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ROLE OF SYNGENEIC Ia$^+$ ACCESSORY CELLS IN THE GENERATION OF ALLOSPECIFIC CTL RESPONSES

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Under conditions in which antigen dose is suboptimal, the recognition of Ia determinants is a necessary component of the generation of allospecific CTL responses. With monoclonal anti-Ia antibodies used as blocking reagents, it is demonstrated that the recognition of alloantigens may proceed via two pathways. Alloantigens can be recognized in the context of syngeneic Ia determinants in a similar fashion to conventional antigens. If, however, the Ia$^+$ cells are removed from the responder population, the generation of such responses involves the recognition of allogeneic Ia determinants directly. A non-T, non-B, adherent accessory cell was identified as the critical syngeneic Ia$^+$ cell required for CTL responses in these cultures.

The generation of both humoral (B cell-dependent) and cell-mediated (T cell-dependent) effector immune responses appears to follow similar pathways of cell-cell interaction. Optimal stimulation of antibody-forming cells or cytolytic T lymphocytes (CTL)[2] requires the cooperation of Ly-1$^+$ helper T cells (T_h) with precursors of the effector lymphocytes. The activation of T_h in turn involves the recognition of antigenic determinants on the surface of nonlymphoid adherent accessory cells in an as yet undefined association with Ia glycoproteins. For responses to non-MHC[2] antigens, the relevant Ia molecules are "self," that is, coded for by the genome of the responding cell population (1, 2). In contrast, a large number of studies have suggested that for CTL responses to cells bearing allogeneic MHC antigens, the allogeneic Ia molecules on accessory cells in the stimulator population are of critical importance in triggering T_h to cooperate with CTL specific for allo-H-2K and H-2D determinants (3–5). Recently, several pieces of evidence have raised questions concerning the real importance of allogeneic *vs* syngeneic Ia determinants in alloresponses. It has been found that proliferative responses to cells bearing only H-2K or H-2D differences nonetheless require Ia$^+$ accessory cells in the culture (6). Furthermore, this laboratory has demonstrated that stimulation of CTL by pure H-2K^k alloantigen incorporated into liposomes requires active antigen processing and presentation to T_h by accessory cells bearing syngeneic Ia antigens (7, 8). Taken together, these findings strongly suggest that T_h triggering by both conventional antigens *and* by allo-MHC gene products may readily occur via presentation in the context of self rather than allo-Ia. The present set of experiments in which viable cells rather than liposomes were used as antigen provides further support for this hypothesis. The implications of these findings for current concepts of alloresponsiveness are discussed.

MATERIALS AND METHODS

Mice. BALB/c (*H*-2^d), B10.BR, C3H, and AKR (*H*-2^k) mice, 6 to 12 wk of age, were purchased from the Jackson Laboratory, Bar Harbor, ME.

Monoclonal antibodies (Mab). Monoclonal antibody 10.2.16 (Salk Cell Distribution Center, La Jolla, CA) is an anti-Ia-17 murine antibody with specificity for the A chain, i.e., it is specific for the I-A^{k,s,f,r,u} subregion (9, 10). This hybridoma was originally produced by fusing splenocytes from a CWB mouse immunized with C3H cells with the NS-1 myeloma. M5/114.2 is a rat Mab with specificity for I-A^d, I-E^d, I-E^k, and I-A^b molecules (described in Reference 11). Supernatants from rapidly growing cell culture flasks were used 3 to 4 days after fresh passage and were added to cultures for blocking studies at a final concentration of 20%.

Preparation of accessory cells and B cells. Splenic adherent cells (SAC) were prepared as previously described (12). Briefly, 5 × 10^7 spleen cells were incubated in 100-mm glass petri dishes in RPMI 1640 supplemented with penicillin/streptomycin, 2 mM L-glutamine, 10% fetal calf serum, and 5 × 10^{-5} M 2-mercaptoethanol for 2 to 3 hr at 37°C. Nonadherent cells were removed by gentle agitation and several washes. Adherent cells were detached by incubation at 37°C with 5 ml of a 1/5000 dilution of Versene buffer (GIBCO, Grand Island, NY) for 15 min followed by scraping with a rubber policeman. The cells were then incubated overnight at 37°C on a roller drum and were subsequently treated with anti-Thy-1.2 antibody plus complement and x-irradiated. B cells were prepared by passage of spleen cells over two consecutive Sephadex G-10 columns to deplete macrophages as described (13), followed by treatment with monoclonal anti-Thy-1 antibodies plus complement. Monoclonal anti-Thy-1.2 was purchased from New England Nuclear, Boston, MA, and was used at 10^{-3} dilution. Low-Tox rabbit complement (Cedarlane Laboratories) was used at a 10^{-1} dilution.

Preparation of responder cells. Single cell suspensions of spleen were prepared and when indicated, spleen cells were depleted of Ia$^+$ cells by passage over nylon wool as previously described (14), followed by treatment with the appropriate anti-Ia antibody plus complement. Nylon wool-passed spleen cells (50 × 10^6) were incubated for 40 min at 4°C with 1 ml of hybridoma supernatant. The cells were then washed and incubated for 45 min at 37°C with rabbit complement (Low-Tox, Cederlane Laboratories), diluted 1/6 in L15 medium. Next, 7 × 10^6 responder cells were cultured with 5 × 10^5 irradiated stimulator cells in 16-mm Linbro wells.

^{51}Cr-release assay. After 5 days of culture, cells were harvested and assayed in a 4-h ^{51}Cr-release assay on the indicated target cells. Data were calculated as percent specific release (15).

RESULTS

Effect of monoclonal anti-Ia antibody on CTL responses by whole spleen cells. Ia$^+$ splenic adherent accessory cells are required for primary CTL responses to alloantigen (14, 16, 17). Because this requirement is best demonstrated with limiting numbers of stimulator cells, the importance of syngeneic *vs* allogeneic Ia molecules in CTL generation was reinvestigated under these experimental conditions, with monoclonal anti-Ia antibodies used as blocking reagents. When BALB/c (*H*-2^d) responder cells were cultured with a suboptimal number of B10.BR (*H*-2^k) stimulator cells (5 × 10^5), and a Mab (M5/

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[1] Dr. S. Burakoff is supported by National Institutes of Health Grant AI 17258, and is the recipient of an American Cancer Society Faculty Research Award.
[2] Abbreviations used in this paper: CTL, cytolytic T lymphocyte; DTH, delayed-type hypersensitivity; IL 1, interleukin 1; Mab, monoclonal antibody; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; SAC, splenic adherent cell, T_h, helper T cell.

114.2) specific for I-A gene products of the responder cells was added, a marked decrease in the CTL response to the allogeneic cells was observed (Table I). When the same antibody was added to cultures involving the reciprocal strain combination, such that the antibody was specific for I-A determinants of the stimulator, no inhibition was observed. Thus, under these conditions, only anti-Ia antibody to the responder and not to the stimulator Ia determinants blocked the generation of a response. In the B10.BR anti-BALB/c combination, no blocking was observed with M5/114.2, which is specific for $I\text{-}E^k$ as well as $I\text{-}A^d$ and $I\text{-}E^d$. This suggests that Ia antigens encoded by the syngeneic I-E subregion are not of major importance in the stimulation of these responses.

Effect of monoclonal anti-Ia antibodies on CTL responses of Ia^- T cells. Previous studies have clearly demonstrated a role for T_h stimulation by allo-Ia-bearing accessory cells in CTL responses *in vitro* (3), in contrast to the results reported above. Therefore, to establish whether recognition of antigen in the context of the Ia determinants of the responder is the *only* pathway leading to alloreactive CTL responses under the present conditions, we analyzed the generation of responses in the absence of Ia^+ cells in the responder population. The responder population was depleted of Ia^+ cells by passage of spleen cells over nylon wool and treatment of these partially purified cells with the appropriate anti-Ia antibody plus complement. Responders were then cultured with limiting numbers of allogeneic stimulator cells (5×10^5), and antibody specific for Ia antigens of either the responder population or the stimulator population was added (Table II). In the absence of Ia^+ cells in the responder population, antibody specific for the responder Ia determinants was not inhibitory, as would be expected; however, under these conditions, antibody specific for the Ia antigens of the stimulator cells could block the induction of a response as predicted from previous reports. Thus, 10.2.16, specific for $I\text{-}A^K$ of the stimulator, blocked the induction of CTL, whereas M5/114.2 did not.

Site of action of anti-Ia blocking antibody. The experiments in Tables I and II do not define the site of action of the Mab. Inhibition of whole spleen CTL responses by anti-responder Ia antibody could reflect blocking of T_h specific for alloantigen presented by Ia^+ syngeneic SAC. Alternatively, such inhibition could result from interference with the activity of an Ia^+ T cell involved in the T_h - T_{CTL} interaction. To distinguish between these possibilities, the responding population was physically depleted of B cells and accessory cells, then treated with anti-Ia antibody plus complement to remove any remaining Ia^+ cells (B, SAC, or T). T cell-depleted, Ia^+ SAC were then added back to these responding cells, together with stimulator cells, in the presence or absence of anti-Ia antibody. The results in Table III demonstrate that under such conditions, only anti-responder Ia antibody blocks the CTL responses generated in the presence of the added syngeneic SAC. This clearly demonstrates that Ia present on non-T cells is the target of the blocking antibody, and strongly suggests T_h recognition of alloantigen in the context of syngeneic Ia determinants. Furthermore, the addition of Ia^- responder strain SAC did not permit anti-responder Ia antibody to inhibit CTL generation, although Ia antibody to the stimulator cells did block, consistent with the site of antibody action being the Ia^+ SAC.

To define further the cellular site of antibody blocking in this model and to test directly whether B cell-associated Ia molecules play an important role in these CTL responses, the experiments presented in Table IV were carried out. In these experiments, both responder and stimulator populations were depleted of Ia^+ cells, under which conditions a weak CTL response was observed. The addition of either responder or stimulator-type B cells devoid of SAC to these cultures failed to reconstitute the response; the addition of SAC did permit a response. Together with the data in Table III showing that Ia^+ SAC are the prime targets for Mab blocking of CTL generation, these results point to non-T, non-B accessory cells as the critical syngeneic Ia^+ cell required for CTL responses in these cultures.

TABLE I

Induction of CTL to allogeneic cells is blocked by antibody to responder Ia antigens[a]

Responder	Stimulator	Relevant Specificity of Antibody Added	Lysis of RDM4[b] 33/1	11/1
BALB/c	B10.BR(5×10^5)	—	78	62
BALB/c	B10.BR	$I\text{-}A^d$, $I\text{-}E^d$, $I\text{-}E^k$	23	14
			Lysis of P815	
B10.BR	BALB/c	—	74	54
B10.BR	BALB/c	$I\text{-}A^d$, $I\text{-}E^d$, $I\text{-}E^k$	63	42

[a] 7×10^6 responder spleen cells were cultured with 5×10^5 stimulator cells for 5 days. Where indicated, the monoclonal antibody M5/114.2 was added at the initiation of culture. —, indicates no antibody added.

[b] Percent specific release of ^{51}Cr was assessed at the indicated effector-to-target ratios. The spontaneous release of ^{51}Cr from RDM4 and P815 cells was <15% in all experiments.

TABLE II

In the absence of Ia^+ cells in the responder population, antibody to stimulator Ia antigens blocks the induction of CTL

Responder[a]	Removal of Ia^+ Cells	Stimulator	Relevant Specificity of Antibody Added[b]	Lysis of YAC[c] 60/1	20/1
BALB/c	–	C3H(5×10^5)	—	62	38
BALB/c	–	C3H	$I\text{-}A^d$, $I\text{-}E^d$, $I\text{-}E^k$	24	10
BALB/c	–	C3H	$I\text{-}A^k$	69	57
BALB/c	+	C3H	—	55	23
BALB/c	+	C3H	$I\text{-}A^d$, $I\text{-}E^d$, $I\text{-}E^k$	49	21
BALB/c	+	C3H	$I\text{-}A^k$	18	3
				Lysis of P815	
C3H	–	BALB/c (5×10^5)	—	81	78
C3H	–	BALB/c	$I\text{-}A^d$, $I\text{-}E^d$, $I\text{-}E^k$	81	73
C3H	–	BALB/c	$I\text{-}A^k$	19	9
C3H	+	BALB/c	—	57	43
C3H	+	BALB/c	$I\text{-}A^d$, $I\text{-}E^d$, $I\text{-}E^k$	4	0
C3H	+	BALB/c	$I\text{-}A^k$	77	63

[a] Where indicated, the responder population was depleted of Ia^+ cells by passage of spleen cells over nylon wool followed by treatment with monoclonal anti-Ia antibody plus complement (M5/114.2 for BALB/c, and 10.2.16 for C3H). The responders were then cultured with 5×10^5 irradiated spleen stimulator cells for 5 days.

[b] Where indicated, monoclonal antibody was added at the initiation of culture. M5/114.2 has specificity for $I\text{-}A^d$, $I\text{-}E^d$, $I\text{-}E^k$, and 10.2.16 has specificity for $I\text{-}A^k$. —, indicates no antibody added.

[c] After 5 days, percent specific release of ^{51}Cr was assessed. The spontaneous release of ^{51}Cr from YAC was <18% and from P815 was <12%.

DISCUSSION

It has been proposed by Bach *et al.* (3) that maximal CTL activity to alloantigens is achieved by the coordinate stimulation of the effector CTL by antigens in the H-2K or D regions, and of an MLC reactive helper T cell by I region determinants. We have previously demonstrated that one component in the generation of a secondary CTL response to a purified alloantigen, i.e., $H\text{-}2K^k$ incorporated into liposomes, is the activation of $Ly\text{-}1^+$ T_h cells (7). Antiserum blocking studies indicated that the induction of the helper pathway involves the recognition of this alloantigen in the context of the Ia determinants of SAC syngeneic to the responder population. The experiments described here were designed to determine whether alloantigens are represented and recognized in the context of syngeneic Ia when

TABLE III

The Ia antigens on SAC are involved in antigen presentation

Responder[a]	Removal of Ia$^+$ Cells	Accessory Cell[b]	Treatment of Accessory cell[c]	Stimulator	Relevant Specificity of Antibody Added[d]	Lysis of RDM4[e] 60/1
BALB/c	−			AKR (5 × 10^5)	—	58
BALB/c	−			AKR	I-A^d, I-E^d, I-E^k	10
BALB/c	−			AKR	I-A^k	52
BALB/c	+			AKR	—	40
BALB/c	+			AKR	I-A^d, I-E^d, I-E^k	40
BALB/c	+			AKR	I-A^k	12
BALB/c	+	BALB/c SAC	C	AKR	—	44
BALB/c	+	BALB/c SAC	C	AKR	I-A^d, I-E^d, I-E^k	9
BALB/c	+	BALB/c SAC	C	AKR	I-A^k	45
BALB/c	+	BALB/c SAC	α Ia + C	AKR	—	42
BALB/c	+	BALB/c SAC	α Ia + C	AKR	I-A^d, I-E^d, I-E^k	40
BALB/c	+	BALB/c SAC	α Ia + C	AKR	I-A^k	5

[a] The responder population was depleted of Ia$^+$ cells by passage over nylon wool followed by treatment with anti-Ia antibody plus complement.
[b] BALB/c SAC (8 × 10^5) were added at the initiation of cultures. All SAC were x-irradiated and treated with anti-Thy-1.2 antibody plus complement.
[c] The SAC were treated with monoclonal anti-Ia antibody (M5/114.2) plus complement or complement alone.
[d] Where indicated, M5/114.2 (with specificity for I-A^d, I-E^d, and I-E^k) or 10.2.16 (with specificity for I-A^k) was added at the initiation of culture. —, indicates no antibody added.
[e] After 5 days, percent ^{51}Cr release from RDM4 cells was assessed.

TABLE IV

B cells cannot substitute for SAC in antigen presentation

Responder[a]	Removal of Ia$^+$ Cells	Additions[b]	Stimulator	Removal of Ia$^+$ Cells	Lysis of P815[c] (60/1)
AKR	−		BALB/c	−	62
AKR	+		BALB/c	−	57
AKR	+		BALB/c	+	15
AKR	+	AKR B cells (10^6)	BALB/c	+	18
AKR	+	AKR B cells (3.3 × 10^5)	BALB/c	+	12
AKR	+	AKR SAC (10^6)	BALB/c	+	53
AKR	+	AKR SAC (3.3 × 10^5)	BALB/c	+	31
AKR	+	BALB/c B cells (10^6)	BALB/c	+	20

[a] Both the responder and stimulator populations were depleted of Ia$^+$ cells by passage of the spleen cells over nylon wool followed by treatment with the appropriate anti-Ia antibody plus complement.
[b] Various numbers of SAC or Sephadex G-10-purified B cells were added at the initiation of cultures.
[c] After 5 days, percent specific release of ^{51}Cr from P815 was assessed.

the antigens are on viable stimulator cells and allogeneic Ia antigens are present as well. We demonstrate that recognition of Ia determinants is indeed an important element in the generation of alloreactive CTL, and there does appear to be specific stimulation by allogeneic Ia. Such stimulation, however, is by no means the only pathway leading to the induction of a CTL response, for even in the presence of allogeneic Ia determinants, alloantigens may be recognized just as conventional antigens are, i.e., they are recognized in the context of syngeneic Ia determinants on antigen-presenting cells. Even the stimulation of primary responses by alloantigens, in the form of whole cells that bear allogeneic Ia determinants in addition to allogeneic H-2K and H-2D antigens, can proceed via recognition in the context of syngeneic Ia. Observations recently reported by Schnagl and Boyle (18) are consistent with these findings. They found that the precursors of proliferative cells bind to a monolayer only if both self-plus allo-Ia are present.

In this study we observed that when responder and allogeneic stimulator populations are cultured together, the addition of antibody specific for the Ia antigens of the responders markedly inhibit the generation of a CTL response. Although an alloresponse is generated from whole spleen cultures in the presence of anti-stimulator Ia, it is possible that the specificity of this response is different from one generated when stimulator Ia determinants are recognized in the generation of an allo-CTL response. If the Ia$^+$ cells are removed from the responder population and the only remaining Ia determinants are those of the allogeneic stimulators, a CTL response is still generated. Furthermore, the addition of antibody specific for the Ia antigens of the stimulators blocks the generation of this response. Our inability to observe a role for the stimulator Ia antigens in the generation of responses by whole spleen cell populations when responder Ia determinants are blocked by anti-Ia antibodies therefore is unexpected. Although the explanation for this phenomenon is not yet clear, several possibilities may be considered: 1) anti-Ia antibody does not act by sterically blocking T cell recognition events at the accessory cell surface, but rather triggers a suppressor mechanism in the cultures. This is unlikely because the anti-stimulator antibody should not lose its ability to generate such suppression in the unfractionated cultures but does fail to block under these circumstances. Furthermore, preliminary results of blocking studies employing F_1 stimulator cells are also inconsistent with the induction of nonspecific suppression. 2) The large number of responder SAC relative to stimulator SAC diverts the pre-CTL to ineffective sites for triggering. In this view, Ia$^+$ accessory cells are critical focal points for local CTL-T_h interaction, and accumulation of pre-CTL at responder accessory cells incapable of triggering T_h prevents the active T_h at stimulator SAC sites from having a sufficient number of pre-CTL locally to generate a detectable response. Careful dose-response tests of syngeneic and allogeneic SAC added back to pure T cell responders and stimulators may provide useful information on this point.

The identification of the target of anti-Ia antibody blocking as a non-T, non-B adherent accessory cell is consistent with a number of earlier reports. More recent studies, however, indicate that Ia$^+$ B cells may be capable of eliciting T cell proliferative or T_h responses (19). The failure of Ia$^+$ B cells to stimulate CTL responses in the present experiment stands in contrast to these latter results. This difference may involve problems with the ability of B cells to acquire or process alloantigens in contrast with non-cell membrane-associated antigens, or with the production or elaboration of lymphokines known to play a role in T_h triggering (e.g., interleukin 1 (IL 1)). In this case, the primary nature of the responses studied here, in contrast to the secondary responses employed in the other models, may be a critical difference.

We have not yet identified the precise alloantigens presented to T_h by syngeneic sites. Our studies with H-2K^k containing liposomes show that pure MHC gene products are sufficient for triggering T_h responses via syngeneic Ia presentation (7). These data, however, do not rule out an important role for non-MHC antigens in this process. Both Matzinger and Bevan (20) studying CTL to minor alloantigens, and Smith and Miller (21) studying delayed-type hypersensitivity (DTH) responses to cells and cell extracts demonstrated T cell priming to non-MHC antigens in the context of syngeneic Ia. It may well be that the

ability of allostimulators to generate substantial primary responses relates to the large number of potential antigens involved in T_h triggering if both MHC and non-MHC gene products are efficiently presented under these conditions.

The results reported here may have particular relevance to the stimulation of effector responses to nonlymphoid allografts and to tumors, which both lack (significant numbers of) allo-Ia-bearing stimulator cells. In these cases triggering of T_h by represented antigen is presumably the major if not the only pathway for CTL induction, and the efficiency of such syngeneic T_h triggering should control the level of the CTL response. In the case of grafts, decreasing *syngeneic* Ia presentation could therefore be of benefit, whereas for tumors, increasing T_h triggering around syngeneic accessory cells should be a goal of immunotherapeutic regimes.

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