine this apparent association in another way, we took advantage of recent observations that factors from activated T cells induce Ia expression by Ia-negative macrophages (16, 17). Accordingly, peritoneal washout macrophages were cultured for several days with either a control Con A Sup or a Con A-stimulated spleen cell Sup (both with α-methylmannoside) and the extent of thymocyte binding was determined. As shown in Table 7, peritoneal macrophages cultured with Con A Sup bound three to four times the number of thymocytes bound by macrophages treated with Control Sup or culture medium. This increase was due to an increase in macrophages showing binding from 20 to 30% to 80 to 90%, and in the number of thymocytes bound per macrophage (data not shown). To determine if this increase in binding was due to Ia expression, anti-Ia antibody was included during the induction culture. Again, anti-I-A^k reduced thymocyte binding by 50 to 70% whereas anti-H-2D^d had little or no effect. These results again confirm that the extent of thymocyte binding is directly correlated with macrophage Ia expression and that the binding itself may be in part associated with Ia antigens.

TABLE 6

Thymocyte Binding to Macrophages Treated with Anti-Ia Serum and Complement

Macrophage treatment[a]	Thymocytes bound/100 Mϕ
Untreated	149
C′	121
Anti-Ia^k	129
Anti-Ia^k + C′	24

[a] CBA/J splenic adherent cells were treated with A.TH anti-A.TL serum (anti-Ia^k) with or without complement, and following 6 hr of additional incubation the binding of syngeneic thymocytes was determined as described under Materials and Methods.

TABLE 7

Induction of Thymocyte Binding by Peritoneal Washout Macrophages Cultured with Activated T-Cell Supernatant Fluid

Expt	Peritoneal macrophage culture conditions	Monoclonal antibody	Thymocytes bound/100 Mϕ[a]
1	Medium	None	70
	Control Sup	None	44
	Con A Sup	None	302
2	Control Sup	None	57
	Control Sup	Anti-I-A^k	61
	Con A Sup	None	219
	Con A Sup	Anti-I-A^k	108
3	Control Sup	None	50
	Control Sup	Anti-I-A^k	38
	Control Sup	Anti-H-2D^d	48
	Con A Sup	None	155
	Con A Sup	Anti-I-A^k	75
	Con A Sup	Anti-H-2D^d	168

[a] Adherent peritoneal washout macrophages from A/J mice were cultured for 3–4 days with medium, Control Con A Sup, or Con A Sup from activated spleen cells with or without anti-I-A^k or anti-H-2D^d antibodies and syngeneic thymocyte binding was determined as described under Materials and Methods.

Although the experiments described above clearly show that anti-I-A treatment of macrophages inhibits thymocyte binding, this reduction was generally only around 50% in most experiments. It was therefore important to determine if anti-I-E antibody had a similar effect. Accordingly, CBA/J splenic macrophages were treated with anti-I-A^k, anti-I-E^k, or both together, prior to thymocyte binding (Table 8). It was found that anti-I-A and anti-I-E both had a similar inhibitory effect on thymocyte binding and that the number of macrophage-bound thymocytes was reduced by around 50% by both antibodies. Again, this reduction is reflected both by fewer macrophages showing binding and in the average number of thymocytes bound per macrophage. Interestingly, macrophages treated with both anti-I-A and anti-I-E showed an 80%

TABLE 8

Effect of Macrophage Treatment with Anti-I-A and Anti-I-E Antibody on Thymocyte Binding

Monoclonal antibody[a]	Thymocytes bound/100 Mϕ	Thymocytes bound/Mϕ				
		0	1–2	3–4	5–6	>6
None	253	30	17	17	18	18
Anti-I-A^k	136	63	18	12	5	3
Anti-I-E^k	104	63	21	11	3	2
Anti-I-A^k + anti-I-E^k	48	72	21	6	1	0

[a] CBA/J splenic adherent cells were treated with monoclonal anti-I-A^k (10.2.16) or anti-I-E^k (14.4.4) antibody, or both, and the binding of syngeneic thymocytes was determined as described under Materials and Methods.

inhibition in the number of bound thymocytes, which reflects a more dramatic reduction in the number of macrophages showing binding and in the average number of thymocytes bound per macrophage. Thus, it appears that both the I-A and I-E subregions are involved in binding of thymocytes by macrophages.

DISCUSSION

In this report we examined the capacity of different macrophage populations to spontaneously bind thymocytes *in vitro*. We found that macrophages from different anatomical locations showed varying binding ability; those from the spleen and thymus showed considerably more thymocyte binding than peritoneal, peripheral blood, or bone marrow macrophages. This pattern of binding seemed to directly correlate with previously determined levels of macrophage expression of Ia antigens using these same mouse strains (13–15) and the best binding was observed with macrophage populations with the highest number of Ia-positive cells. In addition, thymocyte binding by splenic macrophages was dramatically reduced following elimination of Ia-positive macrophages by anti-Ia and complement treatment. This conclusion was substantiated in a second approach in which relatively Ia-negative peritoneal macrophages were induced to express Ia by T-cell factors (16, 17); the induced macrophages showed dramatically more thymocyte binding than uninduced macrophages from the same source. A more direct association for Ia in the thymocyte–macrophage interaction was provided by observations that anti-Ia antibody specifically inhibited the extent of binding. It is of interest that both anti-I-A and anti-I-E partially blocked thymocyte binding, but that both antibodies together had a more dramatic inhibitory effect. Taken together, these results suggest that the capacity for macrophages to bind thymocytes is in part dependent on their expression of Ia antigens.

Although our results indicate that around 80% of thymocyte binding is dependent on macrophage expression of Ia antigens, it is not clear if this binding is mediated solely by Ia. The reason for this is that the macrophage binding capacity is sensitive to trypsin treatment ((10, 12) unpublished observations), whereas cell surface Ia is relatively resistant to trypsin. In addition, Ia-positive B cells fail to bind thymocytes ((10) unpublished observations). Thus, it is unlikely that thymocytes bind solely to Ia. More likely is that several cellular structures are actually involved in the thymocyte–macrophage binding event. For macrophages, at least one of these is trypsin sensitive, and another would seem to be Ia.

The significance of thymocyte binding to macrophages is not known. Based on the observations that hydrocortisone-resistant thymocytes bind much less well than hydrocortisone-sensitive thymocytes, Lopez *et al.* (12) suggested that binding may be important in T-cell maturation. It is also interesting that Kyewski and Kaplan have found that thymus reconstitution of irradiated recipients is associated with cluster formation *in vivo* (18, 19). Thus, another possible role for binding could be in the homing and traffic controlling of immature T cells. In another study (20) we found that those thymocytes that bound were functionally immature, while the unbound cells were enriched for alloreactivity. It has also been found that in the absence of antigen, mature peripheral T cells show little spontaneous binding to macrophages ((12) unpublished observations). Thus, one consequence of binding by immature thymocytes may be a selection process to eliminate self-reactivity as proposed by Jerne (1). However, we found that non-macrophage-adherent thymocytes were also

enriched for syngeneic MLR responses (20). Thus, either the genetic selection of immature T cells for mature functional reactivities is imprecise, and allows a substantial number of autoreactive T cells to mature, or there is another selection mechanism that either prohibits their migration to the periphery or suppresses their reactivity. Under conditions allowing functional maturation of immature T cells, it would be of interest to determine if the phenotype of the macrophage to which thymocytes were bound influences their subsequent genetic restrictions. In any case, the spontaneous binding of thymocytes to macrophages provides an interesting model for studying lymphoid cellular interactions. Although this binding is species specific, no strain specificity is yet evident. This latter observation suggests that the involvement of macrophage Ia antigens in thymocyte binding we have described probably represents a relatively nonpolymorphic interaction with Ia. In this light, this interaction would seem to be distinct from the antigen-dependent Ia-restricted interaction between macrophages and mature T cells. In both cases, however, it is clear that macrophage Ia antigens are involved in physical interactions with T cells in both antigen-independent and antigen-dependent systems, suggesting that Ia may play several roles in mediating T-cell and macrophage collaboration.

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